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Dolly Mehta and Asrar B. Malik

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# Signaling Mechanisms Regulating Endothelial Permeability

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**Mehta, Dolly, and Asrar B. Malik.** Signaling Mechanisms Regulating Endothelial Permeability. *Physiol Rev* 86: 279-367, 2006; doi:10.1152/physrev.00012.2005.—The microvascular endothelial cell monolayer localized at the critical interface between the blood and vessel wall has the vital functions of regulating tissue fluid balance and supplying the essential nutrients needed for the survival of the organism. The endothelial cell is an exquisite “sensor” that responds to diverse signals generated in the blood, subendothelium, and interacting cells. The endothelial cell is able to dynamically regulate its paracellular and transcellular pathways for transport of plasma proteins, solutes, and liquid. The semipermeable characteristic of the endothelium (which distinguishes it from the epithelium) is crucial for establishing the transendothelial protein gradient (the colloid osmotic gradient) required for tissue fluid homeostasis. Interendothelial junctions comprise a complex array of proteins in series with the extracellular matrix constituents and serve to limit the transport of albumin and other plasma proteins by the paracellular pathway. This

pathway is highly regulated by the activation of specific extrinsic and intrinsic signaling pathways. Recent evidence has also highlighted the importance of the heretofore enigmatic transcellular pathway in mediating albumin transport via transcytosis. Caveolae, the vesicular carriers filled with receptor-bound and unbound free solutes, have been shown to shuttle between the vascular and extravascular spaces depositing their contents outside the cell. This review summarizes and analyzes the recent data from genetic, physiological, cellular, and morphological studies that have addressed the signaling mechanisms involved in the regulation of both the paracellular and transcellular transport pathways.

## I. INTRODUCTION

The vascular endothelium lining the intima of the blood vessels regulates a variety of functions including vascular smooth muscle tone, host-defense reactions, angiogenesis, and tissue fluid hemostasis. The maintenance by the endothelium of a semi-permeable barrier is particularly important in controlling the passage of macromolecules and fluid between the blood and interstitial space. It is known that loss of this function results in tissue inflammation, the hallmark of inflammatory diseases such as the acute respiratory distress syndrome. The characteristic permeability of transported macromolecules is dependent on their molecular radii as well as the barrier properties of the particular endothelium. This size-selective nature of the barrier to plasma proteins is a key factor in establishing protein gradients (especially in the case of albumin) required for fluid balance of tissues. In addition, plasma proteins, such as albumin, act as circulating chaperones for hydrophobic substances, fatty acids, and hormones, molecules whose transport is crucial for cell functions vital to the organism. Thus the efficient transfer of many water insoluble substances from the blood into the interstitium relies on endothelial permeability, and often on specific carrier proteins. The requirements for continuous transendothelial protein flux and, at the same time, a steep albumin gradient imply that dynamic processes exist in the endothelium controlling protein flux between the vascular and extravascular spaces.

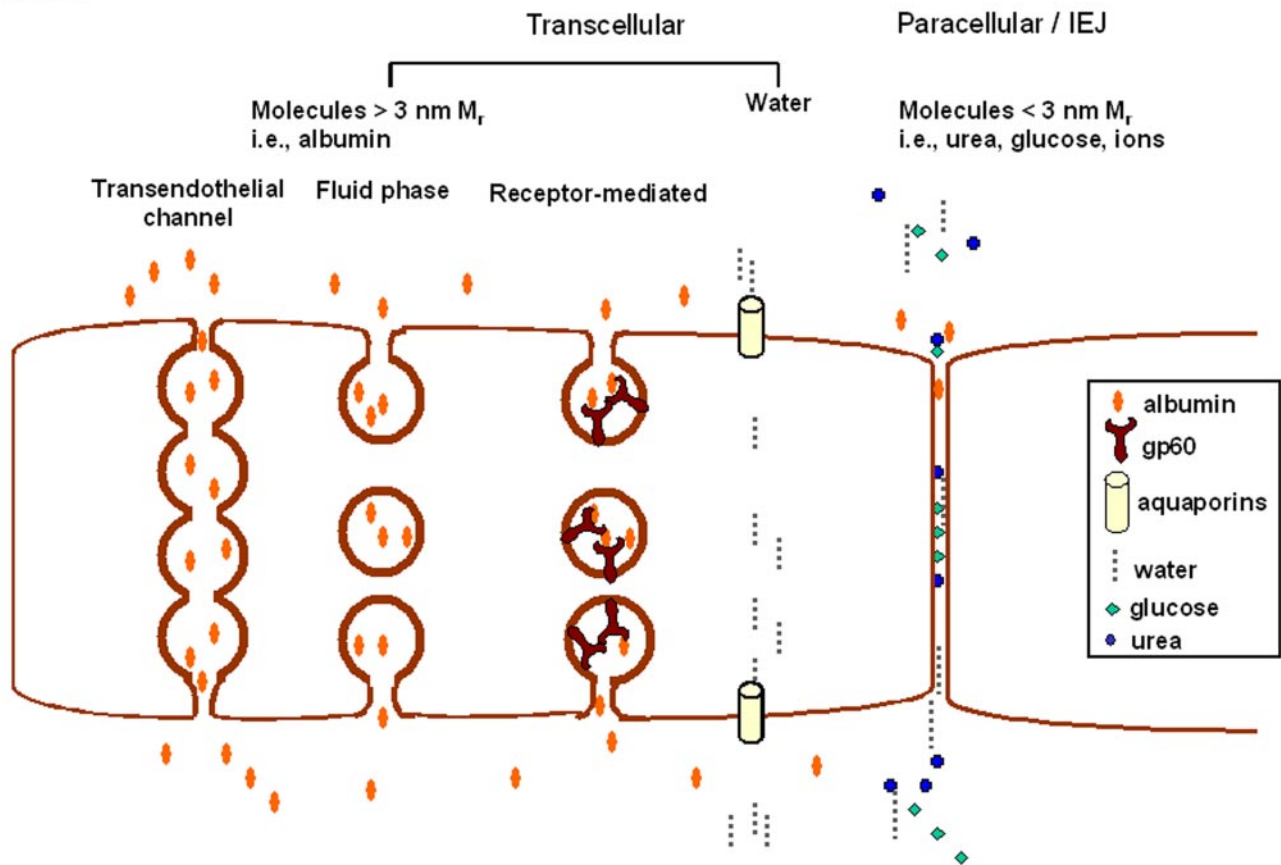
Estimates of the transvascular flux of solutes and fluid indicate that protein transport occurs by a mechanism distinct from that of small hydrophilic molecules. In this regard, the traditional view of the endothelium as a "static barrier" through which proteins leak via interendothelial junctions (IEJ) is an oversimplification. Estimates of the dimensions of unperturbed IEJs are insufficient to allow the unrestricted passage of proteins known to cross the barrier. Moreover, the notion of passive protein leakage through the endothelium has been questioned in light of recent ultrastructural, biochemical, and physiological studies of protein tracers in transit through the intact endothelium. These newer studies have emphasized the role of a vesicular pathway in the mechanism of transfer of proteins with their cargo of small hydrophobic solutes. In this review, we evaluate the evidence that the endothelium controls flux of fluid and solutes across the

vessel wall through highly regulated transport pathways. An emerging general principle is that transport of protein and liquid in the undisturbed endothelium occurs via the transcellular pathway. However, in response to intrinsic and extrinsic stimuli, the endothelium also sets into motion additional signaling pathways that allow transport of solutes through IEJs. This review describes the current understanding of the signaling mechanisms activated in endothelial cells that regulate barrier function via both pathways and raises questions in areas where understanding and important details are in doubt.

Endothelial transport can be thought of in a general sense as occurring via paracellular and transcellular pathways (Fig. 1). The continuous endothelium (as found in pulmonary, coronary, skeletal muscle, and splanchnic vascular beds) is described as being restrictive because solutes with molecular radii of up to 3 nm move passively across the barrier via the paracellular route. The transcellular vesicular pathway is responsible for the active transport of macromolecules as shown for albumin (316, 595, 603, 719, 810, 811, 864). Paracellular permeability is regulated by a complex interplay of cellular adhesive forces balanced against counteradhesive forces generated by actinomyosin molecular motors. The unperturbed endothelial barrier has restrictive properties that are due primarily to closed IEJs. Evidence now suggests that integrin receptor binding to the extracellular matrix (ECM) can also contribute to the barrier function by stabilizing the closed configuration of IEJs. The inflammatory mediators thrombin, bradykinin, histamine, vascular endothelial growth factor (VEGF), and others upon binding to their receptors, disrupt the organization of IEJs and integrin-ECM complexes, thereby opening the junctional barrier (for review, see Refs. 225, 553). Thus the formation of minute intercellular gaps allows passage of plasma proteins including albumin and liquid across the endothelial barrier in an unrestricted manner. The signaling pathways regulating opening and closing of junctions are of great interest as they relate to the regulation of tissue fluid balance and the mechanisms of inflammation, and are discussed extensively in this review.

Caveolae, the vesicular carriers of the transcellular pathway, have been little studied until recently. However, now with identification of a number of caveolae-associated regulatory proteins, dynamin, intersectin, and caveolin-1, there is a growing realization of the fundamental

Luminal side



Abluminal side

FIG. 1. Schematic of transport pathways in continuous endothelium. Under basal conditions, the transcellular pathway can mediate the transport of plasma proteins ( $>3$  nm  $M_r$ ) such as albumin by caveolae via an absorptive (receptor-mediated) or fluid-phase pathway. Transcellular channels can also form transiently in endothelial cells by fusion of multiple caveolae and allow albumin transport. Aquaporins form channels across the lipid bilayer that are highly selective for water molecules and allow their movement across the luminal or abluminal endothelial membrane, thus creating a transendothelial pathway for water. Small molecules including urea and glucose ( $<3$  nm  $M_r$ ) are transported around individual endothelial cells via the paracellular, i.e., interendothelial junction (IEJ) pathway.  $M_r$ , molecular radius.

importance of this pathway in plasma protein transport. The process of transcytosis is initiated by the interactions of plasma proteins such as albumin with specific “docking molecules” (864) in cell surface caveolae that are subsequently released into the cell upon scission. Transcytosis of albumin, a constitutive process, is of particular interest because of its potential for controlling the tissue albumin concentration and hence in regulating the transvascular oncotic pressure gradient (see Ref. 553). Caveolae traverse the cytoplasm reaching the basolateral surface where they release their contents by exocytosis. A recent review by Tuma and Hubbard (966) addresses aspects of transcytosis; in this review we have emphasized the signaling mechanisms of transcytosis and their role in the regulation of endothelial permeability.

There are several recent comprehensive reviews dealing with the general subject of endothelial permeability discussed from different perspectives (592, 966).

Michel and Curry (592) in particular have elegantly addressed the role of the endothelial glycocalyx (termed the “fiber matrix”) in regulating endothelial permeability. Tuma and Hubbard (966) in a thorough review of the subject have drawn attention to the still incompletely understood processes of caveolae- and clathrin-mediated endocytosis and transcytosis in various organs. Although both reviews are seminal with respect to their own emphases, neither focuses on the signaling mechanisms involved in the regulation of endothelial permeability, the thrust of our review. Our objectives when we set out on this venture were twofold: 1) to provide a fresh perspective to the field and 2) to highlight those areas where signaling pathways are beginning to be better understood as well as those areas where a great deal of work is needed. Thus we have evaluated the evidence concerning the signals that control transendothelial liquid and protein transport via the paracellular-junctional and transcellular-



vesicular pathways. Because a great deal of work has recently been carried out using genetically modified mouse models, we have also discussed the data that bear on and inform the signaling mechanisms regulating vascular endothelial permeability in an *in vivo* setting. The reader should be made aware that we have not been timid about speculating, and wherever possible, critically analyzing the findings because we wish to draw attention to uncertainties in the field and, hopefully, to stimulate debate. In writing this review, we have attempted to be as inclusive as possible in citing the most significant work that addresses endothelial permeability regulation; however as is invariably the case given space limitations and the need to emphasize specific areas, we have not been able to refer to all of the published findings. For this, please accept our apologies in advance.

## II. STARLING AND KEDEM-KATCHALSKY EQUATIONS DESCRIBING ENDOTHELIAL PERMEABILITY

A recent review covered extensively the theory of fluid and solute exchange across vessels (592). We do not attempt to recapitulate this review, but instead emphasize the critical importance of the two equations defined below as their understanding is necessary to the appreciation of the signaling mechanisms regulating endothelial permeability.

The magnitude of fluid movement is dependent on the net balance of Starling forces (896) across the endothelial barrier according to the relationship

$$J_v = (L_p S)[(P_c - P_i) - \sigma(\Pi_c - \Pi_i)] \quad (1)$$

where  $J_v$  is volume flux of fluid (ml/min);  $L_p$  is hydraulic conductivity ( $\text{cm} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1}$ );  $S$  is capillary surface area ( $\text{cm}^2$ );  $P_c$  and  $P_i$  are capillary and interstitial fluid hydrostatic pressures, respectively (mmHg);  $\Pi_c$  and  $\Pi_i$  are capillary and interstitial colloid osmotic (oncotic) pressures, respectively (mmHg); and  $\sigma$  is osmotic reflection coefficient of vessel wall ( $\sigma = 0$  if membrane is fully permeable to transported molecular species and  $\sigma = 1$  if membrane is impermeable). Kedem-Katchalsky (450) later derived equations that represent the overall flux of solute molecules, which is the sum of convective and diffusive components

$$J_s = J_v(1 - \sigma)C_s + PS(\Delta C) \quad (2)$$

where  $J_s$  is transvascular solute flux (mg/min),  $P$  is permeability (cm/s),  $\Delta C$  is the difference in solute concentration across the endothelial cell monolayer, and  $C_s$  is mean concentration of the solute within the hypothetical "pore" (which in endothelial cells is likely to be the cleft

formed at the IEJ);  $J_v$ ,  $S$ , and  $\sigma$  are the same terms used in Equation 1.

Besides quantitatively describing vessel wall permeability to liquid and solutes and the relative contributions of diffusion and convection to overall solute transport, these equations have been useful in laying the foundations for the techniques used to experimentally measure endothelial permeability. Table 1 lists the key methods that rely on these principles and describes the measurement of the specific constants derived from these equations.

## III. PERMEABILITY OF THE ENDOTHELIAL BARRIER

The function of exchange vessels is to allow the unimpeded transfer of dissolved gases, ions, and solutes across the vessel wall. The vast majority of these substances are low in molecular weight and higher in concentration in the plasma than in the interstitium; thus passive diffusion is the chief transport mode for these solutes. The vessel wall is restrictive to high-molecular-weight substances such as proteins because tissues do not usually consume these rapidly, and there are important reasons to retain them in the circulation.

The monolayer of endothelial cells forming the innermost layer of the exchange vessels presents a cellular barrier to permeation of liquid and solutes. The vascular endothelium transports solutes with a range of molecular radii ( $M_r$ ) from 0.1 nm (sodium ion) to 11.5 nm (immunoglobulin IgM) (reviewed in Ref. 757). On the basis of the seminal finding that the transport of lipid-insoluble solutes ( $M_r < 3$  nm) across continuous endothelia decreased with increasing  $M_r$  of the permeating solute, it was concluded that the endothelial barrier behaves like a molecular sieve with an average pore radius of 3 nm (674). However, the relationship between  $M_r$  and transcapillary exchange of solutes is more complex. The permeability of the endothelial layer decreased by four orders of magnitude with an increase in  $M_r$  from 0.1 to 3.6 nm ( $M_r$  of albumin). Interestingly, with an increase in  $M_r$  from 3.6 to 6 nm, endothelial permeability was found to be independent of solute radius (749, 855). This suggested that the pathway for transport of macromolecules differs from that of small solutes (Fig. 2). The estimated area of the barrier made up of IEJ discontinuities of  $<3$  nm could account for the permeability of small molecules, such as water, hexoses (glucose, mannitol, and fructose), amino acids, and urea (592). These findings are the basis for the generally accepted view that there is a paracellular permeation path that allows liquid and small solutes to move across the continuous endothelium. The transcytosis pathway (see sect. x) accommodates larger molecules such as plasma proteins regardless of their  $M_r$  and thus serves to extend the range of transported species. A sig-

TABLE 1. Principles and techniques describing measurement of endothelial permeability in various models

Preparation	Technique	Parameter Determined	Description	Reference Nos.
Cell monolayer	Cell monolayer	$L_p$ ( $\text{cm} \cdot \text{s} \cdot \text{cmH}_2\text{O}^{-1}$ )	Volume flow ( $J_v$ ) is determined as a function of hydrostatic pressure difference ( $\Delta P$ ) across monolayer of area $S$ ( $L_p = J_v/S \Delta P$ )	303, 838
	Transwell tracer (e.g., $^{125}\text{I}$ -albumin)	$P$ (cm/s)	Transmural albumin flux $J_s$ is determined as a function of albumin concentration difference across monolayer of area $S$ ( $P = J_s/S\Delta C$ )	855
Single vessel	Split drop	$L_p$	Volume flow ( $J_v$ ) is determined as a function of hydrostatic pressure difference ( $\Delta P$ ) across microvessel wall; $J_v$ is measured as change in length of liquid column (split drop of oil) in vessel lumen	76
	Modified Landis	$L_p$	Volume flow ( $J_v$ ) is determined as a function of hydrostatic pressure difference ( $\Delta P$ ) across microvessel wall; involves occlusion of the isolated vessel and measurement of distance between two marker cells in the vessel lumen	197
	Intravital fluorescence	$P$	$J_s$ is calculated from the ratio of fluorescent light intensity in plasma to that in interstitium; $P = J_s/S (C_p - C_i)$ , where $S$ is vessel wall surface area and $C_p$ and $C_i$ are concentrations of tracer in plasma and interstitium, respectively	1069
Isolated organ	Gravimetric method	$K_{f,c}$ ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{cmH}_2\text{O} \cdot \text{g}^{-1}$ )*	Determined from change in organ weight resulting from a change in hydrostatic pressure	290, 455
	"Single-sample" method using tracer (e.g., $^{125}\text{I}$ -albumin)	$PS$ product ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ )†	Calculated from $A/Ct$ , where $A$ , $C$ , and $t$ are tissue tracer concentration (cpm/g), perfusate tracer concentration (cpm/ml), and exposure time; single-sample method assumes negligible interstitial tracer concentration	454
	Dual tracer method (e.g., $^{125}\text{I}$ - and $^{131}\text{I}$ -labeled albumin)	$\sigma$ , $PS$ product†	<i>Tracer 1</i> is used to measure the diffusive flux of albumin and <i>tracer 2</i> is the total convective flux following an increase in pulmonary venous pressure; $\sigma$ and $PS$ are calculated using Kedem-Katchalsky's equation	455
In situ organ	Dual tracer method (e.g., $^{125}\text{I}$ - and $^{131}\text{I}$ -labeled albumin)	$PS$ product†	<i>Tracer 1</i> is provided intravenously for 30 min and <i>tracer 2</i> is a vascular volume marker for the final 5 min; $PS$ is calculated from the difference between total and vascular uptake	337
In situ lung microvessels	Lymphatic flux analyses	$\sigma$ , for total plasma protein	Protein concentration ratios in lymph and plasma ( $C_l/C_p$ ) are determined at different rates of capillary filtration and analyzed using Kedem-Katchalsky or modified Patlak equation (i.e., $C_l/C_p = 1 - \sigma$ ); assumes lymph and tissue fluids are identical	542, 758

$L_p$ , hydraulic conductivity;  $P$ , permeability;  $K_{f,c}$ , filtration coefficient;  $\sigma$ , osmotic reflection coefficient. \* $K_{f,c}$  is identical to the term  $L_p S$  in Equation 1 (see text). † $PS$  is defined in Equation 2 (see text).

nificant amount of water (up to 40% of the total hydraulic pathway) (592) crosses the barrier through a transcellular route in vascular endothelial cells and epithelial cells (such as alveolar epithelial cells) by water-transporting membrane channels, the aquaporins (725, 1001). Thus, unlike macromolecules, water traverses the endothelial barrier both by paracellular and transcellular routes.

The vascular endothelium is composed of a monolayer comprising not only endothelial cells but also subcellular ECM proteins, indicating that under normal conditions the permeability of the endothelium depends on the combined properties of the cell layer and the ECM barriers in series (51, 77, 623, 729, 730). However, separate contributions of each component to the overall barrier function have been determined only in a few studies.

The measurement of ECM  $L_p$  in cultured bovine microvessel endothelial cells (730) showed that 40% of total  $L_p$  is attributable to ECM. Moy et al. (623) quantified the contributions of cell-cell and cell-ECM adhesion to endothelial barrier function in terms of the electrical resistance of confluent endothelial monolayers and the underlying ECM using an anti-VE-cadherin blocking antibody that interferes with cell-cell adhesion. Large IEJ gaps formed under these conditions prior to endothelial cell detachment from the substrate, thus providing information about the contribution of ECM to permeability. The ECM accounted for 50% of the total transendothelial electrical resistance, a finding similar to earlier results with fibroblast monolayers (1040). These studies suggest, surprisingly, that the endothelial cell monolayer and the under-

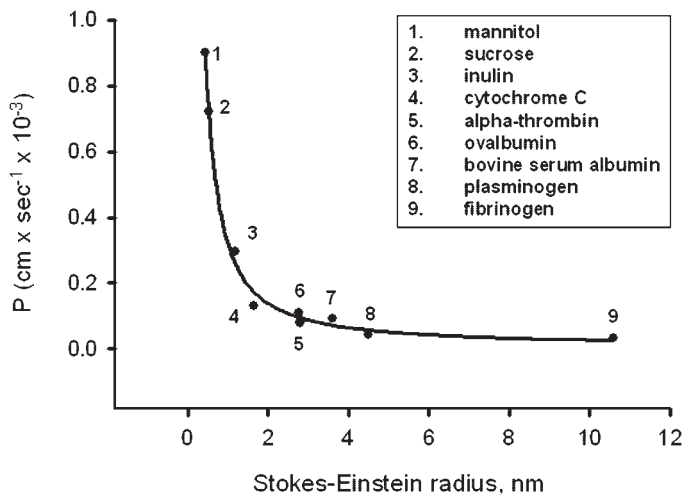


FIG. 2. Permeability of endothelial monolayer to molecules of different Stokes-Einstein radii. Permeability ( $P$ ) of the endothelial cell layer decreases by a factor of 4 with a 36-fold increase in  $M_r$  from 0.1 to 3.6 nm. However, endothelial permeability plateaus when  $M_r$  increases from 3.6 to 12 nm, suggesting that the pathway for transport of these macromolecules differs from that of small solutes. [Modified from Siflinger-Birnboim et al. (855).]

lying ECM each contributes approximately one-half of the total barrier function. It is important therefore that the ECM and its interactions with the endothelium be considered as an essential component of the endothelial barrier.

#### IV. HETEROGENEITY OF ENDOTHELIAL PERMEABILITY

Even though endothelial cells from different vascular sites have many features in common and originate from the same embryonic precursor cells, the hemangioblasts, variations in permeability have been reported in experiments focusing on different regions of the endothelium (11, 29, 124, 173, 193, 194, 325, 339, 686, 861, 911, 935). These positional differences in endothelial permeability have been found in cultured cells obtained from these sites (98, 214, 561, 817, 854) as well as in intact vessels

(15, 458, 492, 560, 607, 680, 864). Baseline vessel wall filtration coefficient ( $K_{f,c}$ ) measurements of isolated lungs of various species indicated that total  $K_{f,c}$  is on average 19% arterial, 37% venous, and 42% microvascular (15, 607, 680) (Fig. 3A), with the exception of experiments from Khimenko and Taylor (458) where 96% of total  $K_{f,c}$  was reported to be the result of the venular segment. This discrepancy could be due to the stop-flow ischemic condition in their studies, as this may have directly increased venular permeability. Aside from this exception, these studies in general indicate that under baseline conditions the arterial segment of the lung is more restrictive to liquid flux than either the venular or capillary segment.

Studies in monolayers of cultured endothelial cells from pulmonary microvessels showed that this segment was the most restrictive to albumin (213, 452, 561, 817). Permeability of  $^{125}\text{I}$ -albumin was found to be three- to fourfold less in confluent monolayers of pulmonary microvessel endothelial cells than those from mainstem artery or vein (561) (Fig. 3B). Transendothelial electrical resistance was also 10-fold greater in pulmonary microvessel endothelia than in large vessel endothelia (98).

Although the basis for segmental variations in liquid and albumin permeability is not completely clear, evidence suggests the involvement of both intrinsic and extrinsic factors (see Ref. 11). Chi et al. (163) using microarray analysis showed a marked variation in genes expressed in endothelial cells isolated from large and small vessels. ECM proteins collagen 4 $\alpha$ 1, collagen 4 $\alpha$ 2, and laminin were associated with microvessel endothelia (163, 917), whereas a greater contribution of fibronectin, collagen 5 $\alpha$ 1, and collagen 5 $\alpha$ 2 was seen with the large vessel endothelia (163). It is possible, therefore, that specific interactions of endothelial cell integrins with ECM are determinants of segmental permeability (208, 211, 730, 884, 934). Of particular note are the differences in genes regulating expression of ECM proteins, integrins, lin-1, isl-1, and mec-3 (LIM) kinase (LIMK), a guanine exchange factor (GEF) for Rho GTPase (Vav), and myosin light-chain kinase (MLCK) in large versus small vessel endothelia (163). Expression of LIMK, MLCK, Vav, and myosin

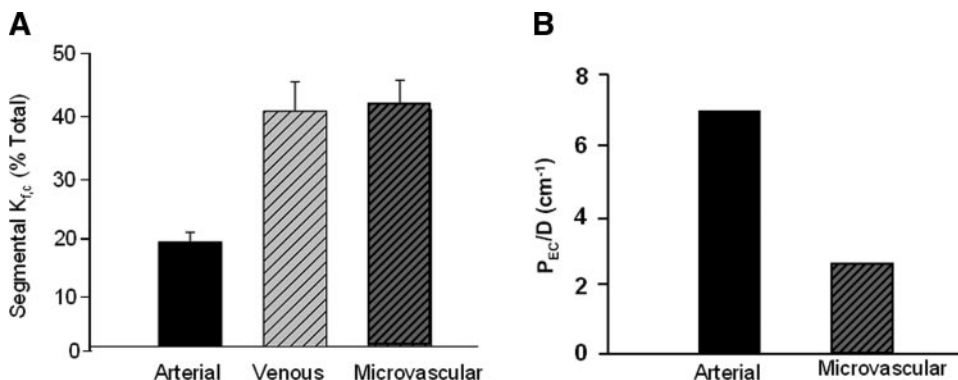


FIG. 3. Segmental heterogeneity of endothelial permeability. A: in situ differences in filtration coefficient ( $K_{f,c}$ ) of three segments as percentage of total  $K_{f,c}$  in rabbit lungs.  $K_{f,c}$  values are calculated by multiplying hydraulic conductivity ( $L_p$ ) by vascular surface area. [Modified from Parker and Yoshikawa (680).] B: permeability-diffusion coefficient ratios ( $P_{EC}/D$ ) of albumin across cultured bovine arterial or microvascular endothelial monolayers of equal surface area (37°C). [Modified from Siflinger-Birnboim et al. (855).]

was found to be higher in endothelial cells from microvessels (163), raising the possibility that extensive cytoskeletal remodeling and qualitative and quantitative differences in cell-ECM attachments in microvessel endothelia regulate the restrictiveness of their junctions. Thus epigenetically determined modifications in endothelial cells from different sites could contribute to the observed segmental variations in barrier properties.

Ultrastructural comparisons of confluent endothelial monolayers from microvessels, veins, and arteries of the lung vasculature have helped to explain the observed differences in endothelial permeability. Microvessel endothelial cells from lungs exhibited better developed IEJs than those in large vessels (817, 862, 863, 869). With the use of the microperoxidase tracer ( $M_r \sim 2.0$  nm), 25–30% of IEJs in postcapillary venules have intercellular gaps of 3.0 nm, whereas IEJs of arterioles and capillaries were impermeable over their entire length to molecules with diameters  $>2.0$  nm (863, 866). There is also evidence that caveolar density is highest in capillaries (584, 816, 863, 868), which may be yet another factor contributing to the observed more restrictive nature of IEJs toward albumin (discussed in sect. xA).

Intracellular signals regulating the endothelial barrier may also vary at specific locales in the circulation. Endothelial cells from microvessels compared with cells from large vessels showed distinct profiles for thrombin-induced intracellular  $\text{Ca}^{2+}$  transients (170); higher basal cyclic nucleotide levels; responsiveness to cAMP-increasing agents isoproterenol, forskolin, and rolipram (192, 900, 1109); and oxidant production (340). While these *in vitro* differences are real, a potential concern is that these cultured endothelial monolayers may have undergone a phenotype drift and may no longer reflect their *in situ* characteristics.

Because the endothelium is continuously exposed to fluid shear force at its apical side and this force differs along the length of a single vessel, it is evident that in addition to the aforementioned intrinsic factors, mechanical stress represents an important extrinsic factor capable of modifying regional barrier properties. Shear stress is known to alter both the organization of IEJs and cell-ECM interactions (203, 412, 851). Mechanical forces can also alter barrier properties of the endothelium by activating intracellular signaling events. Exposure of cultured cells and lung capillaries to mechanical stress resulted in increased intracellular  $\text{Ca}^{2+}$  and the generation of inositol trisphosphate ( $\text{IP}_3$ ) (485, 647, 826), activation of Rac (390, 970), RhoA-dependent reorganization of actin cytoskeleton (89, 851), and  $\beta_1$ -integrin-dependent increase in caveolin-1 phosphorylation (734; see Refs. 412 and 851). These intracellular signals induced by mechanical stress to which the vascular segments are differentially exposed may contribute to differentially modifying the barrier function of these segments.

## V. STRUCTURAL DETERMINANTS OF ENDOTHELIAL BARRIER FUNCTION

The endothelial monolayer is embedded in a complex meshwork of interacting proteins, glycoproteins, proteoglycans, and glycolipids. We discuss below the importance of these cell surface structural components in modulating endothelial barrier function.

### A. Glycocalyx

The glycocalyx is a negatively charged, surface coat of proteoglycans, glycosaminoglycans, and adsorbed plasma proteins lining the luminal surface of the endothelium (548) (see Ref. 726). The apparent thickness of the glycocalyx varies between 20 and 3,000 nm depending on the dye used (ruthenium red, alcian blue, or osmium tetroxide), detection method (electron or fluorescence microscopy), vessel type (capillaries, arterioles, or venules), and tissue (skeletal muscle or heart) (347, 400, 872, 980, 1005, 1006). The negative charge repels red blood cells (133, 200, 774, 827), suggesting that the glycocalyx can modulate oxygen delivery in a charge-dependent manner. The glycocalyx can be shed following exposure of postcapillary venules to formyl-methionyl-leucyl-phenylalanine (fMLP), a neutrophil-activating peptide (626, 627). This implies that the glycocalyx shields the endothelium from leukocyte attachment, although the role of the glycocalyx as a regulator of neutrophil-endothelial interactions has not been directly addressed.

The glycocalyx may also contribute to the overall function of the endothelial barrier by limiting the passage of macromolecules to the endothelial cell surface. The frog mesenteric capillary endothelium is twofold more permeable to the positively charged globular protein ribonuclease (molecular mass 13.7 kDa) than the same-sized negatively charged  $\alpha$ -lactalbumin (molecular mass 14.2 kDa) (6). This difference might be attributed to the anionic sites on the glycocalyx. Albumin (molecular mass 67 kDa) and fibrinogen (molecular mass 340 kDa) were found to permeate the glycocalyx at the same rate despite their threefold difference in molecular mass (1005), reflecting the albumin pI of 4.9 vs. pI of 6.1 for fibrinogen; thus the charge restriction imposed by the glycocalyx may determine accessibility of selected proteins. In electron micrographs of mouse capillaries perfused with cationic ferritin, the glycocalyx appeared to generate heterogeneous microdomains on the luminal cell surface due to a nonuniform distribution of negative charge (865). Together, these findings indicate that the glycocalyx could play a role in “gating” differentially charged macromolecules at specific regions of the endothelial cell surface. In addition, the possibility exists that the binding of macromolecules could itself alter the structure and charge dis-



tribution of the glycocalyx (398; see also Ref. 726) and thus influence the permeation of plasma proteins, such as the most abundant anionic protein albumin.

Further evidence to support the key contribution of the glycocalyx to endothelial barrier function comes from experiments disrupting the glycocalyx or alternatively neutralizing its negative charge. Degradation of the glycocalyx by pronase resulted in a 2.5-fold increase in capillary  $L_p$  in frog mesenteric arteries, independent of changes in intercellular cleft dimensions (4). A similar increase in endothelial permeability to macromolecules was reported in coronary arterioles or swine skeletal muscle following treatment with pronase (400) and/or heparinase (220). Glycocalyx disruption by photolysis (1006) or tumor necrosis factor (TNF)- $\alpha$ -activated proteolysis of hyaluronan (360) also led to increased permeability of the endothelium to macromolecules. In other studies, neutralization of the apical endothelial negative charge using cationic ferritin (561) or protamine (561, 909) increased the transendothelial permeability of  $^{125}\text{I}$ -albumin, pointing to the key role that the glycocalyx plays in maintaining endothelial permselectivity by charge-selective exclusion of plasma proteins.

On ultrastructural observation, the glycocalyx appears as a meshlike or "fiber matrix" structure with regular spacing of  $\sim 20$  nm (892). Michel and colleagues (196, 892) have used mathematical modeling to relate the fiber matrix geometric properties to measured microvascular permeability. Since the glycocalyx (termed "fiber matrix" in these studies) extends between adjacent endothelial cells and lies in series with IEJs, it has been argued that the fiber matrix represents the primary barrier to fluid and solute exchange across the vascular endothelium (196; see Ref. 592). Adamson et al. (7) showed that a sufficient oncotic pressure gradient to oppose net filtration could develop across the glycocalyx. In this model, the glycocalyx (rather than IEJs) offers the highest resistance to diffusion of solutes through the endothelial barrier. IEJs are treated as insignificant barriers even to macromolecules such as albumin, based on reconstructions of electron micrographs showing that IEJs possess discontinuous regions or breaks in their structure (see Ref. 592). However, experimental evidence for this model is still lacking. Detailed studies of various microvascular beds by electron microscopy using electron-dense macromolecular tracers, such as gold- or haptenized (dinitrophenyl-labeled) albumin, have shown that albumin reaches the IEJ cleft, but does not pass through it (719). This contrasting evidence supports the hypothesis that IEJs rather than the glycocalyx are the primary limiting factor with respect to macromolecular permeability. However, the concern remains that fixatives used in these studies may have altered or even destroyed the glycocalyx; thus these studies by themselves do not provide the most robust test of this hypothesis.

The endothelial glycocalyx may also have another function; it may function as a fluid shear-stress sensor (30, 268, 446, 1042), which may regulate the production of nitric oxide (NO) (268, 608). This finding was dependent on the heparin sulfate and hyaluronic acid constituents of the glycocalyx (268). The released NO derived from endothelial NO synthase (eNOS) may stabilize the endothelial barrier through activation of focal adhesion kinase and recruitment of additional focal adhesion complexes to the basal endothelial cell surface (920, 1094). This assertion is reinforced by findings that the number of such complexes is directly related to increased monolayer electrical resistance (539, 1040). Dull et al. (227) showed that yet another function of the endothelial cell heparan sulfate proteoglycan constituent of glycocalyx is to provoke cytoskeletal reorganization leading to barrier dysfunction. They observed that clustering of cell surface proteoglycans induced by arginine-lysine polymers, a model for neutrophil cationic peptide, increased endothelial permeability. Thus the glycocalyx is implicated in the regulation of endothelial barrier function both by the fiber matrix model (see Ref. 592) and by data demonstrating activation of intracellular signals and regulation of NO production (227, 268, 608, 920, 1094).

## B. Extracellular Matrix

The ECM consists of collagen IV, fibronectin, entactin, laminin, chondroitin sulfate, and heparan sulfates, perlecan and syndecan (reviewed in Ref. 443). It appears in cross-section as a fuzzy band 40–60 nm thick (560). In addition, ECM contains "matricellular" proteins (or matrix-associated proteins): thrombospondin (TSP) and secreted protein acidic and rich in cysteine (SPARC) (reviewed in Ref. 443). The first step in ECM assembly is the secretion by endothelial cells of laminin polymers, which bind predominantly to  $\beta_1$ -integrins (14, 502). Collagen IV polymers (also produced by the endothelium) then interact with laminin polymers in the extracellular space to form a scaffold onto which other ECM proteins are assembled to produce a basement membrane of unusually high elasticity and tensile strength (443). Endothelial cells synthesize and secrete these ECM constituents during angiogenesis and vasculogenesis (12, 269, 410, 443, 1009) and have the capacity to continuously remodel ECM in mature vessels (443, 716, 1008, 1080). The interaction of the ECM with endothelial cell surface integrins (described in sect. VIII B) generates signals that inhibit endothelial cell proliferation and migration, but stimulate cell-cell and cell-ECM adhesion (271). The latter signals are crucial for the formation of the intact endothelial barrier. Thus it is surmised that endothelial cells adherent to the normal ECM are quiescent.

A few studies have addressed the permeability characteristics of the ECM barrier itself. Qiao et al. (729)

showed that treatment of the endothelium with the lectin *Ricinus communis* agglutinin (RCA) reduced transendothelial albumin permeability by strengthening the ECM barrier. This finding was attributed to lectin modification of the ECM, which reduced ECM permeability to albumin. A similar concept appears to apply in vivo (177, 202, 1003). A 40% decrease in plasma fibronectin induced by infusing sheep with trypsin, and reflecting degradation of the ECM fibronectin, resulted in a sustained threefold increase in lung transvascular liquid clearance (177). Because fibronectin can stabilize ECM, this finding is indicative of increased pulmonary transvascular protein permeability resulting from degradation of ECM. Likewise, infusion of thrombin into sheep (202) or hydrogen peroxide ( $H_2O_2$ ) into isolated-perfused rabbit lungs (1003) caused release of fibronectin fragments into the plasma or lymph (202) and perfusate (1003) in association with increased endothelial permeability (202, 1003). The release of fibronectin from ECM coupled with increased permeability provides correlative evidence that remodeling of ECM may result in endothelial barrier dysfunction.

In cultured endothelial monolayers, the release of fibronectin from ECM was also associated with two- to threefold increases in endothelial permeability to albumin (684, 751). In some cases, the increases in endothelial monolayer permeability were prevented by reincorporation of plasma fibronectin into ECM (199, 751, 1049, 1050), demonstrating that ECM fibronectin confers a barrier-protective property by maintaining the integrity of ECM. Other ECM protein constituents were also shown to support endothelial barrier function. Degradation of hyaluran

by hyaluronidase increased permeability of endothelial monolayers as the result of increased  $L_p$  of ECM, suggesting that loss of ECM hyaluran can disrupt the endothelial barrier to liquid (730) (Fig. 4A), but “add-back” experiments have not been carried out as they have for fibronectin. Disruption of proteoglycans of the ECM by elastase has been shown to produce pulmonary edema (687). Enzymatic degradation of collagens, proteoglycans, and fibronectin by the zinc-dependent metalloproteases (MMPs), gelatinase A (MMP-2) and gelatinase B (MMP-9), increased permeability of cultured endothelial cell monolayers (685) (Fig. 4B) and also induced edema in rabbit lungs (688). These findings collectively support the role of ECM as being crucial in regulating barrier properties of the endothelium.

The ECM also plays an important role in remodeling the endothelium after permeability-increasing mediators have disrupted its integrity. This is achieved by the “counteradhesive” proteins such as SPARC (414, 449) and MMPs (1111). VEGF and TNF- $\alpha$  stimulated the production of MMP-2 and -9 and SPARC in endothelial cells (449, 493, 685, 1111). The induced proteins denuded regions of the endothelial-lining layer by degrading the ECM (449, 1111). Endothelial cells surrounding the denuded area expressed a modified set of integrins for the “free” ECM proteins (see review in Ref. 443). Upon binding to integrins, the ECM proteins induced the activation of focal adhesion kinase and mitogen-activated kinase (107, 282, 682, 798, 876) and Rho GTPases (RhoA, Rac, and Cdc42) (41, 132, 217, 682) that facilitated endothelial cell migration and proliferation (645; see also Refs. 217, 645, and

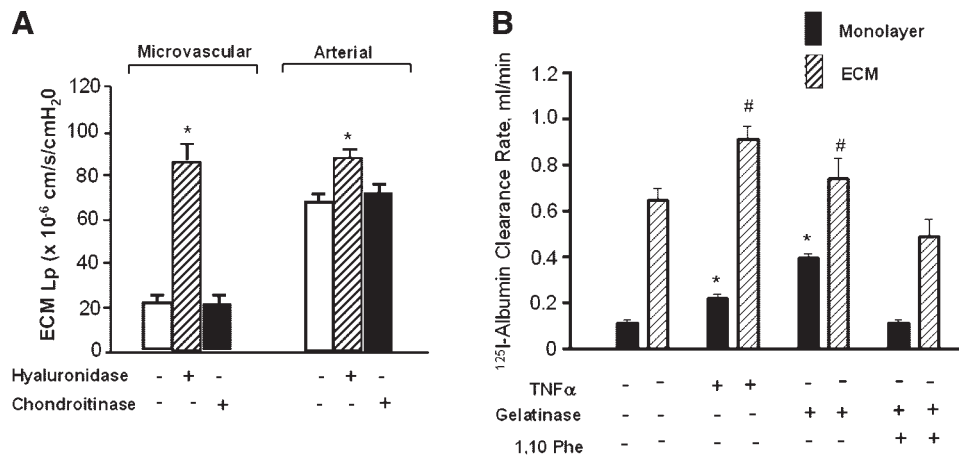


FIG. 4. Endothelial-derived extracellular matrix (ECM) as a determinant of endothelial barrier function. A: degradation of hyaluronan with *Streptomyces* hyaluronidase (6 U/ml for 10 min) significantly increases ECM hydraulic conductivity ( $L_p$ ) of bovine endothelial monolayer. The  $L_p$  response was greater in cells from microvascular segment. Also note that microvascular monolayer forms a tight barrier as revealed by lower basal  $L_p$  compared with arterial monolayer. However, treatment with chondroitinase C, which cleaves chondroitin, did not alter ECM  $L_p$ . These results indicate that the ECM hyaluronan provides a significant barrier to macromolecules. \*Significantly different. [Adapted from Qiao et al. (730).] B: ECM stripped from bovine pulmonary endothelial monolayer (BPMVE) as well as total cell monolayer displayed an increase in  $^{125}I$ -albumin permeability after incubation with gelatinase (membrane metalloprotease, MMP-9) compared with untreated BPMVE. The response was similar to that induced by tumor necrosis factor (TNF)- $\alpha$  exposure. Inhibition of gelatinase with 1,10-phenanthroline (1,10-Phe) reversed the increased permeability of ECM as well as cell monolayer to  $^{125}I$ -albumin, thus indicating a role of MMP-9 in regulating endothelial monolayer integrity. #Significant increase above basal value for total cell monolayer and ECM, respectively. [Modified from Patridge et al. (685).]

756). The regenerated endothelium thereby laid down a new ECM completing the repair process (reviewed in Ref. 519).

ECM is also capable of remodeling the endothelium in response to shear stress acting on the endothelial cell surface. Jalali et al. (426) showed that endothelial cell alignment in response to shear stress required endothelium-derived ECM as a growth substrate. Effective mechanotransduction required the ECM constituents, vitronectin and fibronectin, and the association of Src homology containing (Shc) protein with integrins. Shc activation is probably important in reorganizing the endothelial cell cytoskeleton and focal adhesion complexes in response to shear stress (see Ref. 412). Thus ECM contributes to regulating endothelial integrity and barrier function by orchestrating signaling cues that favor cell adhesion over cell proliferation. It will be important to address whether specific cell-ECM attachments also have a role in triggering IEJs assembly, thus promoting the expression of adherens junctional proteins and contributing to junctional integrity.

### C. Vesiculo-Vacuolar Organelles

Vesiculo-vacuolar organelles (VVOs), described by Dvorak and colleagues (470), are grapelike clusters of interconnected, uncoated vesicles, and vacuoles present in the continuous endothelia lining venules, small veins, and tumor vessels (Fig. 5A) (470, 546; see also Ref. 257). VVOs are enormous cytoplasmic structures that can vary from 80 to 140 nm in diameter (258). VVOs consist of 79–362 vesicles or vacuoles that are 1–2  $\mu\text{m}$  at their

longest dimension and collectively occupy 16–18% of the venular endothelial cytoplasm. The individual vesicles and vacuoles are substantially larger than caveolae (diameter of 70 nm on average) of capillary endothelial cells. Unlike caveolae, however, VVOs are sessile structures that can assemble into transcellular membranous channels in some instances. These channels open into the luminal, abluminal, as well as the lateral endothelial cell surface (257), but their function in regulating junctional permeability is unknown. VVOs are thought to contain caveolin-1, but VVOs appear normal at the ultrastructural level in caveolin-1-deficient mice (see Refs. 257 and 601), suggesting that the assembly of VVOs probably does not require the presence of caveolin-1.

The channel-forming VVOs provide a transcellular permeability pathway to macromolecules such as tracer ferritin (258) because intradermal injection of VEGF and serotonin increased the concentration of ferritin tracer in VVOs and subendothelial space (Fig. 5B). Reconstruction of venular endothelial cells serial electron micrographs from mouse skin further showed that VEGF opened a transcellular permeability pathway via VVOs with patent stomata rather than through IEJs (258). Michel and Neal (593) also showed that VEGF increased macromolecular uptake by a transcellular canicular route. VEGF increased  $L_p$  in isolated coronary venules (54) and isolated perfused lungs (429), indicating that the acute administration of VEGF is capable of increasing microvessel permeability. However, the contribution of transcellular transport of albumin via VVOs and the mechanism of VVO formation in these preparations remain to be investigated.

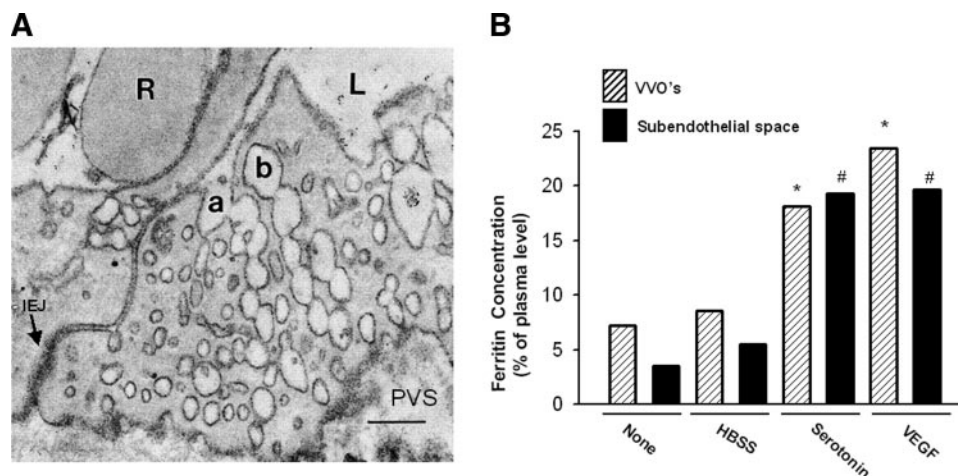


FIG. 5. Vesiculo-vacuolar organelles (VVOs) form transcellular channels. A: 14-nm-thick serial sections through a single VVO in normal mouse skin endothelia shows two sequences (*a* and *b*) of interconnecting VVOs. A closed IEJ is also visible at the left side of panel (arrow), supporting transport via the transcellular route. R, red blood cell; L, lumen; PVS, perivascular space. Bar = 200 nm. [Adapted from Feng et al. (258).] B: amount of tracer ferritin in VVOs and immediate subendothelial space as percent of plasma concentration in normal unstimulated (None) mouse skin compared with skin injected with Hanks' balanced salt solution (HBSS), serotonin, or vascular endothelial growth factor (VEGF). Injection of either serotonin or VEGF increased ferritin concentration in VVOs and subendothelial space, indicating VVOs can provide a route for plasma proteins to cross the endothelium in response to permeability increasing mediators. \*, #Significant increase above none or HBSS for VVOs and subendothelial space, respectively. [Adapted from Feng et al. (258).]



Continuous vesicles within VVOs make contact at stomata (230, 895) that are either patent or closed by diaphragms comprising the glycoprotein PV1 (895). Stan et al. (894) showed by electron microscopy that diaphragms have a central density or "knob." These stomatal diaphragms may act as a barrier between the luminal and abluminal fronts of endothelial cells (258). Permeability-increasing mediators, VEGF, serotonin, and histamine, caused the tracer ferritin to accumulate in VVOs with open diaphragms and in the subendothelial space (258). Diaphragms of VVOs are apparently subject to regulation and may be involved in increased albumin permeability, but the mechanisms of their opening and closing as well as their role in regulating transcellular permeability via VVOs have yet to be identified.

#### D. Development of the Endothelial Barrier

Embryonic precursor cells (EPCs), also known as angioblasts, and hematopoietic cells originate from bipotential stem cells known as hemangioblasts (reviewed in Ref. 33). The origin of these precursor cells is still a matter of debate (344, 802, 977). However, it is known that they are formed extra-embryonically in the yolk sac mesoderm from cell clusters, termed blood islands, probably in response to the endodermally-derived signals VEGF-A (604) and Indian Hedgehog (233) (for reviews see Refs. 100 and 642). Pluripotent stem cells differentiate into hemangioblasts giving rise to an intermediate preendothelial cell type that can differentiate into either a committed cell of the hematopoietic lineage or an endothelial cell (Fig. 6). The molecular determinants of the fate of hemangioblasts are not yet fully elucidated. Evidence thus far indicates that fibroblast growth factor (FGF)-2 is an important mediator responsible for induction of endothelial precursor cells from the mesoderm (see Ref. 714).

EPCs express fetal liver kinase-1 (Flk-1), the type II receptor for VEGF, as the earliest known marker of endothelial cells, evident in mesodermal cells as early as 8 days post coitum (598, 1078). Expression of *fms*-like tyrosine kinase-1 (Flt-1) occurred later during development. As embryonic development progresses, expression of Flk-1 is increasingly restricted to endothelial cells. In response to VEGF, these receptors signal proliferation and migration of EPCs within the ECM resulting in the formation of the primitive vascular plexus through a process termed vasculogenesis (832) (see also Ref. 172). This incipient vasculature is extended by capillaries that sprout from a preexisting vascular network; this process is termed angiogenesis. Angiogenesis results in a characteristically elongated and highly branched vascular plexus (759, 760). In mice, deletion of Flk-1 gene is lethal at embryonic day 8.5 due to lack of hematopoietic and endothelial lineage development (141, 261, 832). Deletion of

Flt-1 on the other hand allows differentiation of endothelial cells, but these cells do not form cell-cell contacts, and the embryos do not survive beyond day 9 because of an inability to develop a functional endothelial barrier (270). The Flk-1-expressing mesodermal cells also have the capacity to differentiate into smooth muscle cells in response to platelet-derived growth factor (PDGF) (1079). Another receptor expressed in EPCs is tyrosine kinase with immunoglobulin-like hoops and epidermal growth factor homology domain-2 (Tie-2), the receptor for angiogenic growth factors angiopoietins (Ang-1 and Ang-2). Loss of Ang-1 or overexpression of Ang-2 impairs normal maturation and stabilization of the embryonic vascular network (559, 910), indicating Ang-1 and Ang-2 have opposing effects on vascular development. EPCs subsequently expressed VE-cadherin and AC133, a novel antigen specifically induced in EPCs (127, 694). AC133 expression disappears once EPCs differentiated into mature cells, and proliferation of endothelial cells virtually ceases at this point and is thereafter absent in the adult (694).

The high proliferation rate of EPCs may distinguish them from endothelial cells that are shed from the vessel wall (33). The proliferative capacity of endothelial cells can be reactivated in response to certain physiological stimuli (e.g., acute wound healing, angiogenesis, cycling endometrium, pregnancy) or pathological stimuli (e.g., tumor growth, rheumatoid arthritis) (759) (Fig. 6). Under these conditions, EPCs can also contribute to vessel growth. However, the relative contributions of EPCs and preexisting endothelial cells to the repair of damaged blood vessel are still unclear (see Ref. 556). Because EPCs have the potential to differentiate in situ into endothelial cells, they may induce vasculogenesis resulting in neovascularization (33). Therefore, this may be an important strategy for reannealing an injured endothelial barrier resulting from inflammation and could thereby restore the vascular integrity and basal level of permeability (165, 365, 556, 1002).

Angiogenic and coagulation factors, proteinases and their inhibitors, junctional adhesion molecules, and ECM proteins and their receptors contribute in a complex way to the mechanism of angiogenesis (see Ref. 556). For example, MMPs and plasminogen activators (urokinase-type plasminogen activator and its inhibitor plasminogen activator inhibitor-1) signal formation of vessels by degrading ECM, which results in the liberation of matrix-bound VEGF and proteolytic activation of chemokines such as interleukin-1 $\beta$  (47, 67, 888). Angiogenesis was inhibited in mice lacking plasminogen or MMP-2 (47, 423). Factors released during intravascular coagulation also were shown to induce angiogenesis (140, 259, 663, 753). Fibrin deposition signaled the migration of endothelial cells, thus inducing angiogenesis (754). Activation of platelets also led to a release of angiogenic factors VEGF,



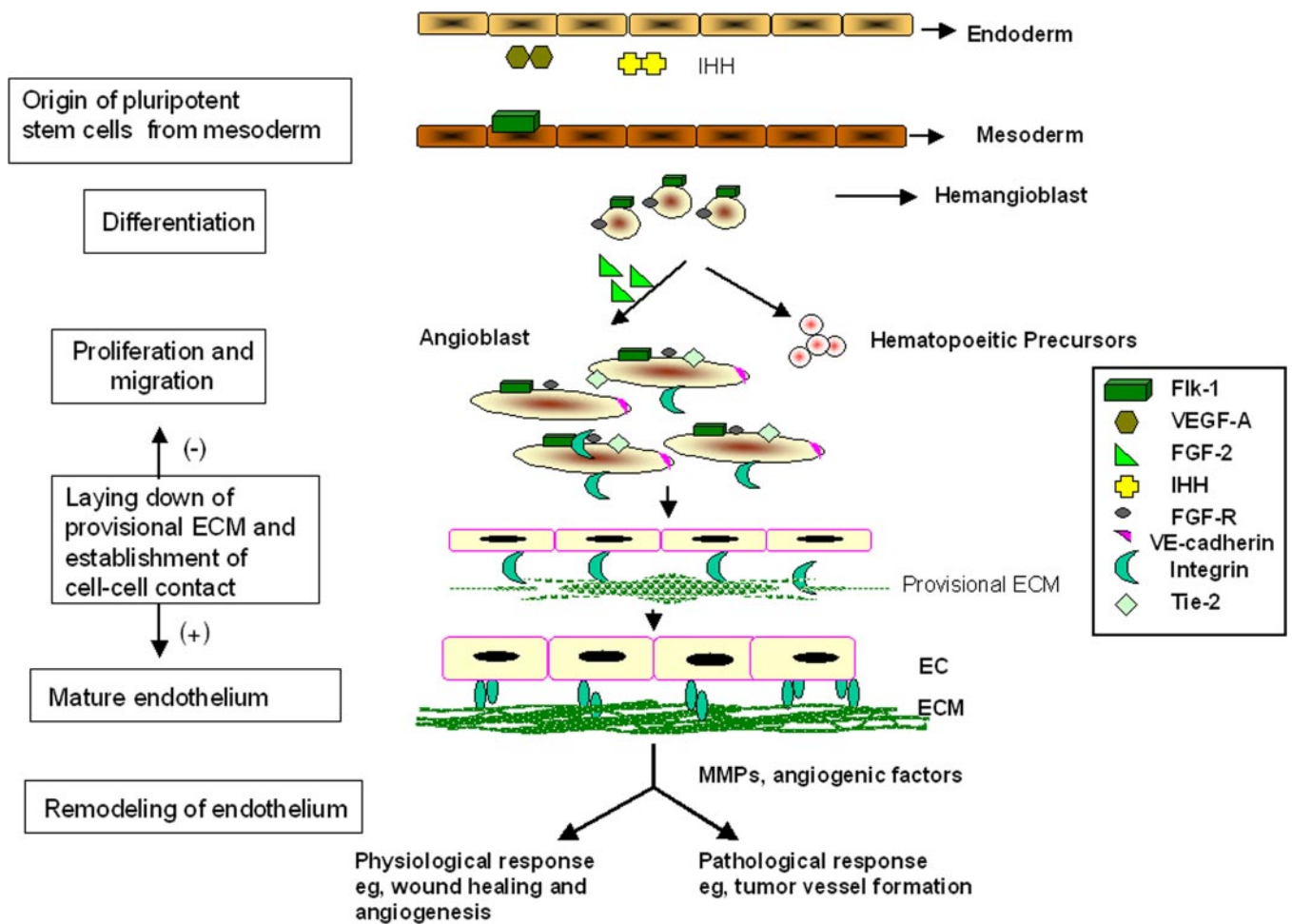


FIG. 6. Model depicting development of endothelial barrier. Pluripotent stem cells form blood islands in the extraembryonic yolk sac splanchnic mesoderm in response to endodermally derived signals, VEGF-A and Indian Hedgehog (IHH). These cells differentiate into bipotential stem cells known as hemangioblasts. Hemangioblasts, in response to fibroblast growth factor (FGF)-2, give rise to an intermediate preendothelial cell type that can differentiate into either committed hematopoietic lineage cells or endothelial precursor cells (EPCs) also known as angioblasts. Expression of fms-like tyrosine kinase-1 (Flt-1), tyrosine kinase with immunoglobulin like hoops and epidermal growth factor homology domain-2 (Tie-2), the receptor for angiogenic growth factors angiopoietins (Ang-1 and Ang-2), VE-cadherin, and integrins occurs later in development on these committed cells. In response to VEGF, the activation of Flt-1, Tie-2, and Ang-1 receptors signal proliferation. After proliferation, endothelial cells migrate within ECM and establish cell-cell contacts that inhibit proliferation (-) while permitting formation of the characteristic endothelial barrier. In response to wound healing or angiogenesis, endothelium is remodeled by activation of MMPs, which degrade ECM resulting in cell detachment and disruption of IEJs. ECM degradation leads to liberation of matrix-bound VEGF, again stimulating proliferation. Platelets also release angiogenic factors such as tumor growth factor- $\beta$ , thrombin, and sphingosine-1-phosphate. These mediators send signaling cues necessary for endothelial cell migration and proliferation. This is followed by laying down of new ECM, which results in wound repair or formation of new vessels (angiogenesis). Proliferating endothelial cells may also generate tumor vessels in response to specific pathological stimuli (described in text). EC, endothelial cells; FGF-R, fibroblast growth factor receptor. See text for details. [Modified from Bohnsack and Hirschi (100).]

PDGF, tumor growth factor- $\beta$ , thrombin, and sphingosine-1-phosphate (S1P) (reviewed in Ref. 122). These mediators may send the signaling cues necessary for angiogenesis by inducing the disruption of IEJs and ECM (212, 634, 780, 1060, 1111; see also Ref. 212).

Interestingly, S1P also has an important endothelial barrier protective effect (300, 511, 780, 794). When administered along with thrombin, S1P suppressed thrombin's effect of increasing endothelial permeability and restored barrier function (300, 794). Similarly, VEGF and Ang-1 cooperate in the formation of blood vessels (see Refs. 293 and 910). However, unlike VEGF, which is known to

increase endothelial permeability, Ang-1 is implicated in inducing the formation of a restrictive barrier (295, 943). In the adult vasculature, Ang-1 opposed VEGF-induced increase in endothelial permeability (295, 942). Overexpression of Ang-1 in skin of adult mice stimulated the growth of nonleaky vessels (943). These findings raise the possibility that S1P or Ang-1 may link angiogenesis with the formation of a stable endothelial barrier. Furthermore, these results point to the potential role of these and other mediators [such as ATP (472)] in reducing endothelial permeability and acting as anti-inflammatory agents (discussed in sect. XIII B).

The neovascularization arising in response to physiological events such as pregnancy differs from the processes forming microvessels in response to pathological stimuli in tumor-driven angiogenesis (556). Tumor vessels are described as “highly disordered, tortuous, dilated and leaky” (556), and it is apparent that the function of VEGF is upregulated in these vessels (231). The contribution of mediators involved in angiogenesis (discussed above) and the formation of tumor microvasculature is an area of intense investigation, and as such it is beyond the scope of this review. It has been extensively covered recently in an up-to-date review (556).

## VI. PHYSIOLOGICAL SIGNIFICANCE OF ALBUMIN PERMEABILITY

The concentration of albumin in human plasma is 3 g/100 ml (~60% of total protein), making albumin the most abundant plasma protein. In addition, the molecular structure and charge ( $pI = 4.9$ ) of albumin facilitates the cotransport of a number of hydrophobic molecules, enzymes, and hormones across the endothelium. In this section, we review the significance of the permeability of the endothelium to albumin.

### A. Albumin Regulation of Tissue Oncotic Pressure and Endothelial Barrier Integrity

The oncotic pressure generated by plasma proteins ( $\Pi_c = \sim 25$  mmHg) is a key factor in maintaining fluid balance across capillaries (499).  $\Pi_c$  plays an important role in fluid reabsorption across the capillary wall, as the plasma protein concentration is greater in vessels than the interstitial space. Plasma albumin accounts for 65% of  $\Pi_c$ , and other plasma proteins, e.g., globulins and fibrinogen, contribute according to their concentrations (71, 1044). Albumin has a plasma half-life of 15–19 days (reviewed in Refs. 701 and 702) and thus must be replaced via resynthesis to maintain  $\Pi_c$ . Interestingly, like IgG, the albumin was shown to bind the major histocompatibility complex-related Fc receptor (FcRn) at low pH and be shielded from degradation, which significantly prolonged the plasma half-life of both proteins (156). Extravasated albumin is recycled into the general circulation by lymphatic vessels, and albumin newly synthesized by hepatocytes is secreted into the circulation (at the rate of 15 g/day in humans) (703). Plasma albumin moves into the extravascular space by crossing the microvessel barrier and entering the interstitial tissue where it serves as the chief interstitial oncotic agent. The endothelial cell layer thus regulates the transport of albumin into the interstitium and in this manner controls the transendothelial oncotic pressure gradient ( $\Pi_c - \Pi_i$ ), the difference be-

tween  $\Pi_c$  and tissue oncotic  $\Pi_i$  pressures, the principle Starling force responsible for fluid reabsorption.

Plasma albumin also has additional functions in mediating endothelial barrier stability (397, 399). This notion is supported by studies in which removal of albumin from the perfusate resulted in a ~1.5-fold increase in capillary wall  $L_p$  (932). Albumin contributes to the maintenance of endothelial barrier function by interacting with the glycocalyx (397, 399) as shown by the finding that loss of adsorbed albumin from the glycocalyx increased the transport of tracer ferritin across the endothelium (808). The basis for this is not totally clear. Albumin's interaction with ECM proteins may also regulate endothelial barrier properties. Kajimura et al. (442) showed that removal of albumin from the perfusate increased  $L_p$  of microvessels and permeability to  $\alpha$ -lactalbumin, supporting a role for albumin in regulating endothelial barrier integrity. However, these may not be unique functions of albumin. Apparently, analbuminemic humans and rats have a normal fluid balance. They have normal  $\Pi_c$  and  $\Pi_i$  values and  $\Pi_c - \Pi_i$  gradient, since they compensate by increasing the production of other proteins (102, 435, 436, 633, 750, 907, 933, 1036). Ultrastructurally, microvessels from analbuminemic rats also appeared normal (D. Predescu, unpublished observations). Compensatory mechanisms increased the production of  $\alpha$ -macroglobulins, immunoglobulin G, and fibrinogen (239) and may thus be able to maintain the  $\Pi_c - \Pi_i$  gradient and restore fluid balance and endothelial barrier integrity.

### B. Albumin as a Chaperone

Albumin has a cargo chaperone function as it binds to many substances in the plasma and facilitates their delivery across the vessel wall barrier. It is not clear whether albumin is cotransported with its cargo molecules in all cases or whether albumin is involved in the transfer of hydrophobic cargo molecules to specific binding proteins on the endothelial cell surface. In the case of fatty acids, evidence favors the latter mechanism (see Ref. 979). Permeation of free fatty acid-conjugated albumin via transcytosis was threefold greater compared with delipidated albumin (28, 294), perhaps on the basis of higher affinity binding of lipidated-albumin to the endothelial cell surface (294). Free fatty acids and other lipids such as S1P conjugated to albumin are required for many vital functions (600, 703, 1087); they serve as an energy source in muscle tissue, substrates in surfactant production and lipid synthesis in lung and adipose tissues, and provide cues in development and tissue patterning as in the case of S1P. Albumin also plays an important role in the transport of drugs such as digoxin to target organs (662). These phenomena have an important clinical impact on drug efficacy, particularly because they have relatively narrow

therapeutic indexes (74). Albumin has also been shown to be a carrier protein for the amino acid tryptophan (675). There is some evidence that albumin also acts as a carrier for thyroid hormone, transporting it across the capillary endothelium (825). These studies showed that endothelial cells take up albumin-bound thyroxin, which after exocytosis, dissociated from its carrier in the pericapillary space, thereby providing free thyroxin to target organs (359).

### C. Other Functions of Albumin

A recent study by Siddiqui et al. (852) using a protein alignment algorithm has shown a structural homology between regions of human albumin and human transforming growth factor (TGF)- $\beta$ 1. In mature human TGF- $\beta$ 1, a 112-residue peptide has marked homology with the human albumin amino acid sequence. A similar 26% homology exists with mouse albumin and mouse TGF- $\beta$ 1 for a 53-amino acid stretch (852). The implications of this finding are not yet clear, but it is possible that albumin has an important “cytokine-like activity” at low levels, a function distinct from its role as a carrier protein and oncotic agent. Intriguingly, Tirupathi et al. (954) showed that albumin mediates the transport of myeloperoxidase (MPO) in endothelial cells by vesicular transcytosis. As MPO plays an important role in host defense and inflammation by regulating the generation of NO-derived oxidants and protein nitrotyrosine formation (see Ref. 116), these findings suggest that the functional consequences of albumin endocytosis go beyond delivery of drugs, hormones, fatty acids, and amino acids into tissue. Albumin-mediated transcytosis may represent a key host-defense mechanism, and as such, interfering with albumin transcytosis may perturb this mechanism and contribute to tissue inflammation in a setting of sepsis.

## VII. ALBUMIN TRANSPORT PATHWAYS

### A. Pore Theory Versus Transcellular Pathway

The “pore model” postulated by Pappenheimer (674) and Grotte (342) has over the years served as a convenient means to describe transendothelial permeability to plasma proteins. The two-pore model describes a very small population of “large pores” (pore radii of 25–30 nm) that accounts for the transport of serum albumin and other plasma proteins across the endothelial barrier. A theoretical estimate of the large pore fraction according to the model is only 0.003–0.1% of that of the small pores (591; for review, see Ref. 592). Considerable attention has been focused on resolving the nature of the large pore system. While this model has proven useful in describing endothelial permeability, large pores (perhaps because of their paucity) have not been detected in the extensive ultrastructural studies carried out (719). Curry and Michel (196) proposed the fiber-matrix model (described in sect. vA) that ascribed molecular sieving properties to the endothelial glycocalyx region in series with IEJs. This model avoids altogether the issue of pores as discrete structural features of the endothelium (reviewed in Ref. 592). However, ultrastructural studies showed that electron-opaque protein tracers (of albumin and other proteins) in transit through the endothelial barrier did not in fact label IEJs. Electron microscopic analysis detected intravascular albumin tracers (11–15 nm diameter gold-labeled or haptenized albumin) on the luminal endothelial membrane, in vesicles or the interstitial space (719). The albumin tracers exited the microcirculation via plasmalemmal vesicles (595, 719) (Fig. 7). As the radius of the neck region of vesicles ( $\sim$ 25 nm) approximates the dimension of large pores (719), it has been proposed that caveolae constitute the postulated large pore system.

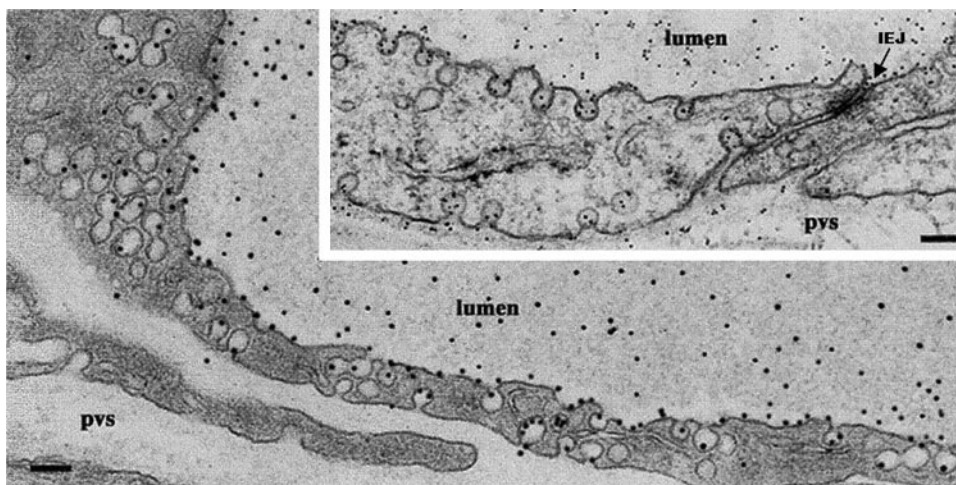


FIG. 7. Transcytosis of dinitrophenyl-conjugated albumin (A-DNP) tracer in lung microvessels. Main electron micrograph: mouse postcapillary venule (bar = 100 nm). *Inset*: pulmonary capillary (bar = 80 nm). Note the marked labeling with A-DNP (diameter 12 nm) of endothelial caveolae in both microvessels at various stages of albumin transcytosis and absence of tracer molecule in well-demarcated IEJs (*inset*). [Adapted from Predescu et al. (721).]



The concept of transcapillary transport of albumin via vesicles has been challenged on various grounds (see Ref. 757). Morphological evidence based on a three-dimensional reconstruction of vesicles failed to establish rigorously the presence of free vesicles within cells. It appeared rather that the vesicles were sessile, interconnected, and continuous with the interstitial space (128). Labeling of vesicles with tracers, the primary evidence of vesicle-mediated transport of macromolecules, could theoretically be attributed to back-diffusion of tracers by abluminal endocytosis after tracer permeation through intercellular junctions (757). Theoretical modeling also suggested that vesicular transcytosis could not account for the observed convective transport of macromolecules through endothelia (95, 209, 350). Finally, the large radii of the caveolae necks may preclude one size-selective macromolecule transport observed in capillaries (592).

Although proponents of vesicular transcytosis have not ruled out all of the arguments mentioned above, this pathway is now much less enigmatic. Wagner and Chen (1021) addressed the issue of vesicle backfilling with tracer into vesicles by following the extravasation of the electron-opaque tracer terbium in capillaries in rete mirabile of eel. Using serial electron microscopy to follow

tracer exit from vessels, they demonstrated that terbium deposits in the interstitium were continuous with endothelial vesicles open to the interstitium rather than to IEJ clefts. Other strategies have also been employed to delineate the role of caveolae in transcytosis, such as cholesterol-binding agents (e.g., filipin or methyl  $\beta$ -cyclodextrin), which prevent formation of caveolae by sequestering cholesterol (433, 603, 816, 1010); uptake of markers for caveolae-mediated endocytosis such as cholera toxin-B, which binds to the caveolae membrane-constituent ganglioside M (433); caveolae-specific antibody TX 3.833 (809); and depletion of the cell surface albumin binding protein gp60 required for caveolae-mediated albumin transport (433, 603, 859, 948, 955, 1010, 1012). These approaches have demonstrated an active transcytosis pathway in endothelial cells that is dependent on caveolae trafficking. Activation of the albumin-binding protein gp60 by cross-linking in cultured endothelial monolayers and in situ increased transendothelial albumin permeability by two- to threefold (Fig. 8, A and B), whereas cell surface depletion of gp60 or pretreatment of cells with methyl  $\beta$ -cyclodextrin (to disrupt caveolae by binding to cholesterol) prevented albumin transport (603) (Fig. 8A). However, gp60 activation had no effect on  $K_{f,c}$

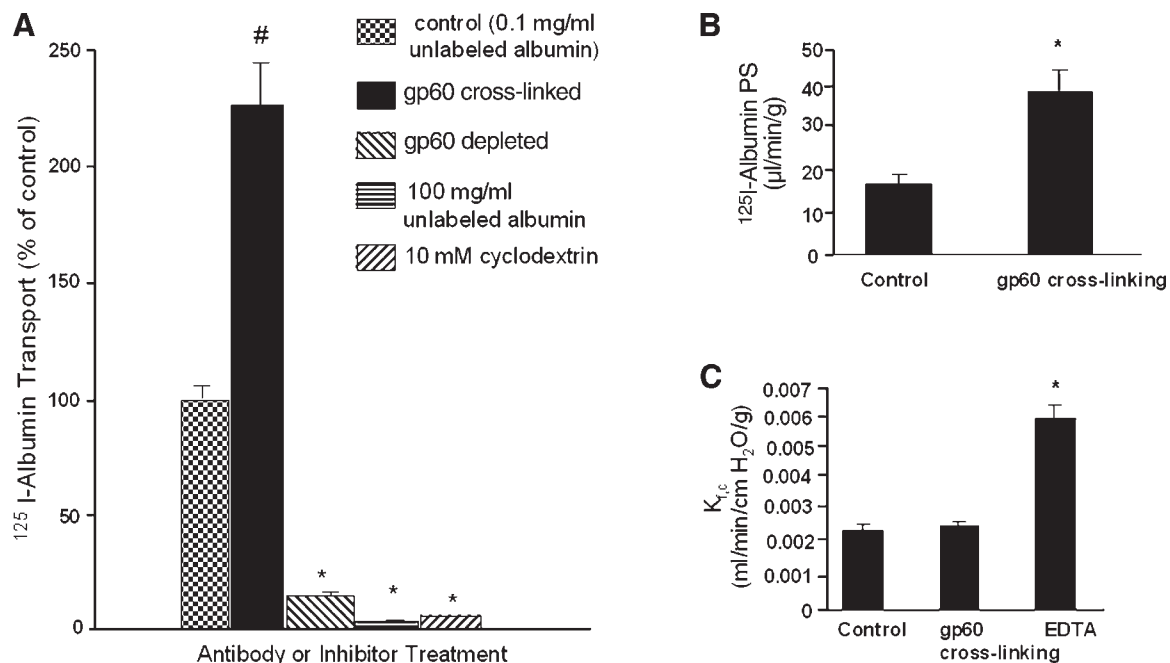


FIG. 8. Role of albumin-binding protein gp60 in regulation of transendothelial albumin transport. **A:** activation of gp60 by anti-gp60 antibody followed by a secondary cross-linking IgG (as described in Ref. 955) increased transendothelial  $^{125}I$ -albumin permeability 2- to 3-fold over a control value of  $38 \pm 15 \text{ nl} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$  (in the presence of 0.1 mg/ml unlabeled albumin), whereas cell surface depletion of gp60 reduced  $^{125}I$ -albumin transport 85%. Transendothelial  $^{125}I$ -albumin flux was also blocked when cells were coincubated with 1.5 mM unlabeled albumin (100 mg/ml) or 10 mM methyl  $\beta$ -cyclodextrin, known to sequester cholesterol, a major lipid component of caveolae. Significant increase (#) or decrease (\*) compared with control values. [Modified from Minshall et al. (603).] **B:** gp60 cross-linking (as in A) also increased albumin transport in Krebs-perfused rat lung microvessels by 2- to 3-fold as determined by measuring  $^{125}I$ -albumin permeability-surface area product. \*Significantly higher than control. **C:** chelation of  $Ca^{2+}$  with EDTA (known to open IEJs), increased capillary filtration coefficient ( $K_{f,c}$ ) in lungs, a response not seen following gp60 cross-linking; thus gp60 activation uncouples albumin permeability from liquid permeability, such that albumin is transported via a nonhydraulic pathway. \*Significantly higher than control or gp60. [Adapted from Vogel et al. (1012).]



indicating that gp60 did not influence liquid permeability (1012) (Fig. 8C). Inhibition of dynamin GTPase function (656, 830) or overexpression of intersectin (724), which was shown to block release of caveolae from the plasma membrane, also prevented albumin transcytosis in endothelial cells (described in sect. x, B1 and B2). These findings from cultured cells and lung microvessels provide support for the role of caveolae as the essential vesicle carriers responsible for albumin transport in endothelial cells.

Studies of caveolin-1 knockout mice (*Cav-1*<sup>-/-</sup>) in which the key structural and signaling protein of caveolae, caveolin-1, is deleted (223, 740, 741, 821) showed a loss of caveolae and of vesicular albumin transport, further supporting the vesicle-based nature of transendothelial albumin transport (Fig. 9). Interestingly, IEJs in capillaries and venules were open in *Cav-1*<sup>-/-</sup> mice and capable of transporting albumin (584, 721, 822). Although open IEJs in *Cav-1*<sup>-/-</sup> mice may represent a compensatory adjustment, they also raise the possibility that caveolae contribute to regulating paracellular endothelial permeability, perhaps by recycling IEJ components to the junctions as well as by regulating the turnover of focal adhesions. For example, dynamin, the large GTPase required for internalization of caveolae (described in sect. xB1), has also been shown to be necessary for the targeting of cadherin-containing vesicles to the junctions (13) as well as turnover of focal adhesion by FAK (250). Likewise, membrane traffic is required for caveolae- and dynamin-mediated endocytosis of the tight junction protein occludin (842). Interestingly, in nonendothelial cells, the down-regulation of caveolin-1 resulted in the dissociation of  $\beta$ -catenin from E-cadherin (547). These studies suggest

that caveolae in endothelial cells may play a role in controlling the paracellular IEJ pathway; thus it is possible that the vesicular and junctional pathways may function cooperatively to control endothelial permeability.

Although the role of caveolae in regulating endothelial permeability is far from clear, there is now at least the prospect that progress can be made using *Cav-1*<sup>-/-</sup> mice under stress such as with edemagenic agents that activate the endothelium. Additionally other approaches such as fluorescence resonance energy transfer (FRET) employing fluorescent-tagged caveolae-specific probes (21, 931) will allow the visualization and quantification of albumin transcytosis in intact microvessels under physiological and pathological conditions.

## B. Paracellular Pathway

Endothelial permeability to plasma proteins and liquid is increased in inflammation, a condition manifested by protein-rich edema. Majno and Palade (560) followed the uptake of colloidal mercuric sulfide in rat cremaster muscle microvessels after a subcutaneous injection of histamine and serotonin to determine the pathway responsible for increased endothelial permeability. They found deposits of the tracer between endothelial cells of postcapillary venules (7–8  $\mu\text{m}$  in diameter) dependent on formation of 0.1–0.8- $\mu\text{m}$ -wide gaps along IEJs. The defect was pinpointed to the venular endothelium since the smaller 3- to 5- $\mu\text{m}$ -diameter capillaries were not affected. The paracellular pathway allowed transport of albumin and other plasma proteins from the luminal to abluminal side of the endothelium through gaps formed by the open

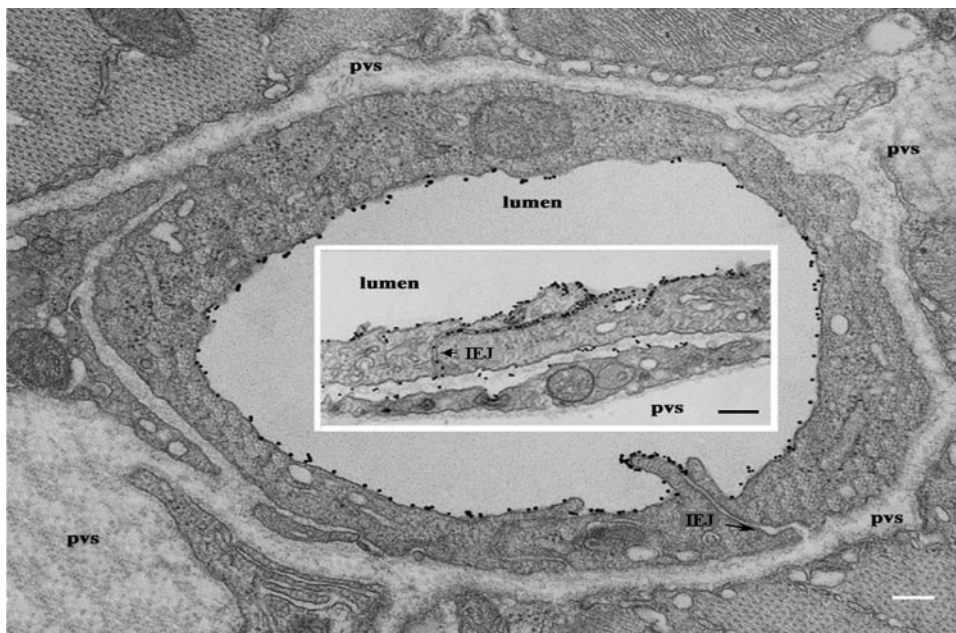


FIG. 9. Phenotype of microvessels in *Cav-1*<sup>-/-</sup> mice. *Cav-1*<sup>-/-</sup> mice were perfused with albumin-gold tracer (Au diameter  $\sim 12$  nm) for 15 min, and tissues were prepared for electron microscopy. Note absence of caveolae in this microvessel segment (A) and atypical presence of tracer in perivascular space (pvs) in B at the level of IEJs. Bar = 525 nm. [Adapted from Predescu et al. (721).]

ing of junctions between endothelial cells (see Fig. 9). It is now clear that many permeability-increasing mediators exert their action by this mechanism; e.g., platelet activating factor (PAF) (718) and VEGF (763) increased endothelial permeability by opening IEJs. The general concepts established by Majno and Palade (560) remain valid, although it is now understood that inflammatory mediators can increase paracellular uptake and transport of macromolecules in the venular as well as capillary endothelium (718, 763). The specific distribution of receptors for the agonists likely determines the sites of gap formation in different endothelial cells. A number of other studies in cultured cells and intact vessels using interventions that directly affect the integrity of IEJs, such as EDTA, anti-VE-cadherin blocking antibody, or inflammatory mediators (e.g., thrombin), have provided evidence of the importance of the paracellular albumin transport in different-sized microvessels (188, 296, 1010, 1011).

### VIII. REGULATION OF ENDOTHELIAL PERMEABILITY VIA JUNCTIONS AND MATRIX INTERACTIONS

The IEJ proteins and integrin receptors provide the endothelial monolayer with adhesive strength to resist separation of cells from the substratum and adjoining cells through their link with the actin cytoskeleton (Fig. 10). This is a primary factor in the albumin-impermeable nature of IEJs under basal conditions (Figs. 1, 7, and 13, *inset*). Given the finding in various cell types including endothelial cells that proteins binding to IEJs also interact with ECM-linked proteins (reviewed in Refs. 57, 216, and 310), the effects of IEJ and ECM components on endothelial barrier function may be additive. In the following section, we discuss the roles of IEJs and ECM proteins in the regulation of the paracellular permeability pathway.

#### A. Functions of IEJ Proteins

Endothelial cells are connected to each other by a complex set of junctional proteins that comprise tight junctions (TJs), adherens junctions (AJs), and gap junctions (GJs). Whereas GJs form transmembrane channels between contiguous cells, TJs and AJs form pericellular zipperlike structures along the cell border through their transmembrane homophilic adhesion (see Ref. 57). We discuss below the role of endothelial junctional complexes in the regulation of endothelial permeability.

##### 1. AJs

AJs identified ultrastructurally as areas of endothelial cell membranes in close apposition (1014, 1057) are of fundamental importance in regulating endothelial barrier

function (see Ref. 57). Endothelial AJs contain vascular endothelial (VE)-cadherin as the major structural protein that mediates homophilic binding and adhesion of adjacent cells in a  $\text{Ca}^{2+}$ -dependent manner. The extracellular domain of VE-cadherin consists of five cadherin-like repeats that oligomerize to form *cis*-oligomers and form *trans*-oligomers between adjacent cells (23, 212, 497, 526). The cadherin cytoplasmic tail contains two functional domains: the juxtamembrane domain (JMD) and the COOH-terminal domain (CTD). These domains interact with three related proteins belonging to the *Armadillo* family, originally identified in *Drosophila*:  $\beta$ -catenin, plakoglobin ( $\gamma$ -catenin), and p120-catenin (Fig. 10). JMD of VE-cadherin binds p120-catenin, whereas CTD binds  $\beta$ -catenin or plakoglobin in a mutually exclusive fashion.  $\beta$ -Catenin or plakoglobin then binds  $\alpha$ -catenin, which links the cadherin-catenin complex to the actin cytoskeleton (57).

VE-cadherin is required for the proper assembly of AJs and development of normal endothelial barrier function. Deletion of VE-cadherin in mice (*VE-cadherin*<sup>-/-</sup>) is embryonically lethal (E9.5) due to immature vascular development (142, 1007). Ectopic expression of a cadherin mutant lacking the VE-cadherin extracellular domain in a human dermal microvascular cell line (995) or mouse endothelial cells (119) resulted in a leaky junctional barrier (119, 995) and increased lung microvascular permeability (119). Disruption of homotypic binding of cadherin proteins by application of VE-cadherin antibodies increased transendothelial permeability to macromolecules (187, 334, 382, 497) (Fig. 11A). Chelation of extracellular  $\text{Ca}^{2+}$  using EDTA likewise increased microvessel permeability in lungs secondary to disruption of cell-cell adhesion; however, permeability could be restored after repletion of media with extracellular  $\text{Ca}^{2+}$  (296). Application of neutralizing VE-cadherin antibodies augmented the increase in microvessel permeability induced by  $\text{Ca}^{2+}$  chelation and prevented reversal of the permeability response following repletion with extracellular  $\text{Ca}^{2+}$  (296) (Fig. 11B).

Although the precise mechanisms of the regulation of junctional assembly by VE-cadherin have not been identified, actin-binding proteins and RhoGTPase appear to be crucial. In nonendothelial cells, E-cadherin has been shown to interact with actin-related protein 2/3 (Arp2/3) (476), which is known to associate with Wiskott-Aldrich syndrome protein (WASP) and cortactin (see reviews in Refs. 597, 1038) as well as vinculin (215). WASP is a downstream effector of the RhoGTPase, Cdc42 (see Ref. 242) (Fig. 12). Thus adhesion induced by cadherin homotypic interactions may activate an "outside-in" signaling pathway resulting in increased actin polymerization by the Cdc42-Arp2/3-WASP pathway, thereby stabilizing AJs. Lampugnani et al. (498) showed that transfection of VE-cadherin cDNA in endothelial cells from VE-cadherin-null

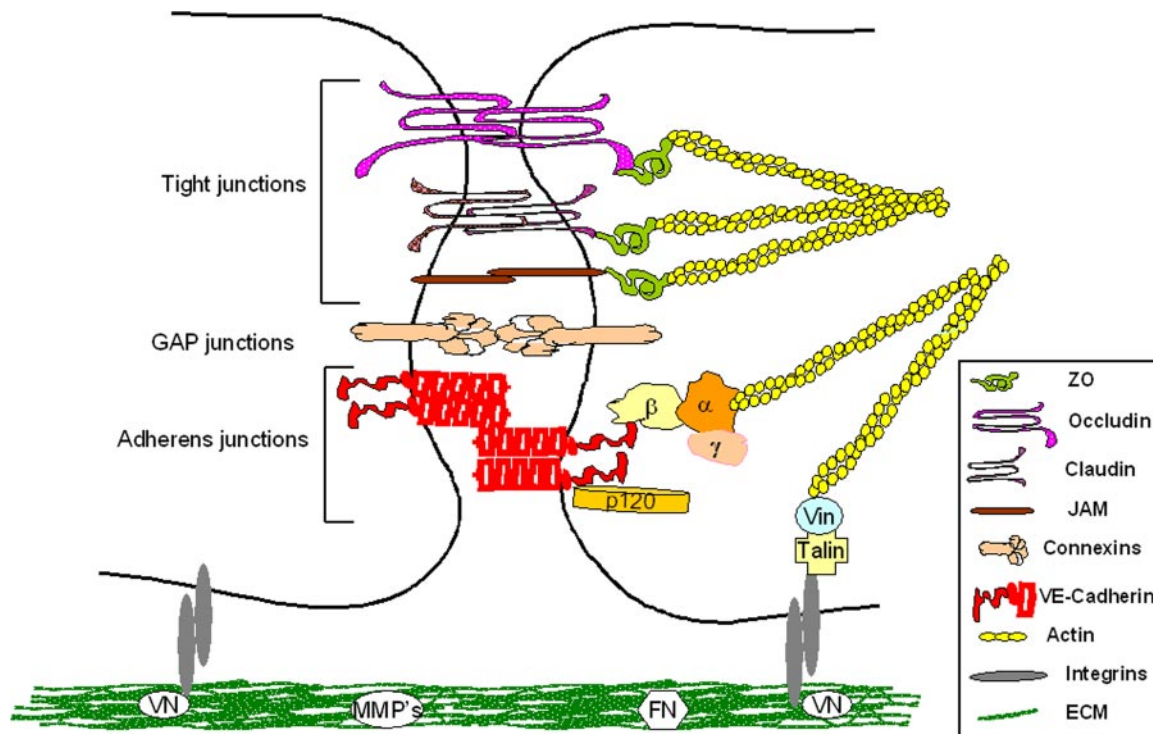


FIG. 10. Structural organization of endothelial cell intercellular and matrix interactions. This figure shows a typical arrangement of IEJs (comprising tight junctions, adherens junctions, and gap junctions) and integrin receptors by which endothelial cells adhere to each other and to ECM to maintain barrier function and intercellular communications. Occludin, claudins, and JAMs are the backbones of tight junctions, whereas VE-cadherin is required for formation of adherens junctions. Connexins form gap junctions. While the extracellular domains of occludin, claudins, and VE-cadherin maintain cell-cell contact, intracellular domains provide junctional stability through their linkages with the actin cytoskeleton via catenins ( $\beta$ ,  $\beta$ -catenin;  $\alpha$ ,  $\alpha$ -catenin;  $\gamma$ ,  $\gamma$ -catenin; p120, p120-catenin) or zona occluden-1 protein (ZO-1). Gap junctions allow the rapid exchange of information in the form of low-molecular-mass second messengers  $\text{Ca}^{2+}$  and  $\text{IP}_3$  between contiguous cells. Integrin receptors link endothelial cells with ECM through matrix proteins which are fibronectin (FN) or vitronectin (VN). The cytosolic domains of integrins are linked with actin cytoskeleton through proteins talin and vinculin (Vin), involved in integrin-mediated signaling. MMPs (membrane metalloproteases) which control remodeling of ECM (described in sect. vB) and modify cell-ECM signaling.

embryos induced actin cytoskeletal rearrangement and activated another GTPase, Rac, via the Rac-specific GEF T-cell lymphoma invasion and metastasis-1 (Tiam-1) (498). Likewise, in another study, engagement of cadherin was shown to activate Rac (650). Expression of a VE-cadherin mutant lacking the extracellular domain activated a related intracellular signaling pathway as evidenced by activation of Cdc42 and actin-dependent formation of membrane protrusions in endothelial cells (474). In contrast, engagement of E-cadherin activated p190RhoGAP, which switched off RhoA by increasing its intrinsic rate of GTPase activity (648). Thus apart from providing IEJ integrity through homotypic adhesion, VE-cadherin may regulate junctional permeability by modulating GTP binding to and GTP hydrolysis of Cdc42, Rac, and RhoA. The net result of the activation state of these Rho GTPases may be to control actin polymerization at the junctions, and thereby to finetune junctional permeability.

Catenins also play an important role in regulating the assembly of AJs. Truncation of the  $\beta$ -catenin-binding cytosolic domain of VE-cadherin was found to be lethal in mice (142), presumably because of the failure of AJs to

associate with actin cytoskeleton and properly assemble AJs. Conditional inactivation of  $\beta$ -catenin markedly reduced the ability of endothelial cells to maintain intercellular contacts (153). Expression of VE-cadherin lacking  $\beta$ -catenin binding sites decreased the strength of cell-cell adhesion and interfered with binding of the actin cytoskeleton to AJs (474, 637, 670). These results fit with the following model:  $\beta$ -catenin and plakoglobin interact with  $\alpha$ -catenin, which in turn is linked to the actin cytoskeleton.  $\beta$ -Catenin and plakoglobin are thus able to stabilize AJs by bridging VE-cadherin and the actin cytoskeleton.

Expression of a VE-cadherin mutant lacking the extracellular domain but with an intact  $\beta$ -catenin binding site was shown to disrupt endothelial barrier function (119). This was attributed to the translocation of  $\beta$ -catenin from the AJs to the expressed  $\beta$ -catenin mutant, resulting in destabilization of AJs and increased endothelial permeability (119). Surprisingly, expression of this mutant also blocked transendothelial neutrophil migration in response to chemotactic stimuli (665). Because the extrajunctional  $\beta$ -catenin pool is known to regulate gene expression by forming a cytosolic complex with wingless/



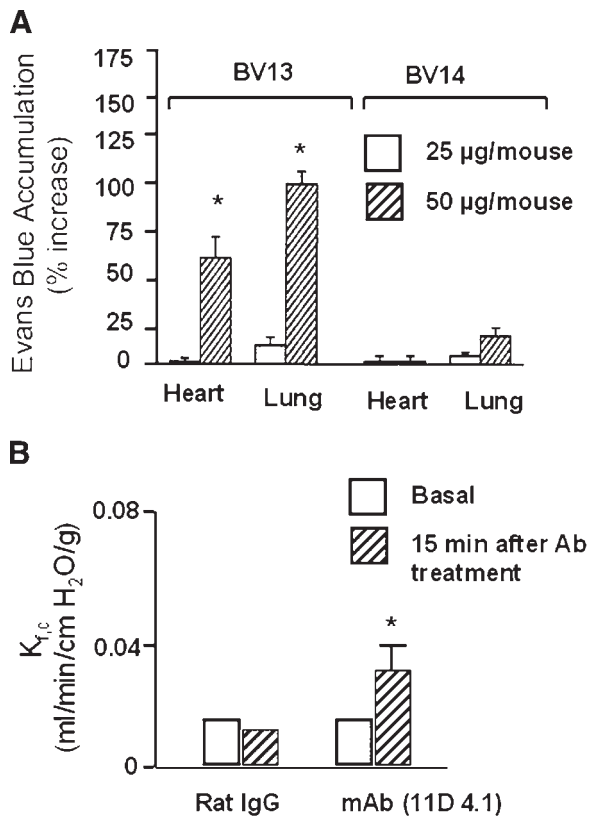


FIG. 11. Disruption of VE-cadherin homotypic adhesion increases endothelial permeability. *A*: an increased accumulation of Evans blue dye in heart and lungs after administration of anti-VE cadherin mAb BV13, but not after isotype-matched nonspecific mAb BV14 in mice; 25  $\mu$ g antibody/mouse (open bars) and 50  $\mu$ g antibody/mouse (hatched bars). \*Significantly different. [Adapted from Corada et al. (188).] *B*: increased liquid permeability in pulmonary microvessels after administration of VE-cadherin monoclonal antibody (mAbD4.1), but not control rat IgG. \*Significant increase above basal value. [Adapted from Gao et al. (296).]

int1 (WNT), adenomatous polyposis coli (APC), and the transcription factor T-cell factor (TCF) (reviewed in Ref. 65) (Fig. 12), it is possible that sequestration of  $\beta$ -catenin by the expressed VE-cadherin mutant may interfere with transcription of endothelial cell surface intercellular adhesion molecule-1 (ICAM-1) required for neutrophil transmigration (see Ref. 252). These observations raise the possibility that AJs “sense” neutrophil migration and thereby control its magnitude by engaging a negatively regulatory signal.

Binding of p120-catenin to VE-cadherin also plays an important role in the organization of AJs, and thus in regulating endothelial permeability. Unlike  $\beta$ -catenin and plakoglobin, p120-catenin does not associate with the actin cytoskeleton (reviewed in Ref. 752). Intriguingly, in nonendothelial cells, p120-catenin was shown to associate with the heavy chain of kinesin (KHC), a family of microtubule motors (159, 1083), raising the possibility that p120-catenin association with microtubules may stabilize AJs and thus the endothelial cell shape (discussed

in sect. ix) (Fig. 12). Moreover, p120-catenin bound to VE-cadherin regulates the contractile machinery of endothelial and epithelial cells by negatively controlling the activity of RhoA GTPase (341, 425, 649). There is an inverse correlation between p120-catenin bound to VE-cadherin and the activation state of RhoA (22, 425) (reviewed in Ref. 24). Studies have also shown that there is a positive correlation between levels of p120-catenin (i.e., the unbound p120-catenin) and the activation of two other members of the Rho family, Rac and Cdc42 (24). Thus p120-catenin via its ability to interact with a microtubule motor protein and by modulating RhoGTPase activity may be a key regulator of AJs stability. However, little is known about the mechanisms of p120-catenin regulation of the activities of these Rho GTPases and how they influence endothelial permeability.

Iyer et al. (425) found that overexpression of JMD of VE-cadherin, depleting endogenous p120-catenin, induced actin stress fiber formation and myosin light chain (MLC) phosphorylation in a RhoA-kinase dependent manner (425). They also showed that knock-down of p120-catenin using siRNA markedly reduced plasma membrane VE-cadherin expression and increased endothelial permeability (425). These findings are consistent with the concept that the p120-catenin bound to VE-cadherin serves as a negative regulator of RhoA function, and thus helps to downregulate endothelial permeability.

As mentioned above, p120-catenin levels have also been implicated in regulating VE-cadherin expression and insertion into the plasma membrane (425). p120-Catenin bound to cadherin’s JMD appears to act as a “shield” that prevents binding of *Hakai*, a ubiquitin ligase, thereby inhibiting cadherin degradation (reviewed in Ref. 1004). Because p120-catenin interacts with the heavy chain of kinesin (KHC) that regulate vesicular trafficking (159, 1083), it is possible that p120-catenin can also control cadherin cell surface expression by influencing trafficking of the vesicular pool of cadherin to IEJs (Fig. 12). In addition, two nonreceptor protein tyrosine kinases (PTKs), one the Src family member Fyn and the other the Frk family member, Fer (939), interact directly with p120-catenin (708). Protein tyrosine phosphatases (PTP $\mu$ ) (1110) and Src homology 2 domain-containing tyrosine phosphatase-1 (SHP-1) (451, 567) also associate with p120-catenin. Binding of these kinases and phosphatases to p120-catenin points to an important role for p120-catenin as a scaffold for these regulatory proteins; thus the possibility exists that they modulate AJs function by regulating the phosphorylation of p120-catenin and other AJ proteins (752). All of these p120-catenin-regulated functions are capable of controlling endothelial permeability; however, their role and relative importance have not been rigorously examined.

The binding of  $\alpha$ -catenin to  $\alpha$ -actinin (468), vinculin (408, 439, 1034), VASP (993), and formin (469) is crucial



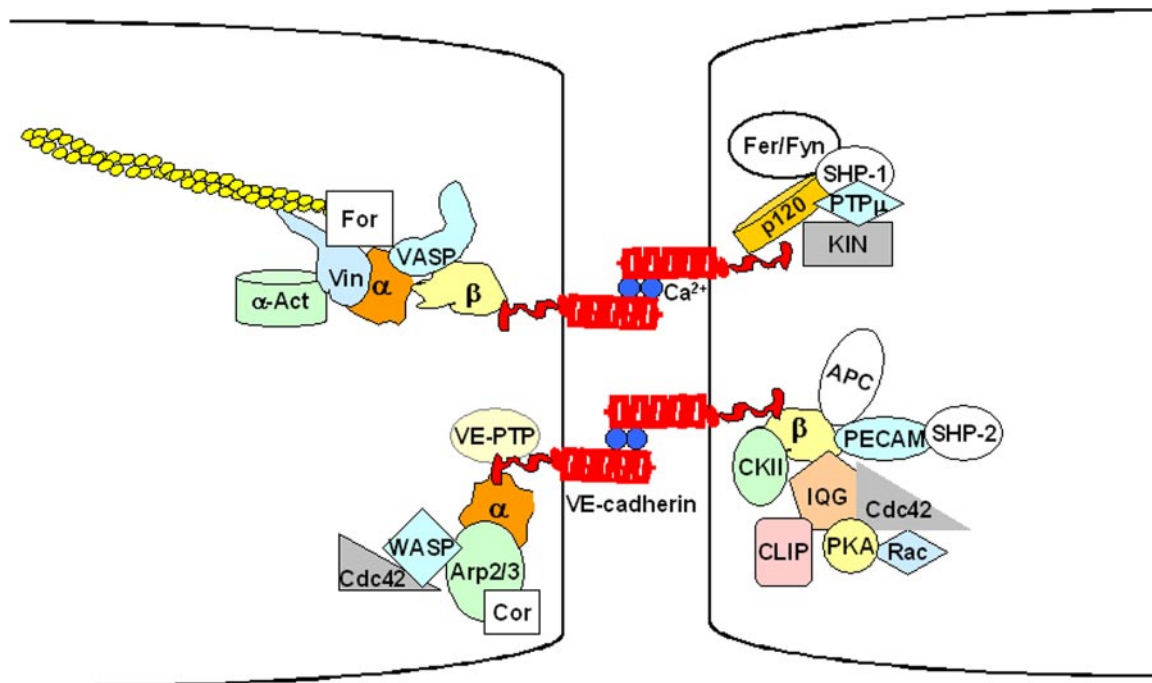


FIG. 12. Adherens junction-associated proteins. VE-cadherin cytoplasmic tail binds  $\beta$ -catenin ( $\beta$ ) or plakoglobin (not shown) competitively. The cytoplasmic tail also binds p120-catenin (p120) at a site separate from  $\beta$ -catenin.  $\beta$ -Catenin by binding  $\alpha$ -catenin ( $\alpha$ ), also links the adherens junctional complex with actin cytoskeleton.  $\alpha$ -Catenin binds actin polymerizing proteins,  $\alpha$ -actinin ( $\alpha$ -Act), vinculin (Vin), VASP, and formin (For). Several junctional proteins IQGAP (IQG), PECAM-1, casein kinase II (CKII), and transcription factors such as wingless/int1 (WNT), adenomatous polyposis coli (APC), and T cell factor (TCF) bind  $\beta$ -catenin. IQGAP also binds Cdc42, Rac, and CLIP (a microtubule motor-associated protein). p120-catenin partners include kinesin (KIN), Src family tyrosine kinases Fer/Fyn, protein tyrosine phosphatases PTP $\mu$ , and Src homology domain containing tyrosine phosphatase-1 (SHP-1). VE-cadherin also binds vascular endothelial protein tyrosine phosphatase (VE-PTP). VE-cadherin is also shown to interact with actin-related protein 2 and 3 (Arp2/3), which in turn interacts with actin polymerizing proteins, Wiskott-Aldrich syndrome protein (WASP), and cortactin (Cor). WASP is a downstream effector for Cdc42. Thus AJs are stabilized through multiple interactions with actin-binding proteins and RhoGTPases (see text for details).

for maintaining the association of E-cadherin with the actin cytoskeleton and for stabilizing AJs (Fig. 12). In addition,  $\alpha$ -catenin by binding with ZO-1 (628) serves to promote interactions among AJs, TJs, and GJs. Actin failed to polymerize in keratinocytes lacking  $\alpha$ -catenin, resulting in leaky junctions (993). These findings suggest the important role of  $\alpha$ -catenin as a component of the AJ complex regulating the integrity of IEJs by mediating actin remodeling.

## 2. TJs

TJs were identified by electron microscopy as points where the outer leaflets of lateral membranes between adjacent endothelial cells were fused (129, 719, 1057) (Fig. 13, *inset*). TJs represent  $\sim 20\%$  of total junctional complexes present in endothelial cells (1057). TJs are composed of claudins, occludin, and junctional adhesion molecules (JAMs). Freeze-fracture studies showed that TJ organization in the endothelium varies along the vasculature. Arterial segments have two to seven highly connected and well-developed TJs, whereas venular segments have one to three discontinuous TJs (807). In contrast to AJs, the role of TJs in regulating endothelial permeability remains incompletely understood.

Occludin was the first integral membrane protein identified that localized to TJs (26, 286). Occludin possesses four transmembrane domains with two extracellular loops that enable it to form homotypic bonds and contribute to TJ assembly. The COOH terminus of occludin associates with the zona occludens protein ZO-1 (described below) in the cytoplasm (160, 287), which links occludin with  $\alpha$ -catenin (628, 805), spectrin (573), and actin cytoskeleton (160, 287, 804) (Fig. 13). Thus occludin through its interaction with ZO-1 and the actin cytoskeleton stabilizes TJs, and presumably transduces information between AJs and TJs. The expression level of occludin was found to correlate with enhanced endothelial barrier properties in different vascular beds (373, 456). The arterial endothelial barrier, which is less permeable than the venular endothelial barrier (807), has an 18-fold greater expression of occludin than the other segments (456). Occludin is expressed at the highest level in endothelial cells of the central nervous system, which has a restrictive epitheloid-like barrier (373). Depletion of occludin using antisense oligonucleotides (456) or disruption of homotypic association of occludin by peptide antagonists mimicking the second extracellular loop of occludin decreased transendothelial electrical resistance

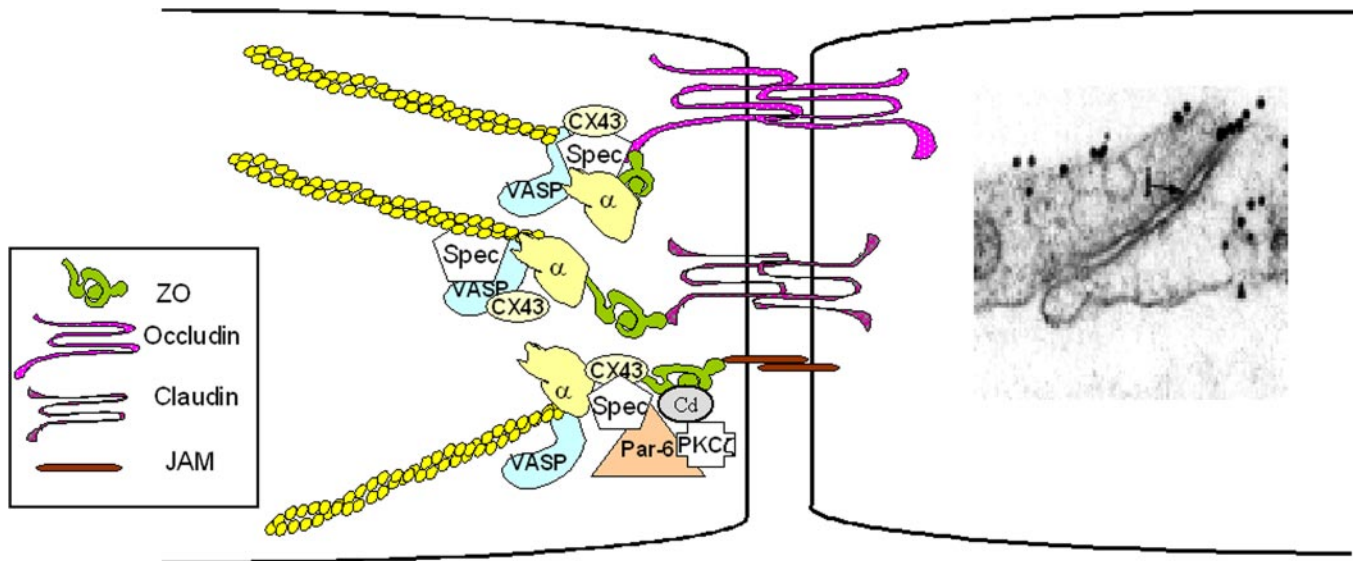


FIG. 13. Protein complexes link zona occludens (ZO) to cytoplasmic signaling molecules and actin cytoskeleton. Tight junctions appear in transmission electron micrograph as "kisses" resulting from fusion of the outer leaflets of lateral membranes at endothelial junctions (*inset*). [Adapted from Predescu and Palade (719).] The cytoplasmic domains of occludin and claudin bind ZO-1, which in turn associates with actin-polymerizing proteins, vasodilator-stimulated phosphoprotein (VASP), and spectrin (Spec) linking tight junctions to the actin cytoskeleton. ZO-1 also binds  $\alpha$ -catenin ( $\alpha$ ) and connexin-43 (CX43), which facilitate communication between tight junctions, gap junctions, and adherens junctions. JAMs, by binding partitioning-defective protein (Par-6), Cdc42 (Cd), and atypical PKC isoform (PKC $\zeta$ ) recruit these signaling molecules to tight junctions.

(96). Deletion of the NH<sub>2</sub>-terminal region of occludin in epithelial cells also caused TJs to become leaky (48, 392), implying that the NH<sub>2</sub> terminus is crucial for regulating barrier function. Ectopic expression of occludin permitted the formation of TJs in a fibroblast cell line expressing only ZO-1 (983). These findings point to an important role for occludin in regulating TJs architecture; however, the picture is more complex *in vivo* since *occludin*<sup>-/-</sup> mice showed no gross changes in TJ morphology and no alteration in intestinal epithelial barrier function (777). It cannot be ruled out that there is a compensatory increase in expression of AJs or other junctional proteins in these mice, which could account for the observed normal epithelial barrier function.

Of the 24-member claudin family, only claudin-5 is expressed in endothelial cells (619, 643). Like occludin, claudins possess four transmembrane domains; however, they show no other sequence similarity. Claudins bind to each other in a homotypic and heterotypic manner to form TJs (289, 963). Also like occludin, the cytoplasmic end of claudin binds ZO-1, which in turn links claudin with the actin cytoskeleton and ZO-1-associated proteins (Fig. 13). Overexpression of claudin in fibroblasts led to assembly of TJs coupled with increased association of occludin (288), suggesting that claudin serves to recruit occludin to TJs. Deletion of claudin-5 in mice resulted in death ~10 h after birth (643). Brain blood vessels formed normally possibly due to compensatory expression of claudin-12. No brain edema was found, and TJs appeared normal but there was increased permeability of the blood-

brain barrier to small solutes (<800 Da) (643). These findings implicate claudin-5 as a key regulator of TJs assembly and blood-brain barrier function.

JAMs, belonging to the immunoglobulin superfamily, are single-pass membrane proteins with a long extracellular "domain" (37–39, 672). Three JAM molecules have been described: JAM-1 (originally known as JAM), JAM-2, and JAM-3 (also known as VE-JAM) (37–39, 672). JAMs have 30–40% amino acid homology, with the expression pattern of the JAMs varying substantially between cell types. JAM-1 is present in epithelial and endothelial cells, JAM-3 is found exclusively in endothelial cells, while JAM-2 is found in high endothelial venular cells (the leakier endothelial subtype of lymphatic vessels) (38, 39). The functions of the JAMs and the basis of their localization to endothelial junctions are not known. JAMs have an interesting functional domain in their cytoplasmic tails, the postsynaptic protein disc large ZO-1 (PDZ) domain (234), which may enable them to recruit the signaling molecules, partitioning-defective protein-6, Cdc42, and protein kinase C (PKC)- $\zeta$  (430) to the TJ complex (Fig. 13). The function of this complex may be to promote endothelial cell polarity during neovascularization. Perturbation of JAM-1 function by a blocking antibody or a recombinant soluble form of JAM-1 prevented the reannealing of TJs in epithelial cells (524, 538). Thus JAMs could also play a similar role in regulating the integrity of the endothelial barrier.

ZOs are members of a family of membrane-associated guanylate kinases (MAGUKs). Three ZO subtypes

(ZO-1, -2, -3) have been identified. ZOs are known to interact directly or indirectly via bridging proteins with claudins, JAMs, and occludin (331) (Fig. 13). The spatial organization of occludins in fibroblasts required the co-expression of ZO-1 (983). Through their PDZ, Src homology 3 (SH3), and guanylate kinase (GUK) domains, ZOs are critical in recruiting signaling molecules to TJs, and thereby linking TJ proteins to the actin cytoskeleton. Studies showed that ZO-1 could serve as chaperones for constituents of the TJ complex (253) (see Ref. 331). ZO-1 also binds to the AJ protein  $\alpha$ -catenin (628), GJ protein connexin-43 (959, 1064), and actin-polymerizing proteins vasodilator-stimulated phosphoprotein (VASP) (179), and spectrin (421, 422). These interactions point to a potentially critical, but poorly understood, role of ZO-1 in orchestrating intercellular adhesion, and hence in controlling endothelial permeability.

### 3. GJs

GJs are formed from clusters of transmembrane hydrophilic proteins called connexons (Cx) (see Ref. 332). Each GJ is made up of two connexons, one contributed by each of the two partner cells. Connexon is made up of six connexin subunits in the form of a hexamer. Endothelial cells express connexins Cx37, Cx40, and Cx43 (988, 989). Connexins contain four transmembrane domains, and the NH<sub>2</sub> and COOH termini face the cytoplasm (see Ref. 332). The intercellular pore formed by the connexons is ~2 nm in diameter, which may be in an open or closed configuration. These intercellular channels typically contain a mixture of any or all of the three endothelial-specific connexins. GJs allow changes in transmembrane potential to propagate rapidly between cells by providing a low-resistance pathway for current flow (150). In addition, GJs are a means of exchanging information between endothelial cells in the form second messengers (e.g., Ca<sup>2+</sup>, IP<sub>3</sub>) (75, 149, 488, 609). Studies in rat pulmonary microvessel endothelia in situ showed longitudinal Ca<sup>2+</sup> "waves" presumably propagated by GJs (1090). Thus GJs provide electrotonic and metabolic pathways for direct cell-to-cell transfer of signaling molecules and ions. Based on the finding that the endothelial cell Cx43 gap junctional plaque surface area is about eight times greater than in vascular smooth muscle cells, the endothelial cell monolayer has been proposed to form a more permissive pathway for the longitudinal conduction of signals along the luminal vessel wall (345). Channel gating in GJs is subject to regulation by phosphorylation of the connexins (134, 527, 906, 1074, 1103, 1104). Serine/threonine and tyrosine phosphorylation of connexins closes the channels and disrupts intercellular communication (134, 527, 906, 1074, 1103, 1104). Phosphorylation also regulates the rate of channel assembly and turnover (reviewed in Refs. 185, 494, 938). Studies using phosphodeficient connexin mu-

tants are likely to be promising as it remains unknown how alterations in phosphorylation of connexins influence endothelial barrier function.

Studies in Cx37 or Cx40 knock-out mice showed that connexins may contribute to maintaining endothelial barrier integrity. Simon and Whorter (870) demonstrated that deletion of either Cx37 or Cx40 in mice had no apparent vascular effects and the mice were viable; however, double knockout mice (Cx37<sup>-/-</sup> × Cx40<sup>-/-</sup>) died perinatally and demonstrated gross vascular abnormalities, most prominently hemorrhaging in several tissues. Interestingly, endothelial cell-specific deletion of Cx43 caused hypotension resulting from a marked elevation of plasma NO level (525). These findings demonstrate the important role of connexins in regulating NO production with possible consequence on endothelial permeability.

Studies showed that Cx43 forms a complex with ZO-1 and spectrin (421, 422, 959, 1064) (Fig. 13), suggesting a link between GJs, TJs, and actin cytoskeleton that may be important for endothelial barrier integrity. Endothelial Cx43 can also associate with neutrophil Cx40 (112, 184, 1097) and vascular smooth muscle Cx40 (532), raising the possibility of connexin-specific communications between these dissimilar cell types. Because challenge with TNF- $\alpha$  or blockade of Cx40 enhanced transendothelial neutrophil migration and reduced barrier function (1097), it is possible that communication between neutrophil and endothelial cells via connexins is a factor contributing to the trafficking of neutrophils across the vessel wall barrier.

A) IEF-ASSOCIATED PROTEINS. A number of IEF-associated proteins may contribute to IEF permeability regulation. The platelet-endothelial cell adhesion molecule (PECAM-1; CD31), a member of the immunoglobulin superfamily of transmembrane proteins, is concentrated in IEFs (44). As PECAM-1 interacts with tyrosine-phosphorylated forms of  $\beta$ -catenin and SHP2 (405) (see also Ref. 404 for review), it is possible that PECAM-1 modulates AJ assembly and function by serving as a reservoir for  $\beta$ -catenin (see Ref. 404) (Fig. 12). Deletion of PECAM-1 had no effect on the development of blood vessels (228), but endothelial cells from these mice recovered more slowly from histamine-induced permeability increase than wild-type endothelial cells (336). Thus PECAM-1 might play a role in restoring endothelial barrier integrity after injury. PECAM-1 is also a ligand for  $\alpha_v\beta_3$ -integrin (126, 707) and anti-PECAM mAb caused activation of  $\alpha_4\beta_1$ - (506) and  $\beta_2$ -integrins (925), implicating a role of PECAM-1 in regulating the function of these integrins. It is likely that the interaction of PECAM-1 with integrins is important in mediating transendothelial migration of leukocytes, rather than endothelial permeability. Leukocyte migration requires the binding of endothelial PECAM-1, mobilized from a surface-connected membranous "subjunctional reticulum" recycling pool (562), to the  $\beta_3$ -integrins expressed on leukocytes (940).



IQGAP GTPase-activating protein-1 (IQGAP-1) is another AJ-associated scaffolding protein (378) that binds actin, activated Rac and Cdc42,  $\beta$ -catenin and E-cadherin, calmodulin (378), and microtubule-associated cytoplasmic linker integral protein-170 (CLIP-170) (for review, see Ref. 118) (Fig. 12). In nonendothelial cells, overexpression of IQGAP impaired E-cadherin-mediated cell adhesion (487). Likewise, recruitment of endogenous IQGAP to intercellular junctions was associated with decreased E-cadherin-mediated cell adhesion (523). Interestingly, calmodulin and E-cadherin compete for binding to IQGAP, and inhibition of calmodulin with structurally diverse membrane-permeant antagonists impaired E-cadherin-mediated homophilic adhesion (523). Activated Cdc42 and Rac1 also inhibited the interaction of IQGAP1 with  $\beta$ -catenin (487). These findings show that IQGAP restricts the accessibility of  $\beta$ -catenin to its partners (i.e., cadherins, p120-catenin, and  $\alpha$ -catenin); therefore, it is possible that activated Cdc42/Rac strengthens barrier function by freeing  $\beta$ -catenin from IQGAP. Additionally, IQGAP through its interaction with actin (569) and CLIP-170 (343) may be important in modulating AJ integrity (118). Thus IQGAP appears to be a potential regulator of AJs, and endothelial permeability, but this issue needs to be explored using strategies such as siRNA knockdown.

Additional identified components of AJs include the receptor-tyrosine phosphatases PTP $\mu$  and vascular endothelial protein tyrosine phosphatase (VE-PTP) (110, 309, 638) and the nonreceptor Src homology protein tyrosine phosphatase (SHP2) (819, 973) (Fig. 12). These phosphatases associate with AJs and may regulate AJ organization and stability by dephosphorylating AJ protein constituents and controlling RhoGTPase activity (638, 819). PTP $\mu$  was shown to associate with and dephosphorylate p120-catenin (1110). Dephosphorylation of p120-catenin may modify AJs integrity by modulating the interaction of p120-catenin with VE-cadherin and controlling p120-catenin regulation of RhoA activity (described above). VE-PTP specifically associates with VE-cadherin via the cadherin extracellular domain (638). Conditional expression of VE-PTP in endothelial cells promoted endothelial integrity, but curiously, this effect was independent of PTP's enzymatic activity (638). Thus VE-PTP association with VE-cadherin may contribute to the strengthening of the AJ barrier by a phosphatase-independent mechanism. Studies have also shown association of SHP2 with VE-cadherin in the endothelial AJ complex (973). This association was found in confluent, quiescent cells but was absent after stimulation with thrombin (973), raising the possibility that dissociation of SHP2 from AJs is an important aspect of the junctional disassembly induced by thrombin. Dissociation of SHP2 may expose junctional proteins to posttranslational modification by tyrosine kinases that in turn can induce AJ disassembly. However, the role of these signaling events in mediating thrombin-

induced increase in endothelial permeability has not been explored. Intriguingly, overexpression of phosphatase-defective SHP2 or inhibition of SHP2 activity by calpeptin (an inhibitor of calpain) activated RhoA (819), suggesting that SHP2 may stabilize AJs by downmodulating RhoA activity. Perhaps this is the mechanism of SHP2 regulation of endothelial permeability.

The actin-polymerizing proteins VASP (993), formin (469),  $\alpha$ -actinin, and vinculin (408, 468) are also localized along the AJs where they form links with  $\alpha$ -catenin. Their propitious localization suggests that they could play a role in stabilizing IEJs. Disruption of preformed actin filaments by C2 toxin (244) or cytochalasin (1031, 1032) is known to increase endothelial permeability in intact microvessels and cultured cells. Moreover, polymerization of actin is required for assembly of AJs, since expression of a formin mutant in epithelial cells (which were devoid of polymerized actin filaments) resulted in impaired formation of AJs (469). Nonmuscle myosin heavy chain II-A (MHC II-A) may be yet another important regulator of AJ assembly. Deletion of MHC II-A in mice resulted in death at day E7.5 because of failure of E-cadherin and  $\beta$ -catenin to localize to cell junctions (183). Together, these findings indicate that multiple actin-polymerizing proteins as well as nonmuscle myosin motor activity provide mechanical stability and are necessary for the proper alignment of AJs proteins in IEJs. The functional redundancy of these proteins indicates the importance of stabilizing IEJs to maintain the integrity of the vascular endothelial barrier.

Desmoplakin was identified as a component of AJs in cultured human umbilical vein endothelial cell monolayers (978). Desmoplakin might provide the important link between AJs and the endothelial intermediate filament vimentin, resulting in an added measure of IEJ mechanical stability (477, 837). The integrins  $\alpha_2\beta_1$  and  $\alpha_5\beta_1$  (496) and the nectin-afadin system (926) have also been localized to IEJs (see Ref. 210), but their role in maintaining IEJ integrity and regulating endothelial permeability is unknown.

## B. Endothelial Cell-ECM Interactions

Endothelial cells express membrane-spanning integrins, the receptors for proteins in the subendothelial ECM. Integrins are bound to ECM at specific cellular sites, termed "focal contacts" or "focal adhesions" (131) that are responsible for the adhesive interactions of the endothelial cell monolayer with ECM. The integrin-ECM interaction in series with IEJs is a critical determinant of cell shape, and therefore of paracellular permeability.

### 1. Integrins

Endothelial cells express a number of integrins primarily on their abluminal surface; each integrin is a het-



erodimer defined by a unique combination of  $\alpha$ - and  $\beta$ -subunits (14, 162, 180, 208, 211, 581, 934). Subunit compositions found in endothelial cells are  $\alpha_1\beta_1$ ,  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_6\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , and  $\alpha_1\beta_5$ . Each subunit is a type I transmembrane glycoprotein composed of a large ectodomain, and in most cases, a small cytoplasmic domain (402, 766, 773) (see Ref. 403). Integrins interact with the Arg-Gly-Asp (RGD) domain of ECM proteins such as fibronectin, fibrinogen, vitronectin, and collagen (162, 180, 496, 504, 709, 880). Competitive disruption of integrin-ECM linkages with the synthetic peptide GRGDSP sequence (Gly-Arg-Gly-Asp-Ser) causes endothelial cell rounding and increased endothelial permeability (199, 731, 769, 902, 1066) (Fig. 14).

Antibodies directed against the fibronectin receptor caused a concentration-dependent inhibition of endothelial cell adhesion to ECM proteins (211) and increased transendothelial transport of the horseradish peroxidase (HRP) tracer (496), supporting an important role for integrin-ECM association in restricting the passage of macromolecules across the endothelial barrier. In intact vessels, peptides inhibiting integrin binding to fibronectin and vitronectin induced concentration-dependent increases in permeability of the venular endothelial barrier (1066). The increase in permeability was prevented by pretreatment of venular segments with fibronectin or vitronectin (1066), corroborating the cell culture results.

The short cytoplasmic domain of integrins is connected to the actin-binding proteins vinculin,  $\alpha$ -actinin, paxillin, talin, zyxin, tensin, and filamin (reviewed in Ref. 310) (Fig. 15). Paxillin interacts with p21-activated kinase (PAK) and PAK-interacting exchange factor (PIX) (968),

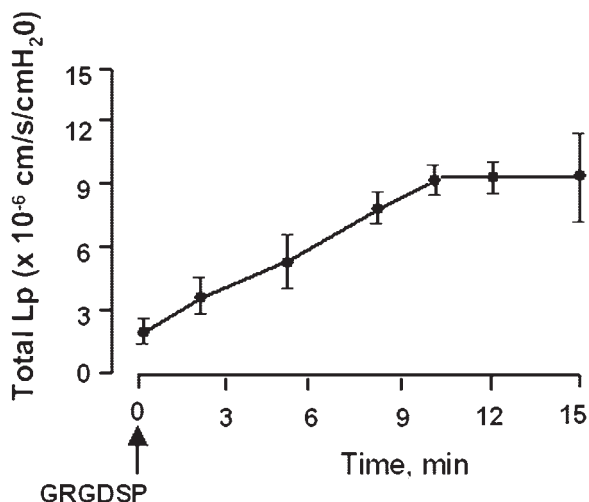


FIG. 14. Role of integrin-ECM interactions in regulating endothelial permeability. Pretreatment of bovine pulmonary microvessel endothelial cells with GRGDSP peptide ( $0.85 \mu\text{M}$ ), which competes with RGD domain of integrins, increased endothelial hydraulic conductivity ( $L_p$ ) maximally within 10 min, indicating that integrin-ECM interactions via RGD domain contribute to maintaining endothelial barrier function. [Adapted from Qiao et al. (731).]

Ablason protooncogene (Abl) (779), and p120 GTPase-activating protein (p120GAP) (see Ref. 123). Filamin binding partners include the Rho GTPases (RhoA, Rac, and Cdc42) and Ral1 (659), RhoA-associated kinase (ROCK) (971), Trio (63), and caveolin-1 (893). Focal adhesion kinase (FAK) binds to paxillin, talin, Src, p190GEF, growth factor receptor-bound protein 2 (Grb2), Arf (ADP ribosylation factor)-GAP containing SH3, ankyrin repeats and PH domain (ASAP1), p130 Crk-associated substrate (p130Cas), GTPase regulator associated with FAK (GRAF), and actin polymerizing proteins (ezrin, WASP) (described below).  $\alpha$ -Actinin ( $\alpha$ -Act) binds zyxin (191), which in turn associates with VASP and profilin (Pro) (746). Tensin contains an SH2 domain through which it can also induce interactions with multiple phosphotyrosine proteins such as Cas and Src (reviewed in Ref. 540). Vinculin interacts with Arp2/3 (215) and phosphatidylinositol 4,5-kinase (PIP5K) (283). The richness of these protein interactions at one site provides the ideal platform for the controlled integration of multiple signaling pathways regulating actin polymerization and the focal adhesion function (Fig. 15). Integrins participate in controlling endothelial cell shape through these protein interactions and transduction of signals in a bidirectional manner from the ECM to actin cytoskeleton and back. Studies showed that ligation of integrins  $\alpha_v\beta_3$  or  $\alpha_v\beta_1$  with ECM proteins resulted in tyrosine phosphorylation of FAK, paxillin, cortactin, and ezrin (78),  $\text{Ca}^{2+}$  influx (80), activation of cytosolic  $\text{PLA}_2$  (79), Rac GTPase (281), and increased capillary  $L_p$  in rat lungs (962). While these studies provide only correlative data, they point to a potentially important role for integrin interactions with ECM in regulating the endothelial barrier function. Further studies involving site-directed mutagenesis of individual integrin subunits and disruption of protein-protein interactions are needed to delineate the function of the specific integrins involved and identify the integrin-regulated "outside-in" and "inside-out" signals influencing endothelial barrier function.

## 2. Integrin-associated proteins

FAK is a non-receptor protein tyrosine kinase that binds to the cytoplasmic domain of the  $\beta$ -subunit of integrins (reviewed in Ref. 799). FAK contains a catalytic domain, interposed between the  $\text{NH}_2$  and  $\text{COOH}$  termini (Fig. 16A). The  $\text{NH}_2$ -terminal domain binds Trio, comprising three domains (two GEF domains for RhoA and Rac and one serine/threonine kinase domain) (582) and also contains a conserved  $\sim 300$  amino acid residue band 4.1, ezrin, radixin, moesin-homology domain (FERM domain) (322). These domains collectively regulate actin dynamics and RhoA activity (117, 364, 582, 1102). In addition, FAK through its  $\text{NH}_2$  terminus binds to and phosphorylates WASP (1071), a downstream effector of Cdc42 (reviewed in Ref. 242) that induces actin polymerization by binding

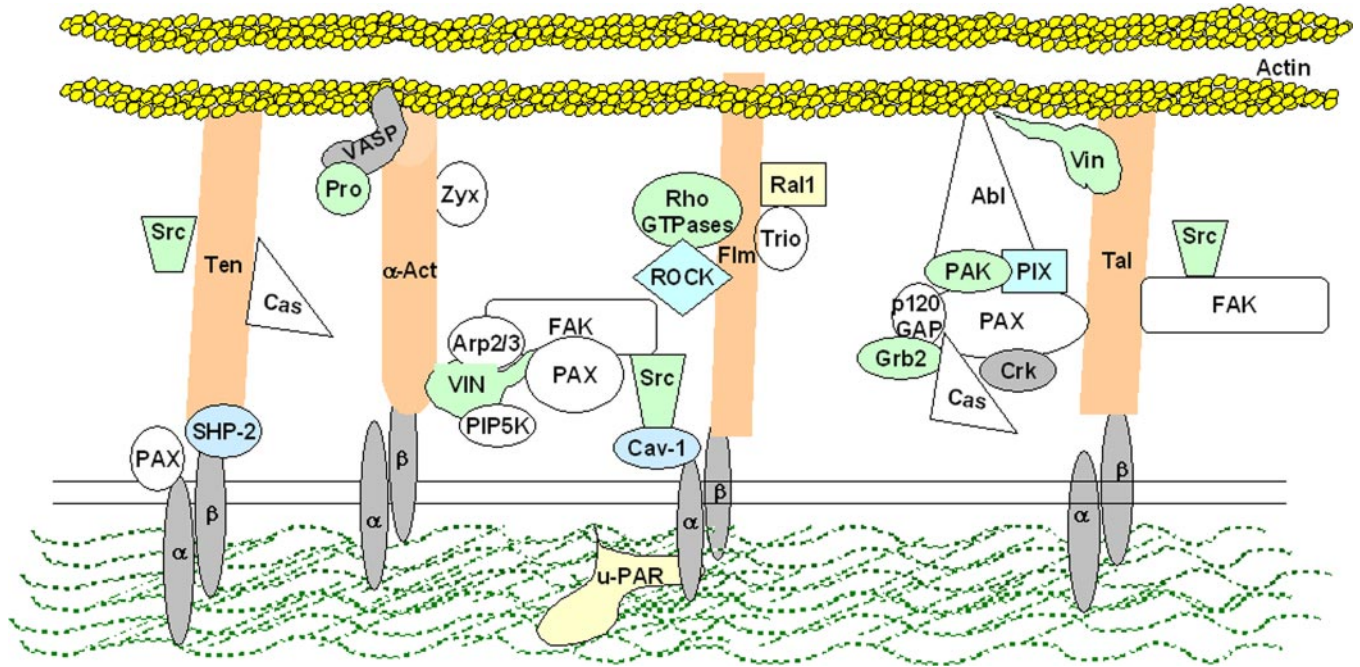


FIG. 15. Model of focal adhesion molecular complexes in endothelial cells.  $\beta$ -Subunit of heterodimeric integrin ( $\beta$ ) binds several focal adhesion proteins of which talin (Tal), filamin (Fln), tensin (ten),  $\alpha$ -actinin ( $\alpha$ -Act), tensin (Ten), and SHP2 are depicted here. Talin interacts with paxillin, which in turn binds p21-activated kinase (PAK), PAK-interacting exchange factor (PIX), growth factor receptor-bound protein 2 (Grb2), p130 Crk-associated substrate (p130Cas, Cas), Abl, and Ras GTPase-activating protein (p120GAP). Paxillin and talin also bind focal adhesion kinase (FAK), which in turn binds a number of other proteins (see Fig. 16). Filamin binding partners include Rho GTPases (Rho, Rac, and Cdc42) and Ral1, rho associated kinase (ROCK) and caveolin-1, and Trio.  $\alpha$ -Actinin ( $\alpha$ -Act) binds zyxin and VASP, which in turn associates with profilin (Pro).  $\alpha$ -Actinin was also shown to interact with vinulin, which binds Arp2/3 and phosphatidylinositol 4,5-kinase (PIP5K). Tensin contains an SH2 domain through which it can interact with multiple proteins such as Cas and Src. The  $\alpha$ -subunit of integrins can interact intracellularly with caveolin-1 (Cav-1) and paxillin (Pax), and extracellularly with urokinase-type plasminogen activator receptor (u-PAR). Thus focal adhesions, through these multiple interactions, serve as a model for regulation of signaling events in response to forces transmitted by the actin cytoskeleton. [Modified from Geiger et al. (310).]

Arp2/3 (see Ref. 597). As discussed above, Arp2/3 interacts with E-cadherin (476), cortactin (see Ref. 1038), as well as vinculin (215). Thus it is possible that FAK provides a crucial link between the endothelial cells and ECM, thereby mediating Cdc42-dependent AJ assembly (475). FAK also contains three proline-rich sequences by which it interacts with the SH3 domain of pp60Src and pp59fyn (151), growth factor receptor-bound protein 2 (Grb2) (798), Arf (ADP ribosylation factor), GAP containing SH3, ankyrin repeats and PH domain (ASAP1) (537), and p130 Crk-associated substrate (p130Cas) (646) (Fig. 16A). A recent study demonstrates that FAK through Grb2 also interacts with the GTPase dynamin, which in turn may regulate FAK-mediated focal adhesion turnover (250). The FAK COOH-terminal domain contains a region (FAT) required for focal adhesion targeting of FAK (for review, see Ref. 799). The FAT domain was shown to be both necessary and sufficient for targeting FAK to the focal adhesion complex (362). FAT domain also binds paxillin (363), talin (157), 190RhoGEF (1102), and GTPase regulator associated with FAK (GRAF) (364) (Fig. 16A). Thus FAK on the basis of its binding to a diverse group of proteins (799) is involved in regulating several key

endothelial processes, including adhesion and contraction, which are closely connected to regulation of barrier function.

FAK has been shown to be activated by tyrosine phosphorylation in response to integrin activation induced by cell adhesion, antibody cross-linking, and several agonists (reviewed in Ref. 799). FAK Tyr-397 is a site of autophosphorylation and high-affinity binding for Src homology 2 (SH2) domains of members of the Src family of kinases, c-Src, and Fyn (713, 793, 1075). This binding could contribute to recruitment of Src family kinases to sites of cell adhesion and also to the catalytic activation of Src through COOH-terminal tail displacement (see Ref. 799), resulting in consequences for endothelial barrier regulation.

Global deletion of FAK in mice is embryonically lethal (E8.5), in part because endothelial cells are unable to migrate and develop a primitive vasculature (impairment in vasculogenesis) (406, 407). A more recent study in mice with an endothelial-specific deletion of FAK demonstrated apparently normal vasculogenesis during early embryonic development (843). However, later in embryonic development (>E12.5) the endothelial-specific FAK

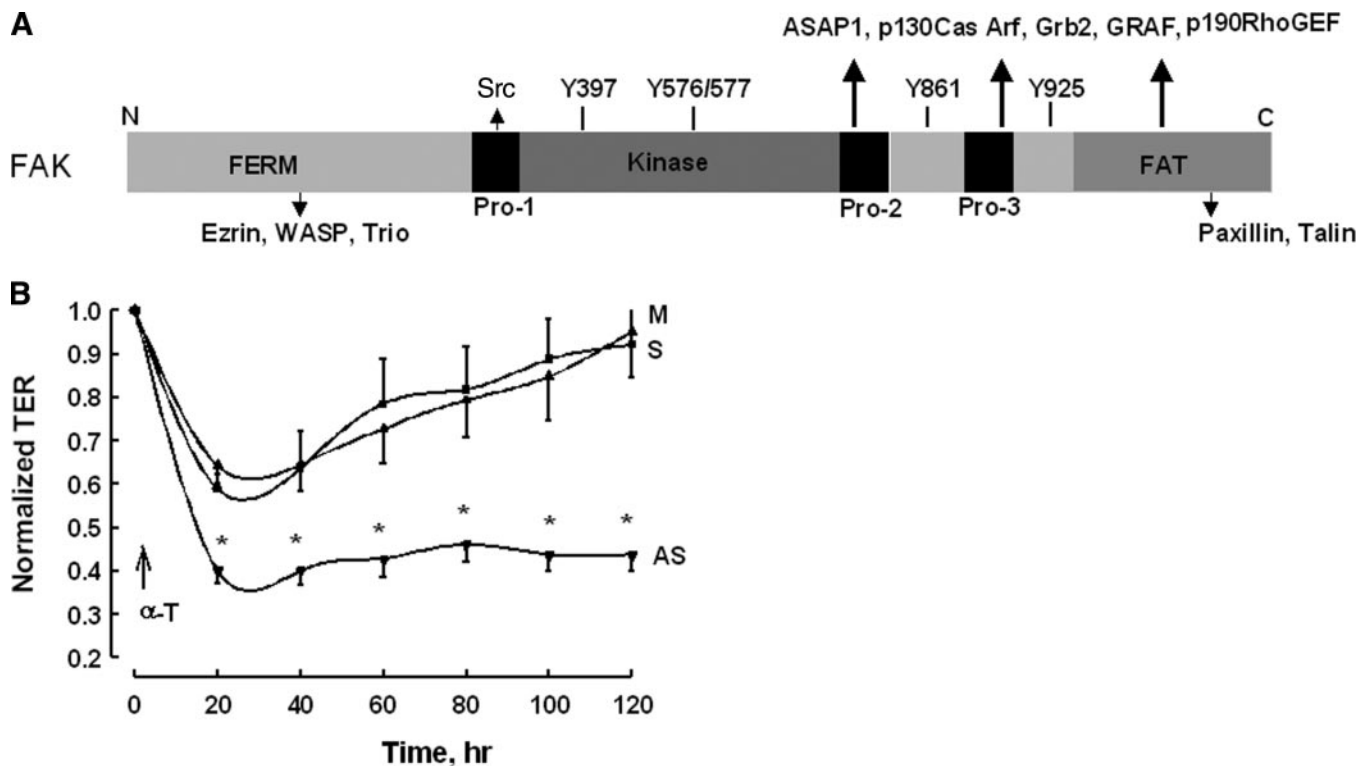


FIG. 16. FAK regulation of endothelial barrier function. *A*: domain structure of FAK. FAK contains a conserved band 4.1, ezrin, radixin, moesin-homology domain (FERM domain), 3-proline-rich domains (Pro-1–3), a kinase domain interposed between Pro-1 and Pro-2 domains, and a focal adhesion targeting (FAT) domain. The FERM domain also binds Trio, so named because of presence of three enzymatic domains (two GEF domains for Rho and Rac and a serine/threonine kinase domain) and WASP. Through Pro-1–3 domains, FAK binds Src, ankyrin repeats and PH domain (ASAP1), ADP ribosylation factor (Arf), Crk-associated substrate (p130Cas), Grb2, and GTPase regulator associated with FAK (GRAF). FAT domain binds paxillin and talin. Thus FAK, by binding several signaling proteins, regulates diverse cellular processes including adhesion and contraction events downstream of integrin binding. [Modified from Parsons (682).] *B*: FAK restores endothelial barrier function after thrombin challenge. Reduction in FAK expression by antisense oligonucleotide prolonged and augmented the thrombin-induced decrease in transendothelial electrical resistance (TER), indicating that FAK facilitates restoration of endothelial barrier integrity after an increase in junctional permeability. \*Statistically significant.  $\alpha$ -T,  $\alpha$ -thrombin; M, mock transfected; S, transfection with scrambled oligonucleotide; AS, transfection with antisense oligonucleotide. [Adapted from Mehta et al. (587).]

deletion demonstrated angiogenic defects, that is, compromised formation of new capillaries (from the primitive vasculature) in the embryo, yolk sac, and placenta. These late-stage angiogenic defects resulted in the formation of a defective vasculature, and ensuing hemorrhage, edema, and developmental delay. An increase in embryonic lethality was evident at E13.5 (843). These findings strongly suggest that FAK is a requirement for normal vascular development.

A variety of permeability-increasing mediators thrombin, histamine,  $H_2O_2$ , and VEGF are capable of inducing FAK tyrosine phosphorylation and recruiting focal adhesions in endothelial cells (1, 137, 587, 795, 996, 1065). In response to these mediators, FAK was presumed to increase endothelial permeability. However, direct perturbation of FAK function using genetic strategies showed that FAK is probably involved in maintaining the integrity of endothelial barrier (587, 732). Depletion of FAK using an antisense oligonucleotide inhibited tyrosine phosphorylation of paxillin, and resulted in augmented and pro-

longed increase in endothelial permeability in response to thrombin (587) (Fig. 16*B*).

Increased activity of FAK has also been implicated in the mechanism of hyperosmolarity-induced strengthening of AJs (732). Exposure of confluent lung microvessel cells to hyperosmolar stress increased FAK activity and VE-cadherin accumulation at cell membrane junctions and induced barrier strengthening (732). In contrast, expression of a kinase-deficient mutant of FAK prevented accumulation of peripheral VE-cadherin and increased transendothelial electrical resistance in response to the hyperosmolar stimulus. Given the evidence that FAK interacts with and activates the Cdc42 effector WASP (1071), it is possible that FAK strengthens endothelial barrier function by promoting the assembly of AJs via Cdc42-dependent mechanisms.

The Src family of non-receptor tyrosine kinases consists of 10 proteins with significant sequence homology (see Ref. 939). All Src family members, except Syk/ZAP, consist of the following domains:  $NH_2$  terminal, Src ho-



mology (SH) 4, unique region, SH3 domain (which generally binds Pro-rich regions), SH2 (binds specific phosphotyrosine-containing motifs), linker region, kinase domain, and a short COOH terminus containing a conserved and critical tyrosine residue. Src kinase activity is normally held in abeyance through intramolecular interactions of its SH2 with phosphotyrosine-527 and of SH3 with a polyproline motif in the linker region, and lack of activating phosphotyrosine-416 in the kinase domain. Src activation occurs downstream of integrin activation that follows integrin clustering. Activated Src interacts with FAK, which in turn modifies residues within the Src kinase domain. Disruption of the intramolecular interactions of the SH2 and SH3 domains leads to Src activation. Auto-phosphorylation of Src on Tyr-416 induced by FAK is necessary for full Src activity (for review, see Ref. 939). Of the various members of the Src family (939), pp60Src kinase has been shown to contribute in the mechanism of increase endothelial permeability in response to production of the superoxide anion ( $O_2^-$ ) (305, 846), thrombin (953), and VEGF (61, 693, 1043). Inhibition of pp60Src activity by PP1 blocked the TNF- $\alpha$  augmentation of thrombin-induced permeability increase in endothelial cells by inhibiting  $Ca^{2+}$  entry (953) and the internalization of plasma membrane-associated cadherin-catenin complex (953). pp60Src also regulates endothelial contraction by promoting tyrosine phosphorylation of MLCK and cactactin (88, 224, 305) and thus signals increased permeability. In addition, pp60Src is involved in neutrophil-induced hyperpermeability through its association with  $\beta$ -catenin and tyrosine phosphorylation of  $\beta$ -catenin in the microvascular endothelium (946). Weis et al. (1043) demonstrated that VEGF did not induce an increase in vascular permeability in mice lacking pp60Src kinase or lacking another Src family member Yes (1043). Because pp60Src activity is also required in signaling caveolae-mediated albumin transcytosis (described in sects. x, *A1* and *B1*, and *xIA1*), it is possible that pp60Src kinase is involved in the mechanism of increased endothelial permeability by regulating both junctional permeability as well as transcytosis.

Proline-rich tyrosine kinase 2 (PYK2) is a  $Ca^{2+}$ -dependent non-receptor tyrosine kinase structurally related to FAK that also binds integrins (42, 517, 785, 1093). PYK2 is highly expressed in pulmonary endothelial cells (927). PYK2 is rapidly phosphorylated following exposure of endothelial cells to agonists (e.g., angiotensin) and mechanical stress (e.g., cyclic strain) (161, 918, 928). However, its role in signaling increased endothelial permeability remains to be established. There is evidence that PYK2 can activate RhoA in fibroblasts (845), suggesting that PYK2 may be involved in regulating endothelial barrier function by this mechanism.

A number of focal adhesion-associated proteins talin,  $\alpha$ -actinin, paxillin, vinculin, tensin, p130Cas, filamin, and

zyxin bind either directly or indirectly with integrins (see review in Ref. 310) (Fig. 15). As these proteins interact with actin filaments, they appear to coordinate signals between focal adhesions and the actin cytoskeleton. These proteins are recruited to focal adhesions upon activation of endothelial cells, and thereby form the focal adhesion complex (FAC) (1, 137, 138, 587, 624, 764, 795, 985). Phosphorylation of these proteins by pp60Src and FAK may regulate their recruitment to focal adhesions (see reviews in Refs. 130, 682). In addition, focal adhesion proteins also interact with components of IEJs. Vinculin and  $\alpha$ -actinin bind to  $\alpha$ -catenin (1034, 1045), which in turn associates with ZO-1 (see sect. VIII A). This interaction of focal adhesion proteins with IEJs may regulate IEJ function, although direct proof of the functional significance of focal adhesion-IEJ cross-talk is lacking.

The transmembrane domains of integrins also participate in protein-protein interaction, and in some cases these interactions may occur at sites distinct from focal adhesions. For example, integrins bind to tetraspan or TM4 proteins (66), glycosylphosphatidylinositol (GPI)-linked urokinase-type plasminogen activator receptor (uPAR), and caveolin-1 (155, 1029, 1030, 1041, 1053) (Fig. 15). The nature of integrin interactions with these proteins and their consequences on endothelial permeability have not been well delineated. Inhibiting caveolin-1 expression was shown to suppress the formation of focal adhesions as well as integrin signaling (1030, 1041). Deletion of caveolin-1 in mice caused abnormal development of basement membranes and increased paracellular endothelial permeability (822), raising the possibility that integrin's interaction with caveolin-1 contributes to maintaining integrin-ECM interactions, and hence, it could regulate endothelial barrier function.

## IX. SIGNALING MECHANISMS REGULATING PARACELLULAR PERMEABILITY

A change in cell shape, i.e., cell rounding, results in the concurrent formation of gaps between endothelial cells and consequently is a primary determinant of increased endothelial permeability to plasma proteins (see Refs. 210, 225 and 553 for reviews). Based on the "tensegrity model" of Ingber (413), endothelial cells maintain their characteristic flattened shape through centrifugal tension generated by interconnected microtubule struts, focal adhesions, and IEJs that resists the centripetal tension generated by actinomyosin cross-bridging (622; see Ref. 225 for review) (M. Konstantoulaki, unpublished observations) (Fig. 17). However, the precise relationship between these opposing forces after stimulation with permeability-increasing agonists and how these forces contribute to the increase in endothelial permeability is un-



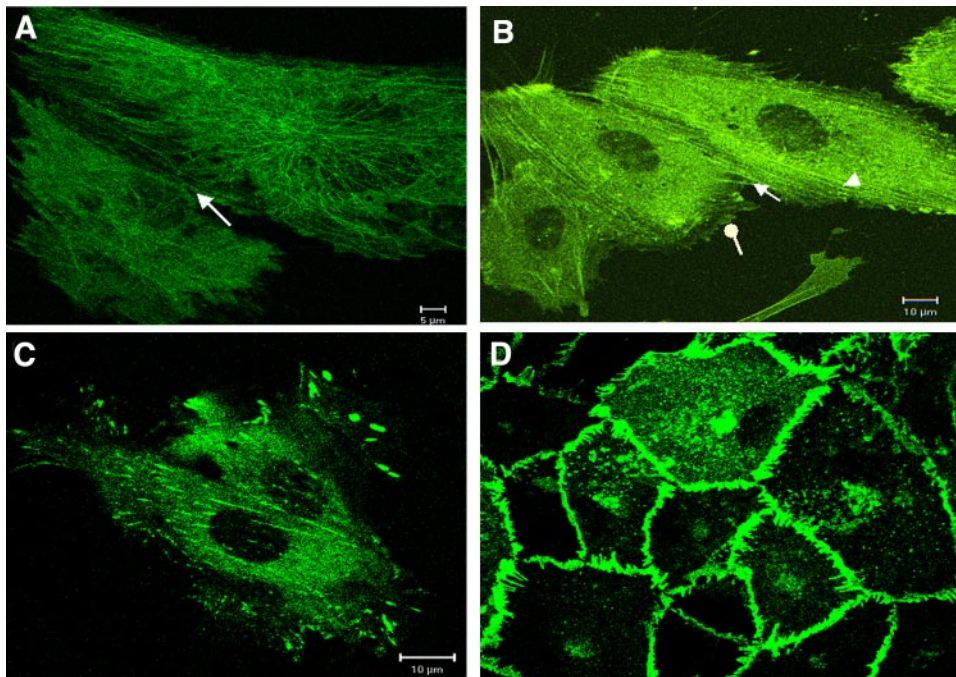


FIG. 17. Microtubules, actin, focal adhesions, and adherens junctions of endothelial cells visualized with GFP-tubulin (A), YFP-actin (B), GFP-FAK (C), and GFP-VE-cadherin (D). Microtubules span the cytoplasm and appear to interconnect with microtubules from adjacent cells (arrow) (A). Actin is organized into filaments concentrated at the cell periphery (arrowhead). Like microtubules, actin filaments at the periphery interdigitate with actin filaments of neighboring cells (arrow) (A). A few lamellipodia and filopodia are also seen at the cell periphery (circle-headed arrow) (B). FAK is organized at focal adhesions (C), and VE-cadherin expression is localized at IEJs (D). Interactions between actin, microtubules, IEJs, and focal adhesions regulate endothelial cell shape crucial for maintaining barrier function (see text for details).

clear. It was however shown that these agonists increase the formation of actin stress fibers (see Ref. 225) and destabilize microtubules (92, 333). Microtubule destabilization was shown to induce the activation of RhoA (90, 93, 982), potentially through the activation of microtubule-associated p190 RhoGEF (982) or via p115RhoGEF (93) and p38 mitogen-activated protein kinase (MAPK) (91), which may result in increased actin stress fiber formation and endothelial contraction. It is possible, therefore, that alterations in microtubule assembly amplify endothelial cell contraction (beyond that induced by actinomyosin cross-bridging) and lead to endothelial cell rounding. Thus the inverse relationship between microtubule stability and actin stress fiber formation may be an important factor increasing junctional permeability by weakening IEJs. In response to actinomyosin-mediated endothelial cell contraction, there is an increase in focal adhesions and strengthening of the endothelial cell-ECM association (587, 622; see Ref. 225 for review). This change occurring at the level of ECM attachment sites during the barrier recovery phase may be essential to the restoration of the flattened endothelial cell phenotype required for barrier function (587). Thus the processes involved in recovery of endothelial permeability are at least at this level the opposite of those responsible for increasing endothelial permeability, although the specifics of the “off” switches signaling barrier recovery have not been delineated. The following sections describe the role of the signaling pathways that regulate the centripetal (contractile) and centrifugal (tethering) forces controlling endothelial permeability during the

phases of increased permeability during barrier dysfunction and of restoration of barrier function.

## A. Paracellular Mechanisms of Increased Endothelial Permeability

### 1. Role of actin-myosin motor

The most direct evidence that endothelial cells contract in response to permeability-increasing agonists and induce opening of IEJs is based on the extensive ultrastructural studies of Majno and Palade (560) and more recently by actual isometric force measurements in endothelial cell monolayers grown on a collagen matrix (471, 617). Upon stimulation with thrombin, the monolayer contractile force doubled from a basal value of 70 to 132 dynes (471). Tension developed by the endothelial cells ( $1.3 \times 10^5$  dynes/cm<sup>2</sup>) was an order of magnitude less than that developed by contracting arterial smooth muscle cells ( $6.7 \times 10^6$  dynes/cm<sup>2</sup>) (471). The primary function of the contractile apparatus in endothelial cells is to regulate endothelial barrier permeability. In the presence of thrombin, the endothelial contractile force rose to its peak level within 5 min and remained elevated for up to 2 h (327); however, the kinetics and magnitude of this response are likely dependent on the specific agonist and its concentration as well as cell surface receptors and their affinities, and even the site of origin of the endothelial cell. For example, transendothelial resistance (TER), exhibits a change directly corresponding to the formation of IEJ gap, in response to histamine and thrombin differ in

magnitude and time of recovery (620, 622). Thrombin (25 and 50 nM) decreased TER, which reached a peak negative value ( $\sim -50\%$ ) within 30 min and recovered to baseline within 2 h (587, 620, 622, 847, 951). In contrast, histamine (10  $\mu\text{M}$ ) induced a short-lived decrease in TER where the maximum decline ( $\sim 20\%$ ) occurred within 2 min and was followed by recovery to baseline by 8 min (620). TER measurement reflects two serial components of monolayer resistance: 1) between adjacent endothelial cells and 2) between the endothelial cells and the substrate (539, 620). In this regard, Moy et al. (620) further showed that in contrast to thrombin, histamine decreases TER primarily by disrupting adhesion between adjacent endothelial cells. However, most studies of TER have not parsed the TER values in these terms, and consequently, the contribution of each component in response to various permeability-increasing mediators has yet to be established.

Stress fibers composed of bundles of polymerized actin and myosin filaments are the primary elements of the contractile machinery of endothelial cells (reviewed in Ref. 225). These fibers assemble in a characteristic manner in cultured endothelial cells in response to permeability-increasing mediators. Actinomyosin-generated contraction requires MLCK-dependent phosphorylation of the regulatory MLC on Ser-19 (mono-phosphorylation) or Ser-19/Thr-18 (diphosphorylation). On thrombin stimulation of human umbilical vein endothelial cells, isometric contraction increased 2- to 2.5-fold within 5 min while MLC phosphorylation increased from a basal value of 0.54 to 1.61 mol  $\text{PO}_4/\text{mol}$  MLC within 60 s primarily as the result of diphosphorylation of MLC (327). This shows that diphosphorylation of MLC is an important determinant of endothelial contraction. MLC phosphorylation has also been shown to regulate the permeability of intact isolated postcapillary venules and pulmonary microvessels in response to histamine and thrombin (1011, 1095; see Ref. 1094). There is evidence in saponin-permeabilized endothelial monolayers that p21-activated kinase (PAK) can modulate phosphorylation of MLC and tension development (326, 1099); however, whether it has a role in increasing endothelial permeability in response to specific agonists remains to be established.

The precise relationship between MLC phosphorylation and IEJ gap formation in endothelial monolayers is not clear, but its understanding is important to establish the basis of increased paracellular permeability. The maximal increase in MLC phosphorylation in response to an agonist such as thrombin precedes maximal tension development and TER decrease (327; see Ref. 225). Cell separation invariably occurred at the level of IEJs (210, 473, 475, 620, 622, 1056). After formation of IEJ gaps, only strands of AJs remained linked with actin filaments (473, 495, 1056). Thus there is a temporal relationship between

the generation of the contractile force induced by MLC phosphorylation and IEJ gap development.

It is useful to examine the sequence of signaling events in response to permeability-increasing mediators that open IEJs by the cell contraction mechanism. The earliest known event following activation of the protease-activated receptor (PAR)-1 receptor by thrombin is a rise within seconds in intracellular  $\text{Ca}^{2+}$ , and this is thought to be the essential signal responsible for the phosphorylation of the  $\text{Ca}^{2+}/\text{CaM}$ -dependent MLCK (see sect. IXA1A for details of MLCK activation). MLCK causes actin-myosin stress fibers to develop force (327). Intracellular  $\text{Ca}^{2+}$  rise, MLCK activation, and endothelial cell contraction all precede the formation of gaps between IEJs. In addition to MLC phosphorylation, evidence indicates a role for actin polymerization in endothelial contraction and the increased permeability response (16, 244, 256, 302, 327, 550, 587, 706, 795, 898, 913, 941, 944, 1017). Disruption of the basal level of actin polymerization by C2 toxin (244) or cytochalasin (1032) increased microvessel permeability (1032) and induced pulmonary edema (244), presumably by interfering with the actin cytoskeleton and endothelial cell shape. Inhibition of actin polymerization also prevented force generation (471) as well as an increase in endothelial monolayer permeability in response to permeability-increasing mediators such as thrombin (302, 471, 587, 1016). Stimulation of endothelial cells with thrombin increased polymerization of actin filaments as determined by the conversion of monomeric (G) actin to filamentous (F) actin (236, 587, 944, 1016), whereas inhibition of actin polymerization by cytochalasin D or latrunculin abrogated the contraction response (327, 587, 622). These results provide support for the key role of actin polymerization in inducing endothelial contraction. After stimulation with thrombin, endothelial cortical actin content declined and actin reorganized to form stress fibers (236, 327, 587, 782, 795, 987, 1016); that is, actin was redistributed and the F-actin content increased. However, the specific sites of actin polymerization during endothelial cell contraction remain unclear. Actin stress fibers make contact with integrin-associated focal adhesions (i.e., talin, vinculin, paxilin, and FAK, each of which can, in turn, associate with the barbed ends of actin filaments) as well as the protein constituents of AJs and TJs (discussed in sect. VIII, A and B). Because of the complexity of the linkages of actin with TJs and AJs, actin polymerization occurring at specific sites may influence the function of both AJs and TJs, thereby affecting endothelial permeability. The following section discusses details of the mechanisms that induce phosphorylation of MLC and subsequent actin polymerization.

A) ROLE OF MLCK. MLCK is a  $\text{Ca}^{2+}/\text{CaM}$ -dependent enzyme that phosphorylates MLC at Ser-19 or Ser-19/Thr-18 (327); activation of MLCK was shown to produce endothelial cell contraction and result in barrier dysfunction in



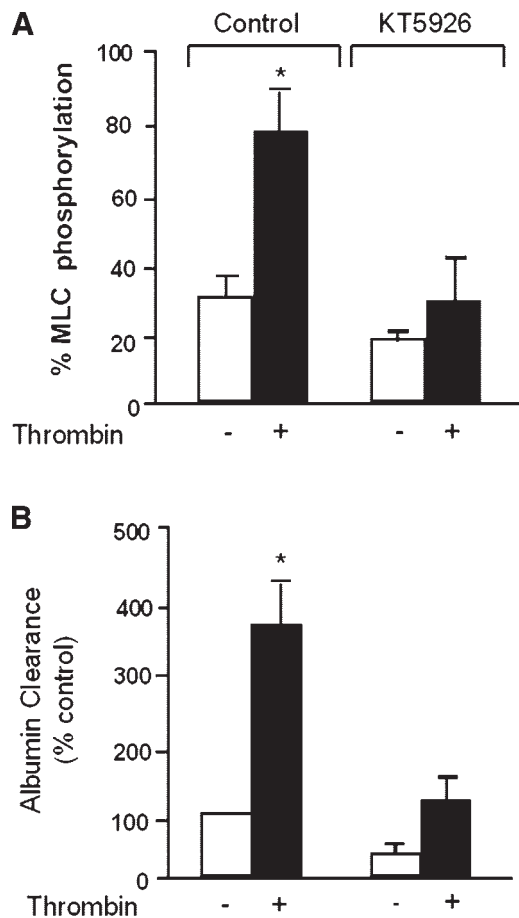


FIG. 18. Role of MLCK in regulating endothelial contraction and permeability. Pretreatment of bovine pulmonary arterial endothelial cells with KT5926, an inhibitor of MLCK, inhibited thrombin-induced MLC-phosphorylation (A) and transendothelial albumin permeability (B). \*Statistically significant. [Modified from Garcia and Schaphorst (301).]

response to inflammatory mediators thrombin and histamine (reviewed in Ref. 225) (Fig. 18, A and B). Pharmacological inhibition of MLCK using KT5926 or ML-7 prevented the increase in vascular permeability (298, 945, 1095).

In human endothelial cells, MLCK (EC MLCK) is a 214-kDa protein encoded from a single gene on chromosome 3. The same gene also encodes the smaller (130–150 kDa) smooth muscle MLCK isoform (see Ref. 225). Comparisons of  $V_{max}$  and  $K_m$  of EC MLCK and smooth muscle MLCK revealed similar values for substrate affinity ( $K_m$ ) and  $V_{max}$  (88). However, EC MLCK was found to have a lower affinity for the regulators,  $Ca^{2+}$ /calmodulin;  $K_{0.5(CaM)}$  of EC MLCK is twofold greater than that of the smooth muscle isoform (88). Structurally, EC MLCK contains all the domains present in the smooth muscle form, but in addition, EC MLCK has a unique 922-amino acid  $NH_2$ -terminal domain containing consensus sites that may be phosphorylated by multiple protein kinases, including

cAMP-dependent protein kinase A (PKA) (299, 999), PKC (99, 787), PAK (326, 781), Src (88), and  $Ca^{2+}$ /CaM-dependent protein kinase II (CaMKII) (999) (Fig. 19).

EC MLCK phosphorylation on Ser/Thr by PKA (999) or PAK (781, 1099) reduced its catalytic activity (326, 781, 999). Thus the contractile activity of EC MLCK is critically dependent on Ser/Thr phosphorylation. There is also evidence that extracellular signal-regulated kinase (ERK-1 and -2) belonging to the family of mitogen-activated protein kinases (MAPK) can phosphorylate MLCK (465), and thereby regulate MLC phosphorylation (465, 565). However, ERK regulation of EC MLCK activity remains to be fully established. In addition, EC MLCK  $NH_2$ -terminal domain contains SH2 sites for protein-protein interactions and sites for pp60Src-catalyzed tyrosine phosphorylation (Fig. 19) (88, 299, 305). EC MLCK forms a complex with pp60Src and cortactin (224, 305). Cortactin is both a Src substrate (1061, 1062) and an actin-binding protein (reviewed in Ref. 1039). Association of the pp60Src-cortactin-EC MLCK complex is regulated by pp60Src phosphorylation of either EC MLCK at Y464 or Y471 or cortactin at Y421, Y466, and Y482 (224). However, in vitro assays showed that the interaction of EC MLCK with cortactin had no effect on MLCK enzymatic activity, whereas it inhibited EC MLCK binding to F-actin (224). These findings suggest that EC MLCK apart from its contractile activity has an important role in regulating actin reorganization. Further studies using phosphodeficient mutants of EC MLCK and EC MLCK mutants unable to bind cortactin are needed to address the functional importance of the phosphorylation and cortactin interactions in regulating endothelial contraction.

The earliest evidence that MLCK was intimately involved in regulating endothelial barrier permeability via phosphorylating MLC came from permeabilized bovine pulmonary artery endothelial cell monolayers (1072, 1073). These monolayers retracted upon addition of MLCK plus calmodulin but not when either was added alone. Subsequent studies established the role of MLCK in regulating endothelial permeability in cultured monolayers as well as in in vivo models of vascular permeability using pharmacological inhibitors of MLCK (298, 457, 679, 841, 1095, 1106; also see Ref. 1094). In addition, expression of the constitutively active form of MLCK was shown to elevate basal permeability of venules and venular endothelial cells in culture (945). Increased MLCK activity and MLC phosphorylation in endothelial cell monolayers were required for transendothelial PMN migration in response to chemotactic agents, presumably secondary to barrier breakdown (304, 375, 776, 945). The development of a strain of MLCK selective knock-out mice lacking only the long isoform, *EC MLCK*<sup>-/-</sup>, supports the in vivo role of EC MLCK-induced endothelial cell contraction in the mechanism of increased vascular permeability (1022). *EC MLCK*<sup>-/-</sup> mice were protected against increased lung



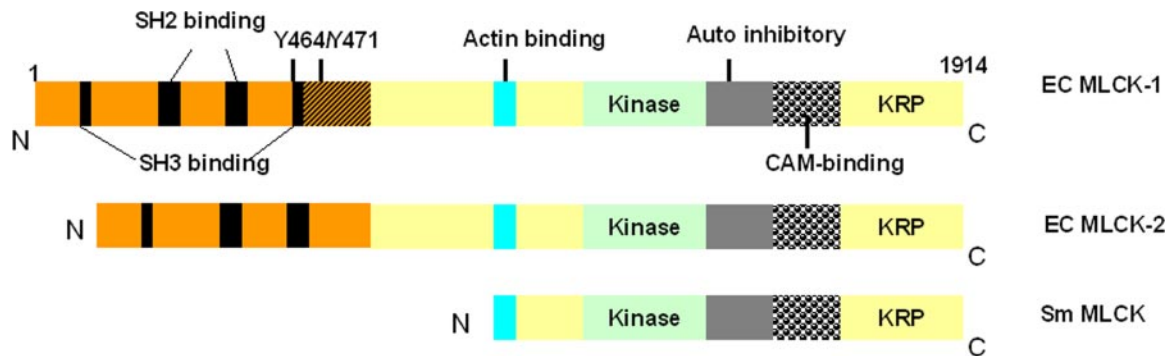


FIG. 19. Domain structure of EC MLCK. EC MLCK contains the following domains: actin-binding, kinase, calmodulin binding (CAM), and kinase-related proteins (KRP) (identical to those in the smooth muscle MLCK isoform). In addition, EC MLCK has a unique 922-amino acid NH<sub>2</sub>-terminal domain, which contains consensus phosphorylation sequences for protein kinases such as cAMP-dependent protein kinase A (PKA), SH2 and SH3 domains for protein-protein interactions, and sites for Src-catalyzed tyrosine phosphorylation (Y464/Y471). Endothelial cells also express several smaller splice variants of EC MLCK such as EC MLCK2, which lacks tyrosine phosphorylation sites. [Adapted from Dudek and Garcia (225).]

vascular permeability induced by intraperitoneal injection of the bacterial product lipopolysaccharide (LPS) (1022). Other studies showed that the thrombin-induced increase in lung microvessel permeability was prevented in *EC MLCK*<sup>-/-</sup> mice (S. Vogel, unpublished observations), indicating a critical role of endothelial contraction induced by EC MLCK in mediating the increase in vascular permeability. Thus *EC MLCK*<sup>-/-</sup> mice may prove to be useful in sorting out the intracellular signaling pathways activated by MLCK that mediate increased vascular permeability in the setting of inflammation.

**B) ROLE OF SERINE-THREONINE PHOSPHATASES.** Phosphoprotein phosphatases (PPP family) regulate endothelial cell contraction by dephosphorylating phosphor-MLC, and thus have an important influence on barrier function. The PPP family includes type 1 (PP1) and type 2 (PP2A, PP2B or calcineurin, and PP2C) enzymes. These phosphatases can be distinguished on the basis of substrate specificity, divalent cation requirements, and their sensitivity to pharmacological inhibitors such as okadaic acid and calyculin (176, 844). Okadaic acid, a toxin isolated from black sponges, inhibits PP2A more potently than PP1 or PP2B and has no effect on PP2C (83, 416). Calyculin inhibits both PP1 and PP2A with equal potency *in vitro* (416). PP1 and PP2A have no divalent cation requirement for their activity, whereas PP2B is a Ca<sup>2+</sup>-dependent phosphatase containing a calmodulin-binding subunit (924). Studies showed that the presence of calyculin but not okadaic acid resulted in an increase in MLC phosphorylation levels and increased endothelial permeability (839, 1000), presumably as a result of inhibition of PP1 activity. Calyculin also increased basal endothelial permeability in intact vessels (1095). Inhibition of PP1 with calyculin did not further increase thrombin-induced MLC phosphorylation (839, 998), which was interpreted to mean that thrombin inhibited a calyculin-sensitive phosphatase. However, phosphorylation of the regulatory subunit of PP1 by Rho

kinase, a specific target protein of the GTPase RhoA (described in sect. *IXA1D*), inhibited PP1 activity and resulted in increased MLC phosphorylation (93, 247, 248, 997). Interestingly, inhibition of PP2B with pharmacological agents had no effect on basal endothelial permeability, but augmented the thrombin-induced increase permeability response as the result of prolonged increase in MLC phosphorylation (998) as well as PKC- $\alpha$  activity (554). As PKC- $\alpha$  was shown to regulate RhoA activation (381, 586), it will be important to elucidate whether PP2B activity (like PP1) is functionally linked to the RhoA pathway, and thereby regulates endothelial barrier function. In nonendothelial cells, calyculin increased the level of phosphoserine/threonine on  $\beta$ -catenin (829), raising the possibility that calyculin-sensitive protein phosphatases may also regulate endothelial permeability by affecting additional relevant targets other than MLC. These findings show that both PP1 and PP2B are required for modulating endothelial barrier function by their ability to dephosphorylate MLC, and perhaps also IEJ components such as  $\beta$ -catenin.

**C) ROLE OF TRANSIENT RECEPTOR POTENTIAL CHANNELS.** An increase in cytosolic Ca<sup>2+</sup> has been established as the initial pivotal signal that precedes endothelial cell shape change and the opening of IEJs that results in barrier dysfunction (see Refs. 641, 952 for reviews). As discussed above, increased cytosolic Ca<sup>2+</sup> resulting in actinomyosin-induced cell contraction is a well-accepted pathway of increased endothelial permeability (reviewed in Ref. 225). After cell stimulation with inflammatory mediators, an increase in cytosolic Ca<sup>2+</sup> concentration is apparent. There is an initial transient peak as the result of Ca<sup>2+</sup> release from endoplasmic reticulum (ER) stores, which is followed by a more sustained response secondary to Ca<sup>2+</sup> entry via plasmalemma channels (see Refs. 641 and 952). Ca<sup>2+</sup> entry refills ER stores and sustains Ca<sup>2+</sup> signaling. Proteins of the transient receptor channel (TRPC) family

are nonselective cation channels present in endothelial cells that increase intracellular  $\text{Ca}^{2+}$  and play an important role in regulating actin-myosin motor activity and endothelial barrier function.

Expression of the various TRPCs differs among human endothelial cells. The majority of human endothelial cells highly express TRPC1 and TRPC6, whereas TRPC3, TRPC4, and TRPC7 are expressed only weakly (641, 952). TRPC1, TRPC4, and TRPC5 are activated by depletion of ER stores and are therefore called store-operated channels (SOCs), whereas TRPC3 and TRPC6 are activated independently of store depletion and are referred to as receptor-operated channels (ROC). SOCs and ROCs provide the primary structures by which  $\text{Ca}^{2+}$  enters endothelial cells. Multiple heterologous combinations of TRPCs (TRPC1 with TRPC4 or TRPC5 and TRPC3 with TRPC6) combine to produce tetrameric channels with unique properties. Although the role of ROC in the mechanism of increased endothelial permeability is not established, ROC has been implicated in thrombin-induced activation of RhoA (874). As RhoA is known to increase endothelial permeability (reviewed in Ref. 225), ROC-mediated  $\text{Ca}^{2+}$  entry may play an important role in regulating endothelial barrier function. In contrast, SOC activation mediated by TRPC1 or TRPC4 has been clearly shown to regulate endothelial barrier function (10, 121, 677). Antisense depletion of TRPC1 in cultured endothelial cells (121) or inhibition of TRPC1 with an anti-TRPC1-blocking antibody (10) reduced  $\text{Ca}^{2+}$  entry by 50% and also decreased endothelial permeability in response to thrombin (10). Overexpression of TRPC1 in endothelial cells increased  $\text{Ca}^{2+}$  entry and in turn the formation of actin stress fibers and increased endothelial permeability (677). In addition, the *in vivo* relevance of TRPC-induced  $\text{Ca}^{2+}$  entry in regulating permeability of microvessels has been demonstrated using *TRPC4*<sup>-/-</sup> mice (949) (Fig. 20C). The PAR-1 (thrombin receptor) activation-induced increase in lung microvessel permeability in mice was reduced by 50% using lanthanum ( $\text{La}^{3+}$ ), a known  $\text{Ca}^{2+}$  channel blocker (949), indicating  $\text{Ca}^{2+}$  entry by this pathway contributed to a portion of the increased vascular permeability response. *TRPC4*<sup>-/-</sup> mice (275) also demonstrated a reduction in the thrombin-induced increase in lung microvessel permeability by ~50%, whereas this response was not affected by  $\text{La}^{3+}$  (Fig. 20C). Microvessel endothelial cells isolated from *TRPC4*<sup>-/-</sup> mice also showed an inhibition of  $\text{Ca}^{2+}$  entry (G. U. Ahmed, unpublished observations) (Fig. 20, A and B), actin stress fiber formation, and increase in endothelial monolayer permeability in response to thrombin (949). These findings collectively demonstrate that thrombin-activated  $\text{Ca}^{2+}$  store depletion and the subsequent  $\text{Ca}^{2+}$  entry via TRPC4 is a determinant of increased endothelial permeability, but that it does not provide a complete understanding of the

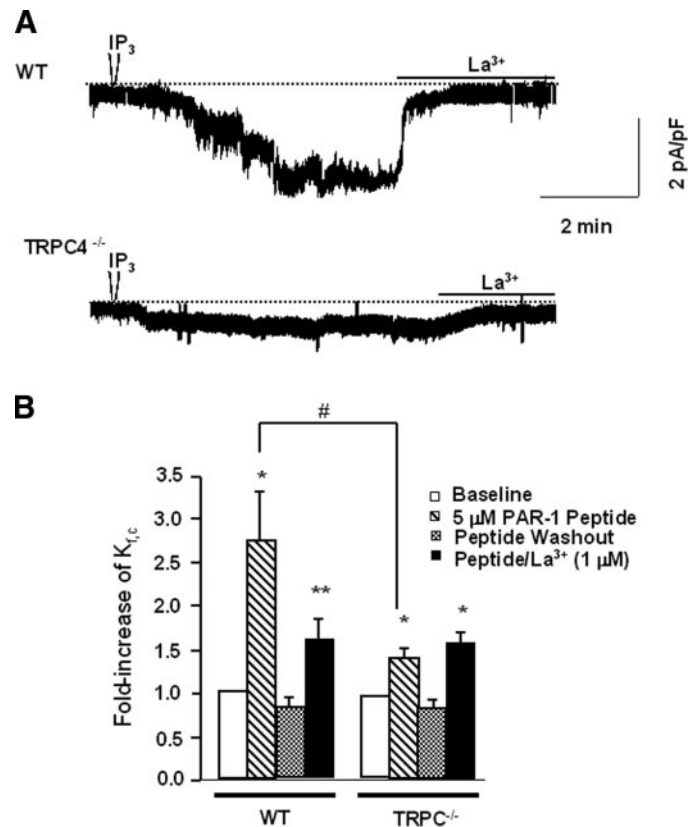


FIG. 20. TRPC4 activation of store-operated  $\text{Ca}^{2+}$  entry (SOCs) regulates microvessel permeability. A:  $\text{IP}_3$ -induced activation of lanthanum ( $\text{La}^{3+}$ )-sensitive SOCs is shown as whole cell continuous current recordings at a holding potential of  $-50$  mV. Traces show that in endothelial cells isolated from wild-type (WT) mice, internal dialysis with  $\text{IP}_3$  induced store-operated current, indicating  $\text{Ca}^{2+}$  entry. Deletion of TRPC4 in mice (*TRPC4*<sup>-/-</sup>) prevented the  $\text{Ca}^{2+}$  entry in response to store depletion by  $\text{IP}_3$ . Scale represents picoampere (pA) and picofarad (pF). B: in WT mice, PAR-1 peptide induced a 2.5- to 3-fold increase in lung microvessel permeability, which was reduced to 50% by  $\text{La}^{3+}$ , the  $\text{Ca}^{2+}$  channel blocker, indicating  $\text{Ca}^{2+}$  entry contributes to the mechanism of increased lung vascular permeability. However, in *TRPC4*<sup>-/-</sup> mice, PAR-1 peptide only induced a 1.5-fold increase in permeability, which was not affected by  $\text{La}^{3+}$ , indicating  $\text{Ca}^{2+}$  entry via TRPC4 contributes to the regulation of microvessel permeability. \*Different from baseline value. \*\*Inhibitory effect of  $\text{La}^{3+}$ . #Significantly reduced response of *TRPC4*<sup>-/-</sup> for PAR-1 agonist peptide compared with WT. [B adapted from Tiruppathi et al. (949).]

role of  $\text{Ca}^{2+}$  entry by this mechanism since the permeability response was only partly reduced in *TRPC4*<sup>-/-</sup> mice.

The signals in endothelial cells inducing  $\text{Ca}^{2+}$  entry following depletion of ER  $\text{Ca}^{2+}$  stores are not fully understood. Evidence from human endothelial cells indicates that a coupling between the  $\text{IP}_3$  receptor and TRPC1 could be mediated through RhoA (583) as inhibition of RhoA prevented the thrombin-stimulated association of the  $\text{IP}_3$  receptor with TRPC1 and influx of  $\text{Ca}^{2+}$  (583). RhoA may facilitate the interaction of the  $\text{IP}_3$  receptor with TRPC1 by promoting actin polymerization. Studies also showed that TRPC1 activity was suppressed by de-

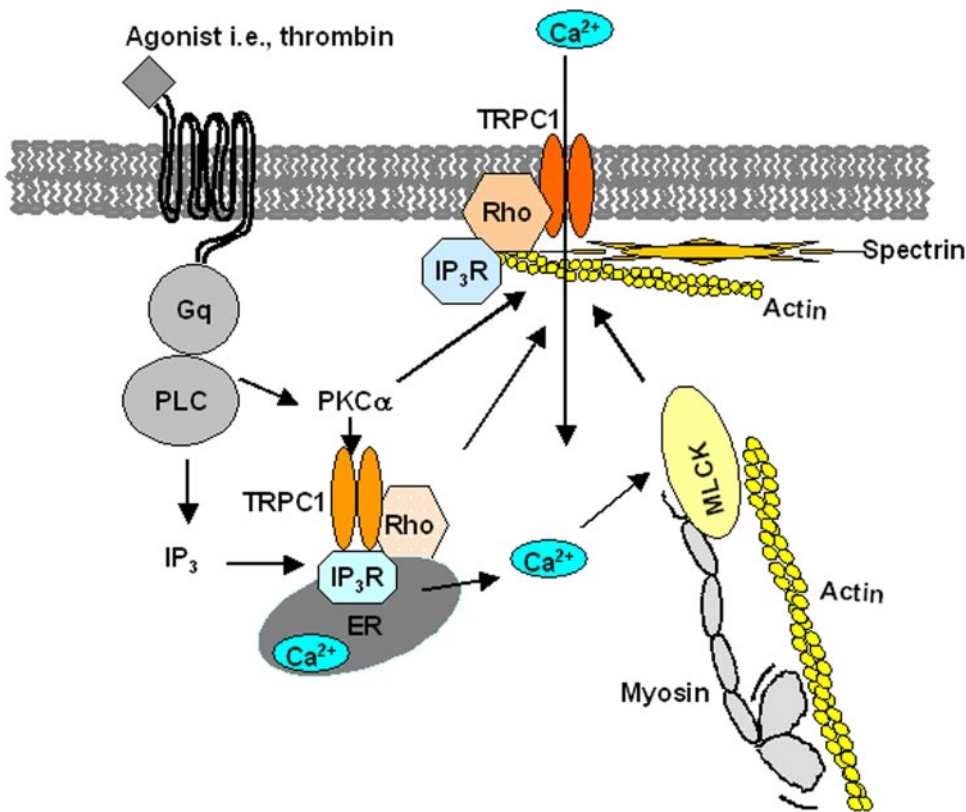


FIG. 21. Postulated mechanisms of Ca<sup>2+</sup> entry following depletion of endoplasmic reticulum (ER) Ca<sup>2+</sup> stores. Coupling between IP<sub>3</sub>R and TRPC1 could be mediated through cytoskeletal linkage (actin filaments and spectrin) or through activation of the small GTPase RhoA downstream of Gq-PLC-PKC-α. Phosphorylation of TRPC1 by PKC-α may directly activate Ca<sup>2+</sup> channels or it may promote interaction of channels and RhoA leading to channel activation. MLCK may regulate store-operated Ca<sup>2+</sup> entry by inducing either phosphorylation of channels or cytoskeletal reorganization. [Modified from Mehta et al. (583).]

polymerization of actin filaments (380, 583) consistent with this model. In addition, SOC-induced Ca<sup>2+</sup> entry was shown to require the interaction of spectrin, an actin cross-linking protein (352), with protein 4.1 (1070) (Fig. 21). Another signaling possibility is that phosphorylation by PKC-α is required for TRPC1 activation (10). MLCK may also be involved in the regulation of SOC-induced Ca<sup>2+</sup> entry as inhibition of MLCK with ML7 prevented the SOC-mediated Ca<sup>2+</sup> entry in endothelial cells (1035). Together, these findings raise several possibilities concerning the mechanism of SOC activation and influx of Ca<sup>2+</sup> in endothelial cells, but the question of which mechanism plays the dominant role in controlling Ca<sup>2+</sup> channel gating in endothelial cells in response to permeability-increasing mediators is unresolved. Moreover, the role of ROCs remains unclear. Studies using TRPC1 phosphodeficient mutants will help to clarify the contribution of TRPC1 phosphorylation (whether by PKC-α or EC MLCK) in the activation of TRPC1-dependent Ca<sup>2+</sup> entry and whether it is involved in mediating increased endothelial permeability. Similarly, studies with TRPC6 mutants (the dominant ROC in endothelial cells) are likely to be profitable in delineating the role of ROCs in endothelial permeability regulation.

D) REGULATION BY RHOA. The Rho family of monomeric GTPases, RhoA, Rac, and Cdc42, has been shown to play an important role in the regulation of endothelial barrier function in response to inflammatory agonists (1057; see

Ref. 1058 for review). While each of these GTPases has a specific effect on the dynamics of actin, RhoA is the major regulator of actin-myosin-induced contraction in endothelial cells (138, 247, 381, 586, 984, 987), and thereby is a key determinant of increased endothelial permeability (Fig. 22).

RhoA, through its downstream effector, Rho kinase (ROCK), stimulates phosphorylation of the myosin light chain phosphatase (MLCP or PP1) regulatory subunit, which attenuates the phosphatase activity (93, 247, 248, 997). Such an inhibition of dephosphorylation of MLC (primarily in the presence of ongoing Ca<sup>2+</sup>/CaM-dependent MLCK catalytic activity) results in a net increase in phosphorylated MLC (93, 247, 248, 997). Inhibition of MLC dephosphorylation may increase the Ca<sup>2+</sup> sensitivity of endothelial cells because phosphorylation of MLC was increased at a given intracellular Ca<sup>2+</sup> level (see Ref. 886). Thus the Rho-ROCK pathway may be able to sustain the endothelial cell contractile response in the presence of low cytosolic Ca<sup>2+</sup> and MLCK activity. In this regard, RhoA activation may modify the set-point for increased endothelial permeability such that the permeability response is greater or more prolonged in the presence of RhoA activation.

Various observations support the concept that the effector arm of the RhoA signaling pathway involves EC MLCK. Inhibition of RhoA by *Clostridium botulinum* toxin (C3 toxin) prevented oxidant-induced MLCK activ-



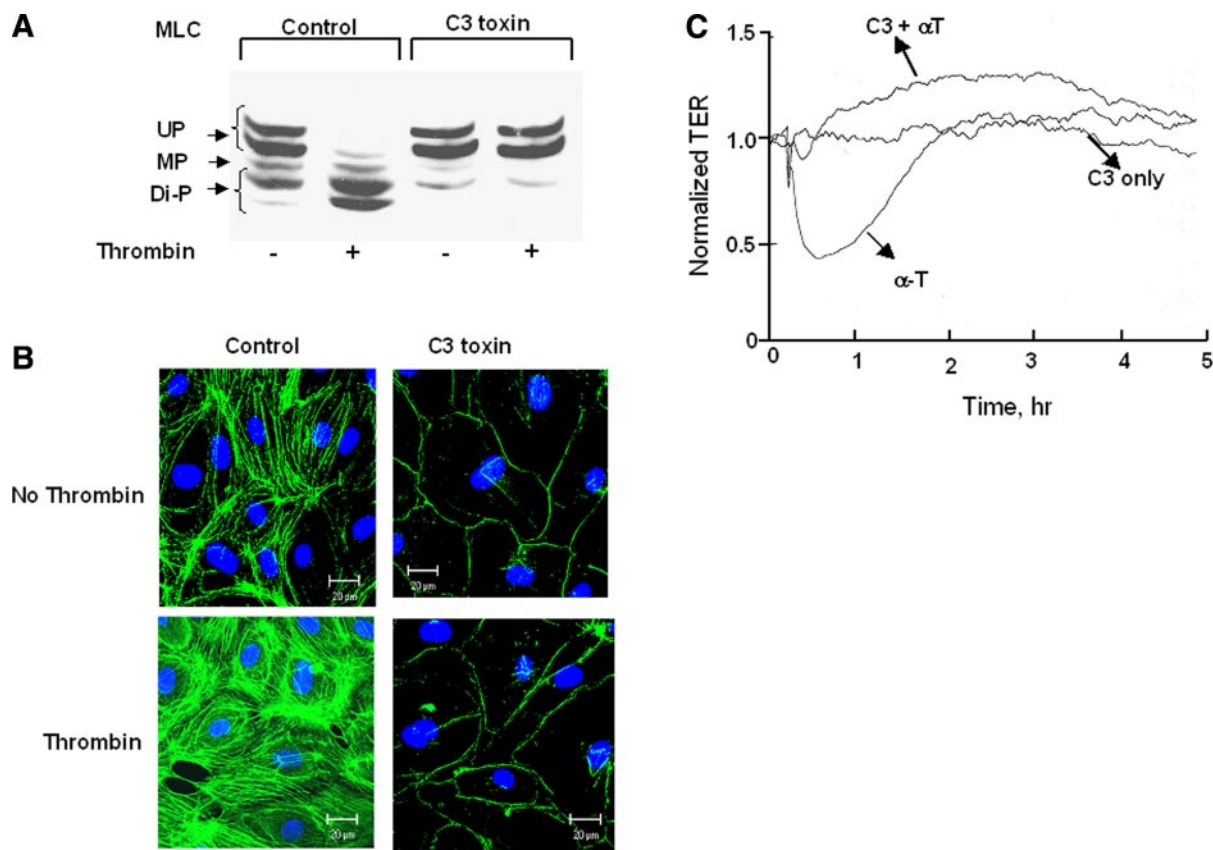


FIG. 22. RhoA regulation of endothelial permeability. Thrombin induces MLC phosphorylation (A), stress fiber formation (B), and a decrease in TER (C) in human pulmonary arterial endothelial cells (HPAEC). Inhibition of RhoA by C3 toxin blocked these responses, indicating RhoA plays an important role in increasing endothelial permeability by inducing endothelial contraction. UP, unphosphorylated MLC; MP, monophosphorylated MLC; Di-P, diphosphorylated MLC; C3, C3-toxin;  $\alpha$ -T,  $\alpha$ -thrombin. Note: 5 bands in the Western blot represent the smooth muscle as well as nonmuscle isoforms of myosin in HPAEC. [B and C adapted from Mehta et al. (583).]

ity (305) and the endothelial contraction response (586). Also, RhoA activation by thrombin increased the cytosolic  $Ca^{2+}$  level by stimulating entry of  $Ca^{2+}$  across the plasma membrane (583), which may be another factor responsible for the activation of MLCK.

RhoA may also increase endothelial contraction by regulating polymerization of actin through its effectors, diaphanous protein (mDia) and ROCK (761). Cofilin, an actin-depolymerizing protein, is negatively regulated by LIM kinase (558). ROCK and mDia activate LIM kinase, which by phosphorylating cofilin inhibited actin depolymerization (558, 658). ROCK also activated the ERM (ezrin/radixin/moesin) proteins (570) that were shown to function as cross-linkers between cortical actin and the plasma membrane (for review, see Ref. 964).

In contrast to RhoA, the role of Cdc42 and Rac in regulating the actinomyosin machinery in endothelial cells is less clear. There is some evidence that PAK, a downstream effector of both Cdc42 and Rac, inhibits MLCK activity (326, 781). However, it was also shown that a constitutively active PAK can directly induce endothelial contraction by phosphorylating MLC independently of

MLCK (326, 1099). The role of Cdc42-PAK-mediated MLC phosphorylation in the face of MLCK- and RhoA-induced MLC phosphorylation in regulating endothelial contraction remains unclear.

E) REGULATION BY ACTIN POLYMERIZING PROTEINS. As described above, actin polymerization is required for stress fiber formation, and hence endothelial cell contraction (Fig. 22). Two sequential processes, nucleation and elongation, culminate in the formation of F-actin polymers from G-actin monomers (see Ref. 222). In nucleation, three actin monomers form the nucleation site for polymerization. During elongation, polymerization of actin occurs primarily at the barbed ends by the addition of ATP-actin monomers. Alternatively, the severing of pre-existing actin filaments followed by the same elongation process described above can extend the actin filaments. Thus far, ~162 different proteins have been identified that regulate actin polymerization by inducing nucleation, elongation, severing, or cross-linking of actin filaments (for reviews, see Refs. 186, 222, 994). However, the role of these proteins in regulating endothelial contraction is not yet understood.

Filamin is a potentially important actin filament cross-linking protein (see Ref. 904). The primary function attributed to filamin in platelets is to stabilize the membrane-cytoskeletal interface (353). The apparent role of filamin in endothelial cells is to regulate the distribution of F-actin between the cortical actin band and actin stress fibers, but the mechanistic details are unclear. Maintaining the function of cortical actin at the plasma membrane may be important for promoting apposition with contiguous endothelial cells and, hence, may be required for endothelial barrier integrity. Filamin is known to associate with the cytoplasmic domains of  $\beta_1$ - and  $\beta_2$ -integrins, and this association is required for recruitment of filamin to the cell membrane (Fig. 15) (544, 834). Filamin also associates with E-selectin upon the binding of leukocytes to the luminal surface of endothelial cells (1092), and thus may help to anchor leukocytes to the endothelium during leukocyte recruitment and transendothelial migration. In unstimulated endothelial cells, membrane-associated filamin is constitutively phosphorylated by PKA (355, 1024, 1025). This appears to be a significant event because PKA phosphorylated filamin is protected from proteolysis by calpain (355, 1024, 1025).  $H_2O_2$ , which decreases constitutive activity of PKA, caused the dephosphorylation of filamin (355) and thus may disrupt the cortical actin band and contribute to increased endothelial permeability induced by oxidant exposure. In contrast to PKA, phosphorylation of filamin by CaMKII at the cortical actin band destabilizes the membrane association of filamin. Bradykinin and thrombin are activators of CaMKII (105, 1025), and both mediators cause translocation of filamin from the membrane to the cytosol (355, 1024, 1025), presumably because of a loss of filamin association with the plasma membrane. In the presence of a cell-permeable peptide inhibitor of CaMKII to prevent filamin phosphorylation, thrombin and bradykinin did not induce formation of actin stress fibers and IEJ gaps and the increase in endothelial permeability was prevented (105, 1025). This peptide inhibitor had no effect on MLCK activity or MLC phosphorylation (105), indicating that filamin regulated the increased permeability response independently of MLCK. Studies in nonendothelial cells have shown that filamin can also bind RhoGTPases (Rho, Rac, and Cdc42) and the Ras GTPase, Ral1 (659), ROCK (971), Trio (63), caveolin-1 (893) (see Fig. 15), and TNF receptor-associated factor 2 (TRAF2) (516). The binding of filamin to the Ral1-GTP recruited filamin into the filopodia of fibroblasts (659). Thus filamin may regulate endothelial barrier function by stabilizing cortical actin at the plasma membrane as well as by controlling the organization and distribution of actin filaments at the junctions.

Spectrin is another actin cross-linking protein (reviewed in Ref. 217a). Spectrin binds ZO-1 (421, 422), which interacts with the AJ protein  $\alpha$ -catenin (408, 628), GJ protein connexin-43 (959, 1064), and VASP (179). Spec-

trin through its actin cross-linking activity and interactions with IEJs components may help to stabilize the integrity of IEJs. In endothelial cells, the association of spectrin with band 4.1 was also shown to be required for activation of TRPC4-induced  $Ca^{2+}$  entry (1070; discussed in sect. *ixB1B*); however, it is not clear whether store-operated  $Ca^{2+}$  entry through a spectrin-based interaction controls IEJ barrier function.

Cortactin and WASP are other potentially important actin-binding proteins that regulate actin polymerization. Cortactin and WASP induce actin polymerization by binding the Arp2/3 heptameric complex of actin-polymerizing proteins (reviewed in Refs. 597 and 1039). Arp2/3 was shown to interact with E-cadherin (476) (Fig. 12) and vinculin (215) (Fig. 15), suggesting that cortactin and WASP through Arp2/3 may have a role in the assembly of AJs and focal adhesions. Cortactin is phosphorylated by Src after stimulation of endothelial cells with thrombin, oxidants, or shear stress (87, 305). Phosphor-cortactin in turn associates with EC MLCK (224, 305), but the functional role of this interaction is unknown. In nonendothelial cells, WASP is phosphorylated by FAK (1071), which prevents WASP translocation to the nucleus, and may prolong WASP-induced actin polymerization downstream of Cdc42 (1071). This may be a mechanism by which both FAK and Cdc42 activation occurring concurrently can restore barrier function (described in sect. *ixB2*).

Vasodilator-stimulated phosphoprotein (VASP) is another actin-binding protein that may regulate endothelial permeability. VASP through its poly-proline rich (PPR) domain associates with profilin, an actin monomer-binding protein (745, 778), and in vitro VASP reduces the capping activity of gelsolin (59), indicating that VASP may activate actin polymerization by modulating the function of capping- and actin-binding proteins. Interestingly, VASP is phosphorylated by the serine/threonine kinase PKA such that the phosphorylation induced VASP localization at the IEJ complex where it associated with ZO-1, occludin, and JAM-1 (179, 505). VASP also binds profilin, an actin monomer binding protein (745), and vinculin (747) (Fig. 15). These findings demonstrate that VASP can regulate the formation of stress fibers, lamellipodia, filopodia, and other actin-dependent intracellular structures at cell-cell contacts in IEJs as well as focal adhesions (see Ref. 481). Expression of a truncated VASP mutant lacking the preferred PKA binding site (Ser<sup>157</sup>) resulted in reduction in barrier resealing over 3 h subsequent to barrier compromise induced by  $Ca^{2+}$  switch (5 min of  $Ca^{2+}$  chelation followed by restoration of  $Ca^{2+}$ ) (505). These results point to a potentially important role for PKA activation of VASP phosphorylation in the mechanism of stabilization of IEJs and focal adhesions, and thus in the control of endothelial permeability. In this regard, VASP knockout mice (*VASP*<sup>-/-</sup>) (356) would be informative in studies addressing the functional role of PKA activation of

VASP in regulating endothelial permeability through the stabilization of both IEJs and focal adhesions.

The actin-binding proteins gelsolin and cofilin inhibit actin polymerization in cell-free systems by capping the barbed ends of actin filaments in a  $\text{Ca}^{2+}$ -dependent (at  $\mu\text{M}$  concentrations) (1088, 1089) and pH-sensitive manner (491). After being capped, the growing actin filaments spontaneously depolymerize from their pointed ends. Cofilin has an additional function of breaking down the fully polymerized actin filament. Depletion of gelsolin by introduction of antisense oligonucleotides led to reduced actin stress fiber formation and contraction in platelets (31). Phosphorylation of cofilin by LIM kinase, the downstream effector of Rho-ROCK, inactivated the breakdown of polymerized actin by cofilin (558, 658). Thus gelsolin and cofilin may interfere with actin stress fiber formation by inducing depolymerization of actin. Deletion of the gene encoding gelsolin in mice was not lethal, but produced defects in platelet hemostasis, inflammation, and tissue remodeling (1055). *Gelsolin*<sup>-/-</sup> mice demonstrated increased lung microvessel permeability to protein (60), indicating that regulation of filamentous actin turnover by gelsolin is a potential determinant of endothelial barrier function.

The actin-binding proteins ezrin, radixin, and moesin (ERM), which share 80% sequence homology (786), regulate the formation of lamellopodia. On activation ERMs cross-link actin (via their COOH termini) with the plasma membrane (via their NH<sub>2</sub> termini) (reviewed in Ref. 424). They may be involved in endothelial permeability regulation, although they have not been extensively studied in this regard. ERMs additionally act as signaling molecules. Overexpression of the COOH-terminal domain of ezrin in Sf9 cells induced the formation of long, actin-containing membrane structures (568), whereas depletion of these proteins using antisense oligonucleotides caused the disappearance of actin-dependent membrane protrusions (923). The ERM proteins contain NH<sub>2</sub>-terminal FERM domain (four point-1, ezrin, radixin, and moesin; a multifunctional protein and lipid binding site), central coiled-coil domain, and COOH-terminal actin-binding domain. Because the NH<sub>2</sub>- and COOH-terminal domains interact with each other, it has been suggested that activation of ERM proteins requires disruption of this interaction and protein unfolding (308). Threonine phosphorylation within the COOH terminus was shown to activate ERM proteins (871). RhoA acting through ROCK (570) or phosphatidylinositol 4-phosphate 5-kinase (571) was shown to regulate the phosphorylation status of ERMs (570, 571), indicating that the RhoA pathway is involved in inducing activation of ERM proteins. Activated ERMs were also shown to bind Rho-GDI-1, allowing activation of RhoGTPases (919). A direct interaction between radixin and the heterotrimeric G $\alpha_{13}$  protein has also been demonstrated in fibroblasts (976). This interaction occurred in a G $\alpha_{13}$ -

activation-dependent manner and resulted in the conformational activation of radixin, and as such it represents an additional pathway of activation of ERM proteins. Ezrin also binds through its FERM domain with FAK (715) (Fig. 16). Thus ERM proteins by sequestering Rho-GDI and through their ability to bind G $\alpha_{13}$  and FAK can regulate RhoA activation and focal adhesion formation in endothelial cells. The richness and complexity of these associations are likely to play an important role in regulating endothelial barrier function, although their role has not been examined.

Members of the stress-inducible small heat shock protein family (HSP27) can also regulate actin polymerization in endothelial cells and thus affect barrier function. HSP27 was shown to inhibit actin polymerization in vitro (605). Phosphorylation of HSP27 relieves this inhibition, thereby derepressing actin polymerization (64). Several edemagenic agents such as TNF- $\alpha$ , LPS, thrombin, and H<sub>2</sub>O<sub>2</sub> were shown to induce the phosphorylation of HSP27 in cultured endothelial cells (318, 371, 394, 588). Thus HSP27 phosphorylation by promoting actin polymerization and stress fiber formation may contribute to the regulation of endothelial barrier function. Evidence indicates that p38 MAPK can induce phosphorylation of HSP27 (306, 371, 394), which could be a factor in regulating the HSP27-dependent increase in endothelial permeability.

F) ROLE OF CALDESMON. A major paradox in linking MLC phosphorylation to endothelial cell contractility (and hence increased endothelial permeability due to junction rupture) is the rather consistent observation that edemagenic agents such as thrombin induce a sustained increase in endothelial contraction while only producing transient increases in MLC phosphorylation. In addition, thapsigargin and ionomycin were shown to induce increases in endothelial permeability without increasing MLC phosphorylation (298, 782). Thus targets other than actin and myosin are also likely involved in regulating increased endothelial permeability. Evidence suggests that the low molecular mass form of caldesmon (70–80 kDa, L-caldesmon), widely expressed in nonmuscle cells (see Ref. 883 for review), is involved in the mechanism of endothelial cell contraction (103, 104, 534, 621). Caldesmon binds to actin and inhibits actin-activated myosin ATPase in a  $\text{Ca}^{2+}$ /calmodulin-dependent manner (see Ref. 883). However, phosphorylation of caldesmon relieves the inhibitory action of caldesmon on myosin-ATPase activity, thereby inducing actin-myosin contraction (883). In addition, caldesmon binds actin and myosin, and hence may stabilize contraction when MLC phosphorylation level is low (similar to a latch state in smooth muscle cells) (883). Evidence also indicates that caldesmon regulates the capping and severing activity of gelsolin (described in sect. IXA1D) (419). Thus caldesmon may control endothelial contraction by regulating myosin ATPase ac-



tivity and actin dynamics and by stabilizing the actin-myosin interaction.

Stimulation of endothelial cells with thrombin was shown to induce the translocation of caldesmon from the cytosol to the membrane and cytoskeleton (104). Thrombin also induces the phosphorylation of caldesmon, which colocalizes with actin stress fibers (103, 104, 897). Phorbol esters and pertussis toxin were also shown to induce the phosphorylation of caldesmon in association with increased endothelial permeability (306, 621). The signaling pathways responsible for caldesmon phosphorylation in endothelial cells are believed to operate via CaMKII, ERK and p38 MAPK (103, 104, 306), and PKC (621, 897). Interestingly, caldesmon phosphorylation occurred independently of MLC phosphorylation (103, 104, 621). Pharmacological inhibitors of CaMKII, ERK as well as p38 MAPK prevented caldesmon phosphorylation and prevented thrombin-induced stress fiber formation as well as the increase in endothelial permeability without decreasing MLC phosphorylation (103, 104). As discussed in section 1A5, inhibition of CaMKII also prevented phosphorylation of filamin, and permeability did not increase in response to thrombin or bradykinin (105, 1025), suggesting that filamin may also contribute to the caldesmon-induced regulation of endothelial contraction. However, a concern is that the evidence implicating a role of caldesmon in mediating the MLC-independent component of increase in endothelial permeability has been mostly indirect; thus further studies using caldesmon phosphodeficient mutants are needed to clarify the role of caldesmon in the mechanism of endothelial contraction and increased permeability response.

## 2. Role of FAC

Focal adhesions, consisting of a highly specialized complex array of integrins and other attached proteins, provide the primary means of anchoring the endothelial cell monolayer to the ECM (reviewed in Ref. 130) (Fig. 15). Focal adhesions are connected to actin filaments and are thus able to transmit the contractile force generated by the actinomyosin machinery to the ECM (130). Studies in fibroblasts, myocytes, and smooth muscle cells demonstrated that a force ranging from 2 to 10 nN/ $\mu\text{m}^2$  was exerted on focal adhesions (see Ref. 73). Given the findings that contraction as the result of agonist stimulation (1, 138, 587, 795) and disruption of microtubules (72, 533) induced focal adhesion formation, it is likely that endothelial cell contractility per se is an important determinant of the formation of focal adhesions. Focal adhesions also serve as signaling platforms (reviewed in Ref. 310), and their signaling activity was found to change in response to the applied force (292). Focal adhesions morphologically appear as punctate structures that reorganize characteristically into thick foci (up to a few microns) at the

attachment points of the fibers following stimulation of cells with permeability-increasing mediators (1, 138, 158, 587, 795). Upon thrombin stimulation, the role of focal adhesions is to strengthen cell-substrate adhesion, and also perhaps to sense the force exerted by the focal adhesions against IEJs and thereby to modulate this force (587). This may be a key mechanism by which focal adhesions can control junctional (paracellular) permeability.

A) REGULATION BY TYROSINE KINASES AND FOCAL ADHESION PROTEINS. Most proteins recruited into the FAC require tyrosine phosphorylation for their translocation. The tyrosine kinases, FAK and Src, are essential for the phosphorylation of the protein constituents of FAC. FAK is known to be activated by permeability-increasing agonists, shear force, or clustering of integrin receptors (1, 2, 68, 137, 335, 509, 587, 795, 985, 996). Src-induced tyrosine phosphorylation is believed to regulate the activation state of FAK. Upon integrin activation, FAK undergoes autophosphorylation on Tyr-397 creating a binding site for the SH2 domains of the Src kinases, pp60Src or pp59Fyn (793, 798). Src kinase thereby phosphorylates a number of additional sites on FAK, which further activates FAK. The various proteins phosphorylated by FAK including paxillin, tensin, PI 3-kinase, and p130<sup>CAS</sup> rapidly aggregate to form FAC (see Ref. 792); the total number of proteins that make up FAC is estimated to be ~50. Inhibition of FAK phosphorylation interfered with FAC formation, whereas tyrosine phosphatase inhibitors promoted FAC formation (792), indicating the importance of FAK as a crucial regulator of FAC formation.

Downstream of FAK, data have implicated paxillin as also playing a key role in signaling FAC formation (Fig. 15). Paxillin associates with vinculin (969), p47gag-Crk (86), COOH-terminal Src kinase (CSK) (775), and pp60Src (1047). Thus paxillin acts as an adaptor protein with the apparent function of recruiting other proteins to FAC. Additionally, paxillin was shown to interact with G protein-coupled receptor kinase interacting protein-1 and -2 (GIT-1, GIT-2), and the interaction of paxillin with GIT-1 increased following endothelial stimulation with S1P (850). GIT-1 and GIT-2 are members of the ADP-ribosylation factor-GTPase activating proteins (ARF-GAPs) family that interact with several signaling and cytoskeletal proteins (1048) (see Ref. 967). Vinculin may also facilitate FAC formation through its ability to bind PIP<sub>2</sub>, the product of PIP5K, since blocking the PIP<sub>2</sub> interaction with vinculin by microinjecting anti-vinculin neutralizing antibody prevented the formation of FAC and actin stress fibers (319). Studies have demonstrated a direct interaction between vinculin and the Arp2/3 complex (215) (Fig. 15). Intriguingly, Arp2/3 also interacts with E-cadherin (476) (Fig. 11), raising the possibility that this type of interaction may regulate not only FAC formation but also interendothelial cellular adhesion via VE-cadherin homo-

typic binding. Thus vinculin and paxillin may serve as the nexus, coupling cell-ECM interaction with intercellular adhesion through VE-cadherin and may thereby regulate junctional integrity.

**B) REGULATION BY RHO GTPASES.** Evidence points to the crucial role of the Rho GTPases in regulating the formation of FAC. Inhibition of RhoA by C3 toxin or dnRhoA reduced the level of phosphorylation of FAK, Src, and paxillin and interfered with FAC formation (1, 138). This implied a positive effect of RhoA-GTP on FAC formation. Additional data suggested that RhoA's downstream effector ROCK may be responsible for RhoA-induced FAC formation, because inhibition of ROCK prevented FAK phosphorylation as well as FAC formation (137, 138, 985). RhoA may also activate the formation of FAC by stimulating the recruitment of PIP5K to the membrane (166) and also by activating mDia (635), although the roles of these two proteins have not been tested in endothelial cells. The precise mechanism whereby ROCK activates FAK is not clear, but it could involve actin polymerization as disruption of actin filament polymerization inhibited FAK activation (587) and the generation of mechanical force resulting from engagement of the contractile apparatus (168). Data concerning the role of Rac and Cdc42 in regulating the formation of FAC in endothelial cells are limited. Rac and Cdc42 are known to induce cell-cell contact by stimulating the formation of filopodia and lamellipodia (see Ref. 921). During the formation of cell-cell contacts, an increase in concentration of Rac and Cdc42 at the cell's trailing edge may induce cell-substrate adhesion by stimulating the formation of FACs (341, 644, 645, 1084) (see Ref. 216 for details). SIP, an endothelial barrier-stabilizing lipid mediator, was also shown to activate Rac and FAK and stimulate FAC formation and AJs assembly (585, 850), indicating Rac may contribute to normalization of barrier function by concurrently inducing FAC formation and AJ strengthening. Therefore, the Rho GTPases RhoA, Rac, and Cdc42 induce FAC formation by their ability to promote phosphorylation of FAK, and the formation of FAC in turn promotes adhesivity of endothelial cells to ECM.

**C) REGULATION BY PKC.** Although direct evidence that supports roles of specific PKC isoforms in signaling the formation of FAC is lacking, it is known that PKC- $\alpha$  can regulate the activity of RhoA (381, 586) and thus influence FAC formation downstream of RhoA. Additionally, PKC- $\alpha$  has been shown to phosphorylate pp60Src on Ser-12 (528), which may in turn activate focal adhesion proteins leading to FAC formation.

### 3. Role of cell-cell junctional complexes

IEJs, composed of AJs (cadherin-catenin complexes) and TJs (occludin-claudin-ZO complexes as discussed in

sect. VIII A) (Figs. 11 and 13), are the primary molecular targets of permeability-increasing agonists in endothelial cells, as demonstrated in both cell culture and intact vessel studies (296, 382, 473, 475, 620, 623, 733, 780, 782, 1016, 1096). The details of the mechanisms regulating patency of IEJs remain unclear. It is known that IEJs are stabilized by their linkage to cortical actin filaments (reviewed in Ref. 1086). However, reorganization of actin filaments into stress fibers results in the transmission of the contractile force to IEJs, thereby influencing both junctional organization and function. The phosphorylation status of proteins that make up AJs and TJs may also affect IEJ organization and the function of IEJs. In this regard, tyrosine and serine/threonine kinases appear to play an important role in regulating the strength of the cell-cell contacts (as discussed below). This section addresses the role of the RhoGTPases and tyrosine and serine/threonine kinases in regulating IEJ function, and hence the implications of their regulation of endothelial permeability.

**A) REGULATION OF TJ COMPLEXES. I) RhoGTPases.** Occludin is suggested to be a key molecular target of permeability-increasing agonists. Thrombin and histamine induce the loss of junctional occludin, thus resulting in the disassembly of TJs (372, 1057). Evidence points to a role of the RhoA-ROCK pathway in inducing actin stress fiber formation, which may transmit a contractile force to TJs, and in turn disrupt TJ integrity (1057). Expression of dnRhoA or pharmacological inhibition of ROCK prevented actin stress fiber formation as well as the loss of occludin from junctions, and increased endothelial permeability (as assessed by TER) in response to histamine or thrombin challenge (1057). Observations were also made in ECV cells, an epithelial-endothelial hybrid cell line, in which lysophosphatidic acid (LPA) was used as permeability-increasing agonist (372). In this study the RhoA-ROCK pathway was shown to induce the phosphorylation of occludin, which may also signal the disassembly of TJs (372). Endothelial cells transfected with dnRac had fewer TJs than control cells, whereas a dominant-active mutant of Rac produced the same effect (1057); thus the precise role of Rac in regulating TJ function in the endothelium is not yet clear.

**II) Regulation by phosphorylation.** The permeability-increasing mediators, thrombin, histamine, and VEGF, were found to induce phosphorylation of occludin and ZO-1, which may be a factor in inducing TJ disassembly (267, 372, 693). However, little is known about the specific kinases involved. Studies have implicated Src, PKC, and casein kinase 2 in regulating TJ protein phosphorylation resulting in disassembly of the junctional complex (171, 219, 530, 693, 823, 877). In addition, conventional PKC isoforms have also been shown to phosphorylate JAMs in epithelial and endothelial cells in response to the permeability-increasing agonist thrombin (669). On the other

hand, there is evidence that PKC-induced dephosphorylation of occludin may signal increased epithelial TJ permeability, possibly by activating a downstream serine/threonine phosphatase (171). The basis of phosphorylation-induced changes in the function of TJ proteins has yet to be determined. It is possible that phosphorylation alters the conformation of these proteins, which affects their ability to interact with each other or the actin cytoskeleton, thereby disassembling protein complexes, which might lead to increased endothelial permeability.

**B) REGULATION OF AJ COMPLEX.** *I) RhoGTPases.* The RhoGTPases, RhoA, Rac, and Cdc42, and AJs are involved in a complex relationship as AJs themselves can modify RhoGTPase activity (described in sect. VIII B). Evidence supports the general concept that each of the GTPases RhoA, Rac, and Cdc42 contribute to the regulation of AJ function. The GTPases were found to influence disassembly of endothelial AJs in response to thrombin, histamine, bradykinin, or PAF (5, 138, 1016, 1056) (Fig. 23). Inhibition of Rac with *Clostridium sordellii* toxin (a specific inhibitor of Rac) caused AJ disruption in cultured endothelial cells and increased  $L_p$  of rat venular microvessels (1031), implicating Rac in promoting barrier function by stabilizing AJs. Kouklis et al. (475) showed that the re-assembly of AJs in endothelial cells during recovery from the permeability increase induced by thrombin required the activation of Cdc42. The protein IQGAP is an important modulator of AJs downstream of Rac and Cdc42 (see sect. VIII A3A). IQGAP by sequestering  $\beta$ -catenin prevents it from associating with  $\alpha$ -catenin in epithelial cells, resulting in AJ disassembly (111, 284, 285). Binding of activated Rac and Cdc42 with IQGAP freed  $\beta$ -catenin to interact

with its partners cadherin and  $\alpha$ -catenin (486), thus favoring AJ stability. Thus IQGAP via its binding to Rac and Cdc42 may be a determinant of regulation of endothelial permeability. In another study, reduction in IQGAP expression by siRNA in epithelial cells reduced E-cadherin accumulation at the junctions (651). IQGAP may be an important modulator of AJ stability and junctional permeability; its mechanism of action is not known.

*II) Regulation by phosphorylation.* In several cell types including endothelial cells, strong evidence supports the concept that the specific phosphorylation status of AJ components is linked to the assembly-disassembly state of the junctions (27, 174, 473, 495, 572, 638, 653, 782, 837, 947, 973, 1059). Phosphotyrosine content of VE-cadherin,  $\beta$ -catenin, and p120 decreased when subconfluent endothelial cells became a confluent monolayer (495), indicating phosphorylation status of AJ proteins may regulate their stability. Supporting this notion are the findings in which VEGF and thrombin, known permeability-increasing agonists, increased the tyrosine phosphorylation of cadherin,  $\beta$ -catenin, and p120, which was associated with disruption of AJs (463, 770, 973). Additionally, Src has been shown to play an important role in inducing tyrosine phosphorylation of  $\beta$ -catenin, p120, and cadherin (668, 770). Studies showed that PKC- $\alpha$  can also mediate serine/threonine phosphorylation of AJ protein constituents (473, 782). The mechanisms by which phosphorylation of AJ proteins disrupt IEJ integrity may involve a reduction in the affinity of  $\beta$ -catenin and p120 binding for VE-cadherin (770, 973). It is also possible that phosphorylation of AJ proteins modifies the interactions of VE-cadherin with the actin cytoskeleton, but this question

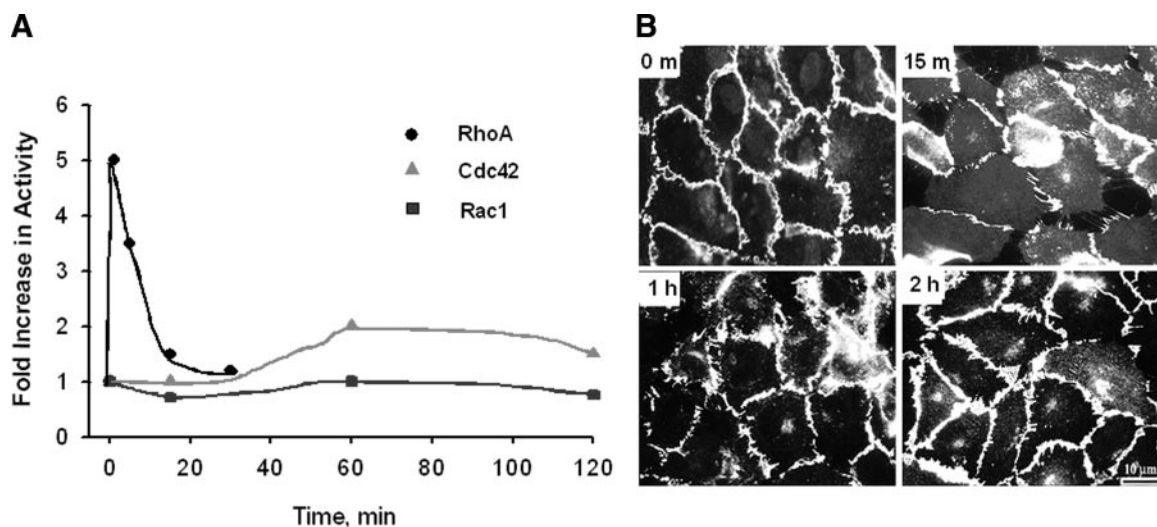


FIG. 23. Activation of RhoGTPases modulates adherens junction assembly. Thrombin induces activation of RhoA within minutes which disrupts adherens junctions (*B*, 15 min). This is followed by activation of Cdc42 at a time during which RhoA activity returns to basal level after thrombin exposure (30 min). The increase in Cdc42 activity is associated with reformation of adherens junctions in endothelial cells (*B*). Thrombin induces a minimal change in Rac activity. Thus thrombin modulates adherens junction assembly by sequentially regulating RhoA and Cdc42 activity. RhoA, circle; Cdc42, triangle; Rac, square. [*A*, Rac and Cdc42 data modified from Kouklis et al. (475); *B* adapted from Kouklis et al. (745).]



has not yet been examined. An important technical issue is how to separate the effects of Src- or PKC-induced phosphorylation and disruption of AJ components from the effects of phosphorylation of other substrate proteins. For example, many Src substrates (e.g., FAK, MLCK, and actin filament-associated proteins such as cortactin, p120-catenin, and  $\beta$ -catenin) affect the organization of actin cytoskeleton, indicating that Src can also modulate intercellular adhesion by regulating the actin cytoskeleton.

Because stable adhesion of endothelial cells to other endothelial cells is associated with decreased phosphorylation of VE-cadherin and catenin constituents (495), it is possible that tyrosine phosphatases also play an important role in maintaining stable IEJs. SHP2, which is found in association with AJs (described in sect. VIII A3A), may help to maintain cadherin and catenins in their dephosphorylated states. Thrombin was found to induce dissociation of SHP2, from the cadherin-catenin complex (973). Likewise, inhibition of phosphorylation by the expression of the protein tyrosine phosphatase LAR (leukocyte common antigen related) in epithelial cells strengthened AJ interaction (629). Together, these findings point to phosphatases as being crucial in regulating the phosphorylation of AJ components under basal conditions. These results also raise the possibility that permeability-increasing mediators such as thrombin induce phosphorylation of catenins and p120-catenin on tyrosine residues by altering the activity of transmembrane tyrosine phosphatases LAR-PTP, PTP $\mu$ , and cytoplasmic tyrosine phosphatase SHP2 (109, 451, 629, 973, 1015, 1110).

#### 4. Role of microtubules

Microtubules are another key constituent of the endothelial cytoskeleton that is important for endothelial barrier function. Microtubules are presumed to resist compression of cells by opposing actin-myosin contractility (411, 413). Microtubules (typically having a diameter of  $\sim 25$  nm) are heterodimers organized by self-assembly of  $\alpha$ - and  $\beta$ -tubulins (molecular mass of 55 kDa each) (see Ref. 1019). Microtubules form a lattice of rigid hollow rods spanning the cell in a polarized fashion from nucleus to periphery. Studies in cell-free systems showed that microtubules undergo "dynamic treadmilling"; i.e., alternating addition and removal of tubulin dimers from the microtubule (reviewed in Refs. 218 and 438). The faster growing end, the plus end, is made up of  $\beta$ -tubulin that binds to and hydrolyzes GTP to GDP.  $\alpha$ -Tubulin constitutes the minus end and binds to but does not hydrolyze GTP. The plus end of microtubules is attached to the cell cortex. These ends exhibit "dynamic instability" because they shift between phases of lengthening and shortening (see Refs. 218 and 438). The minus end is typically joined at a common attachment site in the cell called the microtubule-organizing center (MTOC).

Studies showed that the thrombin-induced increase in endothelial permeability is dependent in part on depolymerization of microtubules in endothelial cells (92, 333). Disruption of microtubules with the depolymerizing agent nocodazole or vinblastine increased endothelial permeability (704, 997) by inducing activation of RhoA signaling (93). RhoA is activated in this context secondary to activation of RhoGEFs (Rho activators) following their release from the microtubule network when tubulin is depolymerized (323, 482, 748, 982). Microtubules by sequestering LIM-kinase may also limit actin stress fiber formation (see sect. IX A1D) (333); thereby microtubules can regulate the state of actin polymerization, and hence endothelial permeability. Unlike actin filaments, endothelial microtubules are not known to associate with any structure identified with the paracellular permeability pathway such as IEJs and FAC. However, there is a potentially interesting association between p120-catenin and kinesins, a family of microtubule-coupled molecular motors (159, 1083), implying that microtubules could be important in the trafficking of cadherin and other AJ components to IEJs (see Ref. 1004), and in this manner regulate AJ permeability. Interestingly, kinesin-1 (KIF 5; a microtubular-associated molecular motor) has also been implicated in the trafficking of signaling complexes to focal adhesions, and thus may modulate the turnover of adhesion sites (484). This finding suggests that kinesin interaction with microtubules could be a determinant of endothelial cell-ECM attachment. Thus it can be speculated that microtubules influence endothelial permeability through their effects on RhoA activation, by regulating vesicular trafficking of AJ proteins to the junctions and by modulating focal adhesion turnover.

#### 5. Role of intermediate filaments

Intermediate filaments (IFs) represent the third major cytoskeletal structure in endothelial cells (for review, see Ref. 905), but their role in endothelial permeability regulation is the least well understood. They are known as IFs because their diameter (10–12 nm) is between 7 nm (of actin filaments) and 25 nm (of microtubules). IFs, together with microtubules and actin filaments, provide cells with mechanical integrity and probably help to maintain endothelial cell shape (413). Unlike microtubules, IFs exhibit no polarity, i.e., elongation can occur from either end of the filament. IFs, consisting of polymers of the protein vimentin and cytokeratin, are found prominently in endothelial cells (19, 691). Phosphorylation of vimentin occurred following stimulation of endothelial cells with agonists known to induce change in cell shape, thrombin, or phorbol esters (106, 897), implying that vimentin, as a PKC substrate and an IF-interacting protein, could influence endothelial barrier by this mechanism. Biochemical studies have shown an association of vimentin with VE-

cadherin, which is disrupted by the permeability enhancing mediator histamine (837). The functional consequence of this interaction in regulating endothelial permeability is not yet clear, but it could be related to the rounding of endothelial cells secondary to release of adhesion-induced tension in the cells. Vimentin knockout mice lack IFs, but do not display any abnormalities in phenotype or cell architecture of their microtubules or microfilaments (178). As the response of vimentin-deficient endothelial cells to permeability-increasing mediators has not yet been examined, this would prove useful in unraveling the contribution of IFs to the regulation endothelial barrier function.

## B. Mechanisms of Recovery of Endothelial Barrier Function

Permeability-increasing agonists typically induce a reversible increase in endothelial permeability (587, 622, 951). The process of recovery of barrier function could reflect reannealing of previously opened IEJs (475), strengthening of adhesion of endothelial cells to ECM (587, 732), or both. Either one or both of these mechanisms could lead to the recovery of endothelial barrier function, the basis of which is discussed in the following section.

### 1. Role of FAK

As described in section VIII B, FAK is a major contributor to the regulation of focal adhesion formation and turnover and is thus a key determinant of vessel wall permeability. Depletion of FAK by antisense oligonucleotides in endothelial cells impaired the recovery of endothelial barrier function following thrombin challenge (587) (Fig. 16), indicating the key involvement of FAK in barrier restoration. Other studies showed that FAK function was required for strengthening the endothelial barrier in response to hyperosmolar stress (732), further supporting the role of FAK in maintaining endothelial barrier integrity. Inhibition of FAK by expression of exogenous FAK related nonkinase (FRNK) (a dn-FAK) in endothelial cell monolayers markedly increased RhoA activity and actin stress fiber formation and produced a sustained increase in endothelial permeability in response to thrombin, suggesting protracted barrier dysfunction (M. Holinstat, N. Knezevic, M. Broman, A. M. Samuel, A. B. Malik, and D. Mehta, unpublished observations). Inhibition of RhoA by C3 toxin restored endothelial barrier function in FRNK-expressing cells in a normal time frame. These findings indicate that FAK-induced downmodulation of RhoA activity is crucial for signaling restoration of endothelial barrier integrity. FAK may downregulate RhoA activity by its association with two Rho GAPs, GRAF (364, 936) and p190RhoGAP (32, 648) (Figs.

15 and 16). Both GRAF and p190RhoGAP were shown to specifically inhibit RhoA activity (32, 648, 936). Moreover, as p190RhoGAP is tyrosine phosphorylated (32, 648), there is the distinct possibility that the GAP activity of p190RhoGAP is modulated by tyrosine phosphorylation induced by FAK itself. A study showed that the engagement of C-cadherin inhibited RhoA activity by activating p190RhoGAP (648); therefore, it is possible that VE-cadherin regulation of p190RhoGAP may also be involved in modulating RhoA activity. Although there are many unknowns, the interaction of p190RhoGAP and GRAF (much less studied than p190RhoGAP) with FAK may signal the downmodulation of RhoA activity and thus promote restoration of the endothelial barrier function.

It is also possible that the effector arm of FAK signaling responsible for restoring barrier function involves Rac and Cdc42. FAK may regulate Cdc42 and Rac function by activating GIT-1, G protein-coupled receptor kinase interactor-1 (1108). GIT-1, a substrate for tyrosine kinases (46), is proposed to complex with PAK-interacting exchange factor (PIX; Cdc42/Rac-1 specific GEF) and bind PAK at adhesion complexes (545), a downstream effector of Cdc42 and Rac (678). Knockdown of GIT-1 led to an additional increase in endothelial permeability in response to thrombin (985), suggesting the functional importance of the FAK-GIT-Cdc42/Rac signaling pathway in endothelial barrier restoration.

### 2. Role of Cdc42 and Rac

Cdc42 and Rac have been shown to regulate the formation and organization of actin filaments associated with membrane ruffles, filopodia, and lamellipodia in fibroblasts, macrophages, and endothelial cells (755, 1058). These structures may induce the formation of IEJs as the edge of the plasma membrane is propelled forward. In addition, Cdc42 and Rac control cadherin-mediated cell-cell adhesion and formation of AJ complexes via modulation of interactions between  $\alpha$ -catenin and the cadherin-catenin complex (reviewed in Ref. 441). Inhibition of Rac-1 using *C. sordellii* toxin increased  $L_p$  of rat and mouse mesenteric microvessels (1031, 1033), indicating that Rac function is required to maintain normal barrier function. Other studies in cultured cells showed that permeability-increasing mediators (such as thrombin) transiently increased RhoA activity but decreased Rac activity and that dnRac perturbed normal barrier function (1017). As shown in Figure 23, thrombin modulates RhoA GTPase activity in a time-dependent manner, as may also be the case with other permeability-increasing mediators. Whereas thrombin induced the transient activation of RhoA, it induced delayed activation of Cdc42 paralleling the time course of endothelial barrier recovery (475, 586, 1017). Activation of RhoA was associated with AJ disassembly followed by Cdc42-dependent AJ assembly during which

Cdc42 activation preceded the reannealing of AJs (475) (Fig. 23). Expression of dnCdc42 (N17Cdc42) significantly delayed the reformation of the VE-cadherin-containing AJs and restoration of endothelial barrier function (475). In addition, N17Cdc42 expression in the mouse lung endothelium markedly impaired endothelial barrier recovery after the permeability increase induced by activation of PAR-1 (thrombin receptor) (475). These findings indicate that the delayed activation of Cdc42 represents a key negative-feedback mechanism responsible for signaling AJ reassembly after an inflammatory mediator-induced increase in endothelial permeability. However, the GEFs, GAPs, and GDIs regulating Cdc42 activation in the endothelium downstream of the agonists remain to be elucidated. Likewise, the effectors facilitating IEJ assembly downstream of Cdc42 activation in endothelium are unknown. Evidence points to WASP, Arp2/3, and WASP-related SCAR (like WASP an activator of actin nucleation/polymerization by Arp2/3) as possible targets of Cdc42 because these proteins can regulate lamellopodia and filopodia formation (see review in Ref. 594) and thus might facilitate AJ reformation.

Emerging evidence indicates that Cdc42 regulates AJ permeability by controlling the binding of  $\alpha$ -catenin with  $\beta$ -catenin and the consequent interaction of the VE-cadherin/catenin complex with the actin cytoskeleton (M. Broman, P. Kouklis, X.-P. Gao, R. Ramachandran, R. Neamu, R. D. Minshall, and A. B. Malik, unpublished data). These results show that Cdc42 is involved in the mechanism of increased vascular permeability induced by the loss of homotypic VE-cadherin interactions. These results are consistent with studies showing that the reannealing of endothelial AJs occurring 1–2 h after the thrombin-induced increase in permeability also depends on Cdc42 activation (475). These dual actions of Cdc42 [i.e., promoting the binding of  $\alpha$ -catenin to  $\beta$ -catenin (Broman et al., unpublished data) and reannealing of (475)] suggest that Cdc42 activation can regulate both AJ disassembly as well as reassembly. This concept is consistent with the versatility of RhoGTPases as effectors (441, 755); thus it is possible that the Cdc42-GTP “switch” integrates both events depending on its spatial and temporal activation in the endothelial cell.

### 3. Roles of PKA and adenylate cyclase

An increase in the concentration of cAMP is known to strengthen endothelial barrier function such that it prevents increased permeability in response to known permeability-increasing mediators in endothelial cells (9, 272, 551, 599, 690, 898, 901) and intact microvessels (357). Evidence indicates that increased levels of cAMP can be endothelial barrier protective through the cAMP-dependent kinase PKA. Activation of PKA inhibited endothelial contraction (551, 689) by preventing the activation of

RhoA (728) and MLCK (299, 999). PKA phosphorylates VASP (179), activating binding to occludin, JAM, and ZO-1 (179), and promoting the formation of a restrictive IEJ barrier. In this manner, the PKA-VASP interaction may increase barrier function by stabilizing IEJs (discussed in sect. IXB1). Studies also indicated that cAMP can strengthen barrier function by PKA-independent mechanisms. cAMP was shown to increase Rap1 (member of the small GTPase Ras family) activity (195) and decrease RhoA activity by inducing the phosphorylation of RhoA (237). Rap1 (see Ref. 143) is particularly enriched at sites of endothelial cell-cell contacts (195) and is implicated in inducing cell adhesion and spreading on ECM proteins (241). However, it appears on the basis of observations by Sayer et al. (788) that the barrier protective effect of cAMP is more complex than previously believed. It was shown that *Pseudomonas aeruginosa* ExoY, which resulted in an 800-fold increase in cytosolic cAMP concentration (a level typically achieved after forskolin or dibutyryl cAMP application to cells), in fact disrupted the endothelial barrier.

cAMP synthesis in endothelial cells is regulated by adenylate cyclase and phosphodiesterases (reviewed in Ref. 616); therefore, it is possible that agents may decrease endothelial permeability by activating adenylate cyclase activity or decreasing phosphodiesterase activity. In this regard, the adenylate cyclase 6 ( $AC_6$ ) isoform expressed in endothelial cells (901), having both high- and low-affinity  $Ca^{2+}$  binding sites (see Ref. 615), represents a potentially important target because a change in cytosolic  $Ca^{2+}$  concentration in response to a permeability-increasing mediator can transiently inhibit  $AC_6$  activity. This would presumably have a permeability-increasing effect. Overexpression of  $AC_8$ , an isoform that reverses the  $Ca^{2+}$ -induced inhibition of cAMP production in endothelial cells, was able to prevent the IEJ gap formation caused by thrombin (170). This finding is consistent with the concept that  $Ca^{2+}$  modulation of  $AC_6$  regulates endothelial permeability. Also, it was shown that  $AC_6$  colocalized with SOCs near the plasma membrane (251) and propitiously at sites of cell-cell contact (170). However, it remains to be determined whether an increase in  $AC_6$  activity has in fact a functional role in restoring endothelial barrier function, and if so, what is the mechanism.

## X. TRANSCELLULAR ENDOTHELIAL PERMEABILITY

### A. Function of Caveolae

Caveolae make up ~15% of the total endothelial cell volume (~10,000–30,000 caveolae/cell) (124, 431, 861) (see also Refs. 584, 721). These distinctive organelles, detected in the early days of electron microscopy, were



referred to as plasmalemmal vesicles by Palade (671) and caveolae intracellular by Yamada (1077). They range from 50 to 100 nm in diameter, and unlike other prominent intracellular features (e.g., clathrin-coated pits), they are not electron dense but appear striated by transmission electron microscopy. The striations are due in part to oligomerization of the caveolae-specific protein caveolin (described in sect. xA1A) (also reviewed in Refs. 743 and 966).

Caveolae were thought to be sessile structures because they were found to be contiguous with the surrounding plasmalemma, with few apparently free caveolae detectable within the endothelial cytosol (see Ref. 757). These findings did not seem to suggest a role for caveolar trafficking in the regulation of endothelial permeability (see Ref. 757) (discussed in sect. viIA). While some data over the years have suggested such a role for caveolae, a recent study has given it powerful support. Pelkman and Zerial (697) have demonstrated the highly dynamic nature of caveolae trafficking. They showed, using HeLa cells stably expressing GFP-caveolin-1, the existence of two subsets of caveolae. One subset of caveolae is transport incompetent and is found as clusters in multicaveolar assemblies communicating with the extracellular space, the classic early description of caveolae. The second subset, the transport-competent caveolae, undergo “kiss-and-run” cycles in a small volume below the plasma membrane. Interestingly, exposure of cells to okadaic acid (a phosphatase inhibitor), SV40, or actin depolymerization agents induced a three- to fivefold increase in caveolae docking events, which was not associated with an overall increase in the number of caveolae at the plasmalemma. Thus, using sophisticated imaging technology such as this, it will be possible to determine how the trafficking of caveolae from the apical to basal side of the endothelium regulates endothelial permeability.

Caveolae by transporting albumin and other plasma proteins across endothelium help to maintain the protein oncotic pressure ( $\Pi_c$ ), crucial for fluid balance across capillaries (499) (described in sects. viA and viIA). In addition, caveolae contain signaling molecules, indicating their involvement in regulating endothelial cell function (described below) (see Ref. 25). Caveolae also help to maintain cholesterol homeostasis and transport hydrophobic substances (e.g., fatty acids and hormones) (described in sect. viB) (reviewed in Ref. 25). In the following section we discuss the proteins that regulate caveolae formation and the trafficking of caveolae-derived vesicles in the endothelium.

### 1. Formation of caveolae

The processes leading to formation of caveolae-derived vesicles in endothelial cells and engagement of the “transcytosis machinery” remain incompletely understood. Unlike clathrin-coated vesicles, caveolae are not

readily detectable in a nascent form. They are usually found as fully developed flask-shaped vesicles attached by a neck to the plasma membrane (743). Based on targeted deletion of the gene for caveolin-1 (*Cav-1*<sup>-/-</sup>) (Fig. 9), the major protein component of caveolae, it has become apparent that caveolin-1 is required for the formation of caveolae in endothelial cells (223, 740; see also Refs. 584 and 721). Interestingly, expression of exogenous caveolin-1 in cell types normally devoid of caveolae such as lymphocytes (273), insect *Spodoptera frugiperda* (Sf21) cells (522), Fischer rat thyroid (FRT) cells (529), human erythroleukemic cell line, K562 (681), and Caco-2 cells (1013) induced the de novo formation of caveolae. Likewise, depletion of caveolin-1 in fibroblasts using antisense oligonucleotides (291) or in endothelial cells using siRNA (K. Miyawaki-Simizu, D. Predescu, J. Shimizu, M. Broman, S. Predescu, and A. B. Malik, unpublished observations) led to the loss of caveolae. Endothelial cells from *Cav-1*<sup>-/-</sup> mice have no caveolae demonstrating the requirement for caveolin-1 in caveolae formation (223, 740; see also Refs. 584 and 721) (Fig. 9). In addition to caveolin-1, the product of a second gene, caveolin-2, has also been implicated in facilitating caveolae biogenesis (201, 279, 796, 889). However, this finding is controversial, since caveolae persist in the endothelium of *Cav-2*<sup>-/-</sup> mice (742). Because all caveolin-1 associated with caveolae is found in the form of oligomers (522, 531), and hence is resistant to solubilization by nonionic detergents (796), it is possible that formation of caveolae requires post-translational modifications of monomeric caveolin-1 that permits oligomerization to proceed (601, 603, 683, 800, 878). Phosphorylation of caveolin-1 (601, 603, 878) and application of glycosphingolipids (835) facilitated caveolin-1 oligomerization and induced the formation of caveolae. Cellular cholesterol was also shown to be required for caveolin-1 oligomerization and recruitment of caveolin-1 to the plasma membrane (266, 557, 613, 614, 772, 835). Oligomerized caveolin-1 stabilized by membrane cholesterol may give rise to the characteristic flasklike invaginations seen at the plasma membrane (reviewed in Ref. 182). Evidence also suggests that association of caveolin-1 with protein cofactors such as Ras are required to induce the formation of caveolae (520), suggesting that there may be an intermediate form of caveolae. These events are followed by recruitment of the large GTPase dynamin to the necks of caveolae, and this recruitment is facilitated by intersectin (724). The “pinchase” activity of dynamin leads to the release of caveolae from the plasma membrane via fission, and ultimately the free cytosolic vesicles fuse to the target membrane at the abluminal side or other cellular domains using the SNARE machinery (described in sect. xB3).

A) ROLE OF CAVEOLIN-1. Caveolin-1 (originally known as VIP21), an integral membrane protein (20–22 kDa), was first identified in Rous sarcoma transformed cells as a

protein richly phosphorylated on tyrosine (324). At the ultrastructural level, caveolin-1 decorates the cytoplasmic aspect of caveolae and appears as ridges (700, 767). The caveolin family of genes consists of caveolin-1, -2, and -3, with caveolin-1 having two isoforms (see Ref. 661). Caveolin-1 and -2 are coexpressed in many cell types, especially endothelial cells, smooth muscle cells, fibroblasts, and adipocytes. In contrast, expression of caveolin-3 is muscle specific. Using cells expressing exogenous caveolin-1, several groups showed that there was a direct correlation between the level of caveolin-1 expression and the number of caveolae (273, 522, 529, 681, 1013). However, there are some tissue-specific exceptions (e.g., endothelial cells from brain microvessels and type II alveolar epithelial cells), where caveolin-1 is expressed, but there are relatively few caveolae (25, 135, 136). It might be that caveolin-1 fails to oligomerize in these cells, thus interfering with formation of caveolae.

Caveolin-1 has three main structural domains: two cytoplasmic domains (NH<sub>2</sub>-terminal amino acids 1–101 and COOH-terminal 135–178) and a central hydrophobic domain (amino acids 102–134) that has a hairpin tertiary structure in the native lipid environment (229, 557) (Fig. 24). Caveolin-1 is subject to two types of posttranslational modifications, phosphorylation and palmitoylation, that regulate its intracellular localization, activity, and its ability to form oligomers. The cytoplasmic NH<sub>2</sub>-terminal domain of caveolin-1 contains three Src-consensus tyrosine phosphorylation sites of which Tyr-14 appears to be the critical functionally relevant residue (521). Phosphorylation of caveolin-1 may be required for its ability to interact with proteins required for the formation of caveolae secondary to caveolin-1 oligomerization and the initiation of endocytosis (507, 602). One of these interacting proteins is dynamin, the large GTPase, i.e., the pinchase, responsible for caveolar fission (1085). Caveolin-1 also contains

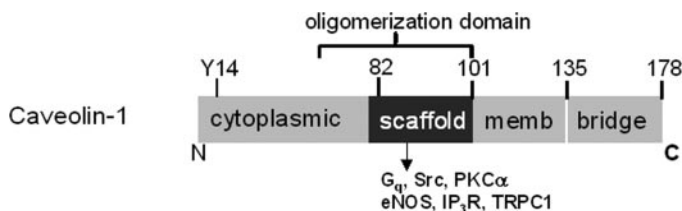


FIG. 24. Domain structure of caveolin-1. Based on amino acid hydrophobicity of caveolin-1 and localization studies of caveolin-1 mutants, the primary sequence is divided into three main domains: 2 are cytoplasmic (NH<sub>2</sub>-terminal amino acids 1–101 and COOH-terminal at 135–178) and a central hydrophobic transmembrane domain (amino acids 102–134) that exists in a tertiary hairpin structure in the native lipid environment. Caveolin-1 forms oligomers due to NH<sub>2</sub>- and COOH-domain interactions forming a caveolin-rich scaffold on the cytoplasmic side of the plasma membrane. Caveolin-1 scaffold domain (amino acids 82–101) interacts with diverse signaling molecules such as G<sub>q</sub>, Src, PKC- $\alpha$ , Ca<sup>2+</sup> channels such as IP<sub>3</sub>R and TRPC1, and endothelial nitric oxide synthase (eNOS) (see text for details). [Modified from Minshall et al. (602).]

Cys residues near its COOH terminus (Cys-156) that can be palmitoylated (221). Although palmitoylation is not required for caveolin-1 targeting to caveolae or caveolin-1 oligomerization (221), mutation of Cys residues resulted in impaired caveolin-1 interaction with Src and prevents phosphorylation on Tyr-14. Additionally, proteins modified by acylation, such as caveolin-1 itself (where the acyl group has been shown to interact with cholesterol) and other proteins localized to caveolae (e.g., clustered GPI-linked proteins, eNOS), could modulate caveolin-1 phosphorylation (508) as well as disrupt binding and trafficking of cholesterol within the cell (972).

Studies have shown that caveolin-1 is required for the caveolae-mediated transcytosis of albumin. Deletion of the caveolin-1 gene in mice (*Cav-1*<sup>-/-</sup>) prevents transcellular albumin transport via transcytosis in mouse embryo fibroblasts (740), mouse capillary (721), and in isolated aortic rings (821). Interestingly, while transcytosis via caveolae was absent in *Cav-1*<sup>-/-</sup> mice, the concentration of albumin in cerebrospinal fluid was found to be unperurbed (223). This finding suggests that compensatory mechanisms can maintain albumin transport across the blood-brain endothelial barrier or that this barrier has a specialized as yet undescribed caveolae-independent albumin transport pathway.

Interestingly, increased permeability due to the paracellular pathway was found in *Cav-1*<sup>-/-</sup> mice, which may be explained by an excess of NO production in these endothelial cells (822), due to derepression of eNOS in the absence of caveolin-1, known to inhibit eNOS by direct protein-protein interaction. In *Cav-1*<sup>-/-</sup> mice, lung capillaries showed defects in the morphology of TJs and additional abnormalities in endothelial cell adhesion to the capillary basement membrane (822). Thus, in most vascular beds, there may actually be a net increase in transendothelial albumin permeability. These findings demonstrate that caveolin-1 is involved not only in caveolar-mediated transcytosis but also can negatively control paracellular permeability by regulation through the signaling functions described below. Studies in *Cav-1*<sup>-/-</sup> mice have brought to light a number of mechanisms: 1) function of the proteins: dynamin, intersectin, and albumin binding protein gp60, forming the endocytic machinery of caveolae; 2) integration of integrin-associated bidirectional signaling (as discussed above in sect. VIII B) in *Cav-1*<sup>-/-</sup> mice; and 3) the mechanism of opening of IIEJs in the absence of caveolin-1 and its significance as a compensation for the loss of caveolin-1. Addressing these issues will go a long way toward clarifying the critical function of caveolin-1 in regulating endothelial transcellular and paracellular pathways and endothelial permeability.

It is important to take stock of the evidence that supports caveolin-1 regulation of several signaling events crucial in mediating transendothelial albumin permeabil-

ity. It serves as a platform for Src, PKC- $\alpha$ , eNOS, and Ca<sup>2+</sup> channels (both IP<sub>3</sub>R and TRPC1), receptors (e.g., EGF and PDGF receptors), and GTP-binding proteins (reviewed in Ref. 535) through its scaffolding domain (amino acids 82–101) (Fig. 24). Caveolin-1 maintains eNOS in its catalytically inactive state (125) (see also Ref. 601), and caveolin-1 is required for localization of store-operated TRPC1 Ca<sup>2+</sup> channels in plasma membrane caveolar lipid raft domains (115). Both of these functions of caveolin-1 are important in regulating the intracellular concentrations of eNOS-derived NO (822, 1107) and Ca<sup>2+</sup> (420). Deletion of caveolin-1 in mice resulted in a marked increase in NO concentration as the result of activation of eNOS (822, 1107). This was coupled to pulmonary hypertension and dilated cardiomyopathy (223, 1107). Additional studies showed that caveolin-1 regulates Rac activity (330), cell migration (58, 330), matrix metalloproteinase (MMP) function (489), and the integrity of IEJ complexes (13, 547, 842). Downregulation of caveolin-1 was shown to dissociate  $\beta$ -catenin from E-cadherin (547), suggesting that caveolin-1 may also contribute to regulating the endothelial barrier by promoting the assembly of AJ complex. Likewise, membrane traffic was shown to be required for caveolae- and dynamin-mediated endocytosis of TJ components, including occludin (842). Thus caveolin-1 may play an important role in endothelial barrier function by regulating Ca<sup>2+</sup> entry via TRPC1, eNOS activity, and assembly of AJs.

## B. Transcytosis: Caveolae Fission, Targeting, and Fusion

Albumin transcytosis is a specialized process required for barrier homeostasis that results in the transport of albumin from the luminal to abluminal side of the endothelium (864). It is initiated by the binding of albumin to specific caveolae-associated proteins (hence the term *receptor-mediated transcytosis*) that are internalized in caveolae (endocytosed) and undergo scission, releasing the vesicle from the plasma membrane (reviewed in Refs. 602, 966). The released caveolae move through the cytoplasm, presumably on tracks, avoiding lysosomes, to the opposite cell surface, where they fuse with the target membrane and release albumin by exocytosis (Fig. 1). The albumin-binding protein gp60 appears to be required for albumin transcytosis in endothelial cells (602, 603, 966 for review) (Fig. 8). Albumin can be transported in caveolae in either the fluid phase or bound to gp60 (433, 602, 867) (Fig. 1). During fluid-phase transcytosis, most of the albumin transported to the basal side is free within vesicles (433; see also Ref. 966). Specific binding of albumin to the albumin-binding protein gp60 localized in caveolae is intrinsically a saturable process (602, 810, 813, 955). This model of transcytosis, initially proposed largely on

the basis of electron microscopic studies (864), has subsequently gained support from functional studies (28, 432, 433, 595, 603, 717, 720, 811, 816, 822, 1010, 1012) (Fig. 8). Studies in cultured rat lung microvessel cells demonstrated that transcytosis of albumin was not saturated under physiological conditions but had the capacity for modulation (433). However, whether this conclusion is relevant to the intact microcirculation needs to be determined. In succeeding sections, we describe the role of proteins that have been implicated in initiating fission, targeting, and fusion of caveolae including dynamin, intersectin, SNARE complex, Rab proteins, actin, and microtubules. Since the subject of endocytosis has been recently reviewed (966), we discuss below the features as they pertain to endothelial cells and the signaling mechanisms that regulate transcytosis.

### 1. Role of dynamin

Dynamin, a mechanochemical enzyme, is a 100-kDa GTPase and exists as a homotetramer (625) that undergoes GTP-dependent self-assembly, resulting in the formation of higher order structures: dynamin rings and spirals (146, 369). Oligomerization of dynamin increases its intrinsic GTPase activity up to 10-fold (965, 1026, 1027). By analogy with myosin ATPase (see Ref. 368), the GTPase activity of oligomerized dynamin generates a constricting force around the collar of vesicles undergoing fission (922, 1028; reviewed in Refs. 803, 916). Expression of a dynamin mutant lacking GTPase activity (K44A mutant) or, alternatively, intracellular injection of dynamin-neutralizing antibodies blocked internalization of caveolae, as quantified by cellular uptake of cholera toxin B (656) and albumin transcytosis assessed with the tracer <sup>125</sup>I-albumin (830). These findings demonstrate the functional importance of dynamin-induced caveolar fission in the mechanism of transcellular albumin transport. However, the mechanism of dynamin recruitment to caveolar necks is poorly understood. Dynamin, described as a “pinchase,” contains five domains: NH<sub>2</sub>-terminal GTPase domain; middle domain (MD); pleckstrin homology (PH) domain that binds phosphatidylinositol lipids, which enables dynamin association with plasma membrane; GTPase effector domain (GED); and COOH-terminal proline-rich domain (PRD) that directly binds to the SH3 domain-containing proteins intersectin and Grb2 (368, 578) (Fig. 25A). On the basis of dynamin’s interactions, it is likely that these proteins regulate dynamin recruitment, self-assembly, and GTPase activity.

Src phosphorylation of dynamin is a key requirement for the dynamin self-assembly and pinchase activity (Fig. 25B). Expression of a dynamin-2 mutant deficient in Src Y231 and Y597 phosphorylation (Y231F/Y597F) in human microvessel endothelial cells interfered with the association of the mutant dynamin-2 with caveolin-1 (830). In



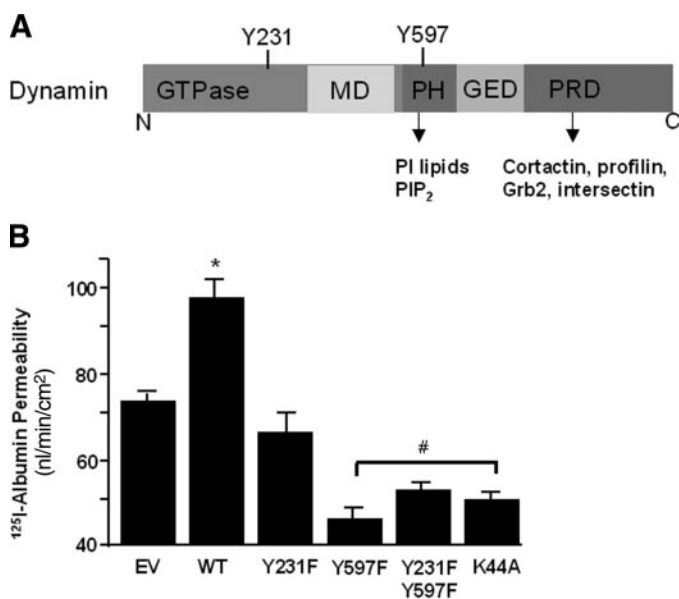


FIG. 25. Dynamin regulation of albumin endocytosis in endothelial cells. *A*: structural domains of dynamin. Dynamin contains an NH<sub>2</sub>-terminal GTPase domain that has a Src phosphorylation site (Y231), middle domain (MD), pleckstrin homology domain (PH), GTPase effector domain (GED), and COOH-terminal proline-rich domain (PRD). PH domain also contains a Src phosphorylation site (Y537) that directly binds SH3 domain-containing proteins such as intersectin, Grb2, and actin-polymerizing proteins (cortactin and profilin). PH binds phosphatidylinositol lipids, allowing dynamin association with plasma membrane. [Modified from Orth and McNiven (667).] *B*: supports a role for dynamin in regulating albumin transcytosis in endothelial cells. In rat lung microvascular endothelial cells expressing wild-type (WT)-dynamin-2, a 30% increase in <sup>125</sup>I-albumin transport was observed compared with control cells transfected with empty vector (EV). However, in cells transfected with a nonphosphorylatable dynamin mutant (Y597F), transendothelial <sup>125</sup>I-albumin permeability was reduced to an extent similar to that seen in cells expressing the GTPase-defective dynamin mutant K44A. In contrast, expression of nonphosphorylatable Y231F mutant had no significant effect on <sup>125</sup>I-albumin permeability. These findings indicate that dynamin-2 phosphorylation at Y597 in PH domain plays a critical role in regulating albumin transcytosis. \*Significantly higher than EV. #Significantly lower than EV, WT, and Y231F. [Adapted from Shajahan et al. (830).]

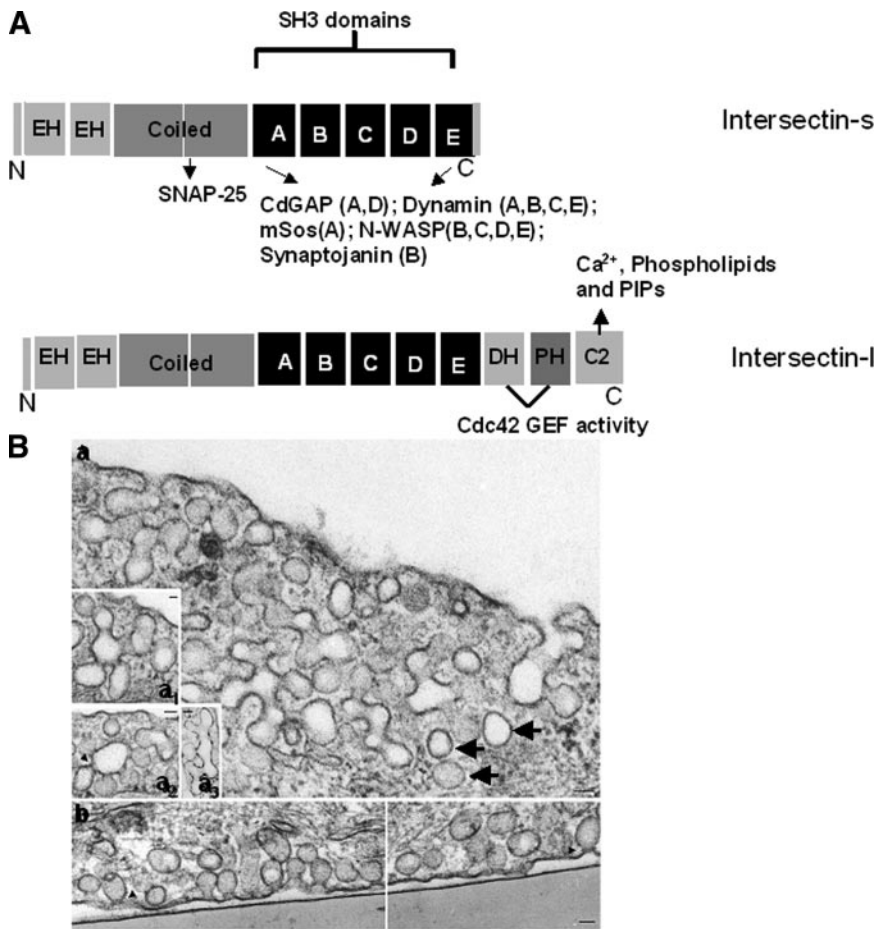
contrast to wild-type dynamin-2 (the ubiquitously expressed isoform of dynamin), the non-Src phosphorylatable mutant failed to translocate to the caveolin-rich membrane fraction (830). Expression of Y231F/Y597F dynamin-2 mutant also blocked albumin endocytosis and transcytosis (Fig. 25*B*). Interestingly, expression of Y231F mutant alone did not impair transendothelial albumin transport, whereas expression of Y597Y did. The Y231 residue is in the GTPase domain of dynamin and Y531 lies in the PH domain. These findings suggest that Src phosphorylation of dynamin does not contribute to its GTPase activity; rather, it is important for recruitment of dynamin to caveolae and for its pinchase action. Further studies using a dynamin mutant lacking the PH domain are needed to clarify this issue.

Predescu et al. (724) identified the role of another important protein partner of dynamin, intersectin. This

was done in studies in which intersectin (described below) was overexpressed in endothelial cells. These intersectin-overexpressing cells exhibited abnormal caveolar morphology with either fused caveolae or unusually elongated necks that were incapable of undergoing fission. Additionally, dynamin's pinchase function was impaired in the intersectin overexpressing cells (724), indicating that intersectin interfered with caveolae-mediated endocytosis by regulating the GTPase activity of dynamin. Because dynamin interacts with the actin-polymerizing proteins, cortactin and profilin, and adaptor protein Grb2 (for reviews see Refs. 483, 667 and 790), it is possible that protein interactions with dynamin may have additional roles such as in regulating actin dynamics required for trafficking of released caveolae (discussed in sect. xB5). Moreover, dynamin interacts (through its PRD domain) with FAK via Grb2 as well as with cortactin (through their SH3 domains). As cortactin in turn binds MLCK, this points to a possible role for dynamin in regulating the organization of actin stress fibers (discussed in sect. IXA1E) and focal adhesion turnover (discussed in sect. VIII B); thus dynamin is a multitasking protein that functions as a pinchase and may also be involved in regulating junction permeability via these protein interactions.

## 2. Role of intersectin

Intersectin is a highly specialized protein with two NH<sub>2</sub>-terminal Eps15-homology (EH) domains coupled to five SH3 domains (1076) (Fig. 26*A*). Intersectin-1 is nerve specific, whereas intersectin-2 is ubiquitously expressed (396, 727, 765, 1076) and has two splice variants, a long and short form (Fig. 26*A*). The long form of intersectin-2 includes all features of the short form with the addition of a COOH-terminal extension bearing a double homology (DH) domain, the canonical organizational feature of Rho GEFs capable of activating RhoGTPases in tandem with a PH domain (395). The GEF domain of intersectin-2 was shown to activate Cdc42, but not Rac or RhoA (395, 882) (Fig. 26*A*). Intersectin-2 is localized at necks of caveolae (724) where it may sequester dimers or tetramers of dynamin; thus intersectin appears to be crucial in regulating dynamin oligomerization and activity at caveolar necks. As stated above, dynamin oligomerization is required for the maximal GTPase activity as the "collar" of oligomerized dynamin around vesicle neck induces fission of vesicles (803, 916, 922, 1026, 1027). Depletion of intersectin using an anti-intersectin-2 antibody prevented fission of caveolae in endothelial cells (724). Thus a crucial function of intersectin may be to regulate dynamin's "pinching" of caveolae, and thereby control caveolae-mediated endocytosis. Also, caveolae in intersectin-overexpressing endothelial cells had an abnormal morphology, either fused together or attached to the plasma membrane by extended necks (724). These modified caveolae localized to



**FIG. 26.** Functional role of the scaffold protein intersectin in regulating caveolae internalization. **A:** structural domains of the short and long forms of intersectin. Both forms of intersectin have 2 NH<sub>2</sub>-terminal Eps15-homology domains (EH), a coiled-coil domain which binds SNAP-25 and SNAP23 coupled to 5 SH3 domains. SH3 domains binds WASP, son of sevenless (Sos), dynamin, synaptojanin, and CdGAP. Letters in parentheses indicate specific SH3 domains that bind with these proteins. The long form of intersectin-2 (intersectin-l) additionally has a COOH-terminal extension bearing a double homology (DH) domain in tandem with a PH domain, an organizational feature of Rho GEFs capable of activating RhoGTPases and a C2 domain which binds Ca<sup>2+</sup>, phospholipids, and inositol phosphates. [Modified from Orth and McNiven (667).] **B:** electron micrograph of endothelial cells overexpressing WT intersectin-short. Intersectin-s overexpressing cells express a large population of caveolae open to the apical (a) or basolateral (b) front of the cells, or apparently free in the cytosol (arrows). Caveolae appear mainly as large clusters of interconnected "grapelike" structures or alternatively with unusual shapes (elongated caveolar necks), that accumulate at the cell periphery. *Insets* in **A** illustrate additional grape-like (a<sub>1</sub>, a<sub>2</sub>) or unusually shaped (a<sub>3</sub>) caveolae clusters. These findings indicate that normal levels of intersectin are required for proper formation of caveolae. [Adapted from Predescu et al. (724).]

the cell surface failed to undergo fission despite the presence of dynamin (724) (Fig. 26B). Overexpression of intersectin-2 thus induced suppression of dynamin activity (724), consistent with a key role of intersectin in regulating dynamin's GTPase activity. Dynamin was also incapable of dissociating from the intersectin-dynamin complex in intersectin-overexpressing cells. The implication of this finding is that suppression of dynamin's GTPase activity may occur after the dissociation of dynamin from the intersectin-dynamin complex.

Unlike dynamin, intersectin remained associated with vesicles following fission (724). Evidence also indicates that the vesicle-associated intersectin interacts with key proteins constituting the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) machinery and synaptosome-associated protein of 25 kDa (SNAP-25) (660) and SNAP-23 (724) (Fig. 26A); therefore, intersectin may also be involved in inducing the coupling of endocytosis with exocytosis, the defining feature of transcytosis.

Intersectin binds actin binding proteins WASP (395, 576), son of sevenless (SOS) (958), a GEF for Ras (445) (Fig. 26A), and regulates MAPK (957) as well as Ras activities (610, 957). Intersectin through its SH3 domains was also shown to bind to Cdc42 GTPase-activating pro-

tein (CdGAP) (427), a GAP for Rac1 and Cdc42 (490), and inhibited GAP activity for Rac1, resulting in Rac1 activation (427) (Fig. 26A). Thus intersectin through its ability to bind SOS and CdGAP and by its GEF activity toward Cdc42 may modulate Cdc42, Rac1, and Ras activities (475, 1058), and thereby regulate endothelial barrier function. In this regard, intersectin is an important nodal point for multiple signaling pathways, which contributes to regulating junctional permeability, in addition to its potential role as a determinant of transcytosis that is described above.

### 3. Role of SNARES

SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) fall into two categories: t-SNAREs (syntaxin and SNAP-25 family members localized on target plasma membranes) and v-SNAREs (vesicle-associated membrane protein, VAMP, localized on vesicle membranes) (Fig. 27). SNAREs may participate, at the basolateral membrane of the endothelium, to mediate exocytosis of albumin and other macromolecules associated with caveolae (see Ref. 311). In endothelial cells, evidence has emerged that caveolae-mediated transcytosis requires a carrier system similar to that reported for

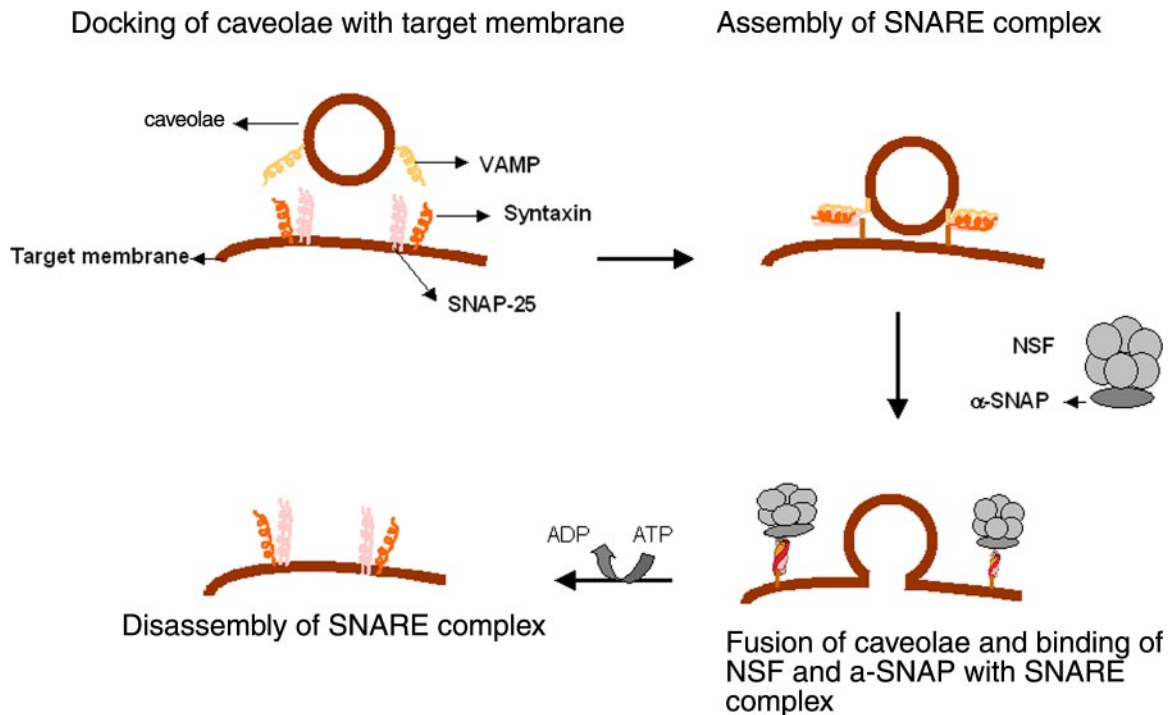


FIG. 27. SNARE machinery is responsible for fusion of caveolae with target membrane. SNAREs comprise pairs of proteins known as v-SNAREs (VAMP) localized on caveolae and t-SNAREs (syntaxin and SNAP25 family members) localized on the target membrane that are central to the process of membrane fusion. These proteins interact to form a SNARE complex resulting in fusion of caveolae with the target membrane. NSF and  $\alpha$ -SNAP are important SNARE regulatory molecules, which catalyze the disassembly of the SNARE complex following membrane fusion, freeing SNARE monomers for future rounds of fusion. (Modified from Chen and Schaller. *Nature Rev Mol Cell Biol* 12: 98–106, 2001.)

exocytosis of vesicles in neurons. *N*-ethylmaleimide (NEM) is an inhibitor of NSF, which regulates exocytosis (see Ref. 1051) (Fig. 27). NSF, a hexameric ATPase, induces disassembly of v- and t-SNARE complexes formed after membrane fusion, thereby recycling SNARE monomers for subsequent membrane fusion events. NSF binds the SNARE complex through soluble  $\alpha$ - or  $\beta$ -SNAP (1051).

NEM was shown to inhibit transendothelial transport of tracer albumin both in situ (717) and in cultured cells (811), consistent with the role of NSF in controlling the assembly of SNAREs. The canonical proteins known to be involved in vesicle docking and fusion, syntaxin (see Ref. 311), Rab5 (reviewed in Ref. 881), VAMP (also called synaptobrevin) (reviewed in Ref. 311), and NSF (see Ref. 1051) were also localized to endothelial caveolae (577, 722, 814). Cleavage of the caveolar VAMP-2 by VAMP-specific neurotoxins (botulinum D and F but not A) (577) and inhibition of SNARE function by botulinum toxins A and B (640) resulted in the impairment of cholera toxin-B or water-soluble styryl pyridinium endocytosis and intracellular accumulation of vesicles in endothelial cells, respectively (577, 640). These studies provide crucial evidence that caveolae require intact v- and t-SNARE apparatus for fusion of caveolae with the plasma membrane during exocytosis.

A fundamental question is how v-SNAREs, present in caveolae, bind t-SNARE at the target membrane. The mechanisms of caveolae-targeting to the basal membrane in endothelial cells are unclear; however, some inferences can be made from studies in neurons. Binding of v-SNARE to t-SNARE involves the spontaneous binding of the interacting domains of the v-SNAREs with the t-SNAREs (388). Docking of vesicles to the basal membrane occurs through the engagement of accessory proteins or vesicle tethering complexes Sec 6/8, conserved oligomeric Golgi (COG) complexes (see Ref. 1052), and syntaxin-associated protein Munc 18 (see Ref. 762). But their precise role in caveolar docking and fusion and in the mechanism of transcytosis in endothelial cells has not been delineated. In addition, intracellular  $\text{Ca}^{2+}$  signaling and PKC activation, known to stimulate exocytosis in neurons, regulates SNARE complex formation secondary to the unfolding of syntaxin (762), but whether this occurs in the exocytosis in endothelial cells is not known. Intriguingly, Fu et al. (277) demonstrated that siRNA-induced knockdown of syntaxin-4 (but not of syntaxin-3) inhibited exocytosis of Weibel-Palade bodies induced by thrombin. Phosphorylation of both syntaxin-4 and Munc 18 by PKC- $\alpha$  was required to induce exocytosis; thus it is possible that a similar phosphorylation step is responsible for exocytosis of caveolae during the process of transcytosis.



#### 4. Role of Rab GTPases

Rab proteins, members of the Ras superfamily of monomeric GTPases, are involved in the regulation of membrane trafficking (reviewed in Ref. 1101). More than 60 Rab GTPases have thus far been identified in the human genome with nearly all involved in signaling vesicle formation, motility, docking, or fusion (see Refs. 828 and 1101), events critical to endocytosis and exocytosis in endothelial cells. Rabs 5, 11, and 27 were shown to regulate clathrin-mediated endocytosis in epithelial cells (reviewed in Ref. 966). Endothelial cells express Rab 1–9, 11, 13, 14, 15, 18, 22, and 30 (212a, 447, 791, 1054). Studies showed that caveolar-mediated transcytosis was sensitive to NEM (717, 723, 811, 814), implying that transcytosis required the engagement of NSF in endothelial cells. In a series of experiments designed to identify NSF-interacting molecules, NSF-myc was incubated with cytosolic and membrane fractions of cultured human endothelial cells (722). These experiments demonstrated that in addition to SNAP-23, SNAREs, and caveolin-1, a number of other fusion-related proteins, especially Rab5, were present in endothelial caveolae. While these data localized Rab5 to caveolae, its role in transcytosis in endothelial cells (using approaches such as siRNA-induced knock-down of Rab5) remains to be addressed.

#### 5. Role of actin and myosin

Cortical actin filaments in the cell periphery are directly linked to and functionally integrated with the plasma membrane (for review, see Ref. 790). The proteins regulating polymerization of actin filaments, Arp2/3 complex, WASP, profilin, cortactin, and filamin (for reviews, see Refs. 667 and 790), also interact with proteins of the endocytic machinery, caveolin-1, dynamin, and intersectin (395, 483). Thus endocytic and exocytic events at apical and basal plasma endothelial membranes may require rearrangement of cortical actin. Myosin molecular motors have been described for axoplasmic transport of vesicles in nerve endings (see Ref. 501). Myosin-V, a member of 18 known types of myosin motors, is involved in anterograde and retrograde movement of vesicles along axons. It is unclear, however, whether vesicle motility in endothelial cells is dependent on myosin-V or other myosin motors. Any description of vesicle motility must take into account the actin barrier in endothelial cells that needs to be traversed. One intriguing possibility is that formation of a cometlike tail (so-called “rocket-based” vesicle motility) by a dynamin-dependent polymerization of actin filaments at the head domain of the comet could drive endocytic vesicles through the actin barrier (666); however, this mechanism has not been explored to explain vesicle trafficking in endothelial cells.

Actin may also have a role in caveolae-mediated endocytosis. Evidence indicates that cortical actin reorga-

nizes in association with the vesicle fission event (536, 640, 696; see also Ref. 695 for review). Imaging of actin in cells using a GFP reporter showed that actin was associated with coated vesicles as they detached from the plasma membrane (589). Disruption of polymerized actin with cytochalasin was shown to hinder caveolae-mediated endocytosis (536, 640, 683). In contrast, in cells stably expressing GFP-tagged caveolin-1, disruption of actin filaments in one case with latrunculin (an actin monomer sequestering agent) triggered the movement of caveolin-1-positive vesicles into the cell's interior (630) and in another case increased random caveolar docking at the target membrane (697). Thus these pharmacological approaches have not been clear in defining the role of actin in endocytosis. Because caveolae are not distributed uniformly over the entire endothelial cell surface, it is possible that localized actin polymerization, occurring at the barbed ends of actin filaments, regulates caveolae-mediated endocytosis by forming hot spots where caveolae are concentrated. Actin cytoskeleton, known to maintain the shape of endothelial cells (see Ref. 413), at the same time may provide a high degree of plasticity at sites of caveolae fission by regulating vesicle trafficking. Thus globally acting agents such as latrunculin and cytochalasin may not be useful in delineating the role of localized actin polymerization/depolymerization in the mechanism of endocytosis.

#### 6. Role of microtubules and molecular motors

Microtubules, tubulin heterodimers, are long filamentous elements that are important for the spatial organization of intracellular organelles as well as intracellular vesicle trafficking. Chinese hamster ovary cells expressing fluorescently tagged caveolin-1 showed movement of caveolae along microtubules ranging from 0.3 to 2  $\mu\text{m/s}$  (630), a finding consistent with a key role for microtubule motors in directed trafficking of vesicles. Disruption of microtubules with nocodazole caused the disappearance of cell surface caveolae in COS cells expressing a GFP-caveolin-1 construct (630). Likewise, endocytosis of fluorescently tagged dextran (molecular mass 70 kDa) was reduced after disruption of microtubules with colchicine (536). These observations raise the possibility that the released caveolae use microtubule tracks to traverse the endothelial cell and consequently the barrier, but this idea has not been rigorously examined. Kinesins and dyneins are mechanochemical motor proteins required for microtubule-based motility (reviewed in Ref. 444). siRNA-induced knock-down of kinesin (KIF 3A or KIF 3B) or dynein (DNCH1) in endothelial cells reduced transendothelial albumin transport (S. Siddiqui and A. B. Malik, unpublished data), pointing to a possible role of kinesin and dynein as important components of transcytotic machinery. However, further studies will be important to

unravel the mechanisms used by these motor proteins to regulate albumin transcytosis.

### C. Role of Endothelial Cell Surface Albumin-Binding Proteins in Transcytosis

Several laboratories have described a role for albumin-binding proteins on endothelial cells in regulating transendothelial albumin transport via transcytosis (28, 316, 317, 595, 813, 860, 948, 955). Albumin binding to the endothelium is a saturable and specific process, and the albumin is subsequently discharged into the subendothelial space within minutes after labeling of the luminal membrane (28, 316, 317, 595, 813, 860). These features of albumin transport fit with the classic ligand-receptor concept in that albumin binds to a saturable and compatible “receptor” on the endothelial cell surface (315, 316, 813, 818). Four albumin-binding glycoproteins of varying molecular masses (18, 31, 60, and 75 kDa) have been identified in endothelial cell membrane fractions that may be involved in albumin binding, uptake, and transport (314–316, 812, 818, 859, 948). Additional studies have ruled out gp18 and gp31 as receptors in mediating albumin transcytosis because they may instead be scavenger receptors for the uptake of denatured and modified albumin forms (602, 812, 818). Although this process has not been well investigated, denatured albumin may be internalized by this mechanism and targeted to lysosomes for degradation. In contrast, several studies have supported a role of gp60 as an albumin-binding protein responsible for the specific albumin binding that promotes transcytosis (432, 603, 812, 948, 955, 1010, 1012). Cy3-fluorophore-tagged anti-gp60 antibody showed a punctate distribution on the cell surface of pulmonary microvascular endothelial cells, typical of the profile of clustered cell-surface receptors (955). Interestingly, gp60 was shown to colocalize with both albumin and caveolin-1 (Fig. 28). Activation of gp60 by cross-linking using an anti-gp60 antibody (955) caused up to threefold increases in the uptake and transcellular transport of  $^{125}\text{I}$ -albumin tracer in endothelial cells (433, 603, 955) (Fig. 8). Albumin uptake was also markedly reduced after depletion of cell surface gp60 by preincubating endothelial monolayers with an anti-gp60 antibody (603, 955), suggesting that the albumin “receptor” could be internalized. Although these findings are consistent with the role of gp60 as an albumin “receptor,” in endothelial cells responsible for albumin endocytosis via caveolae, many details are unknown.

A study has described two binding affinities of albumin for gp60: high-affinity ( $K_{D1} = 13.5 \text{ nM}$ ) and low-affinity ( $K_{D2} 1.61 \mu\text{M}$ ) binding sites in rat lung microvascular cells (433). The proposed model based on these findings is that gp60 exists in clustered and unclustered

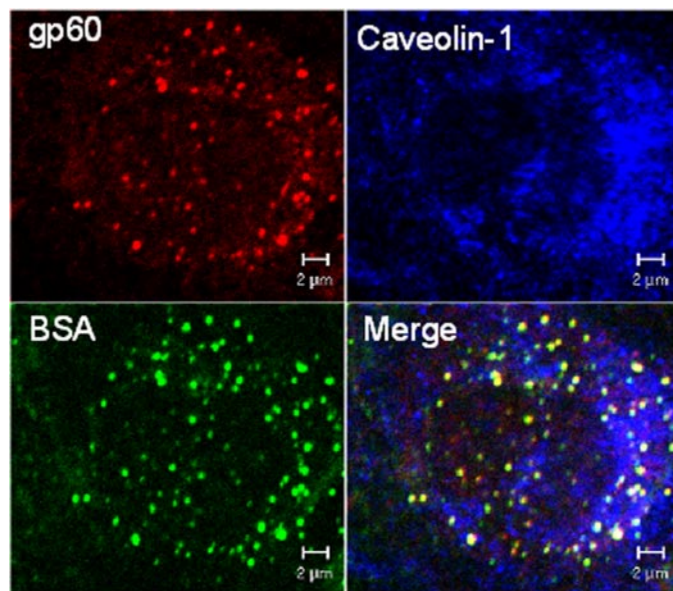


FIG. 28. Colocalization of albumin with gp60 and caveolin-1. High-magnification confocal image ( $<1.0 \mu\text{m}$  in thickness) of rat lung microvascular endothelial cells shows coincident (yellow) staining of gp60 (red) and albumin (green) and coincident (white) of gp60, albumin, and caveolin-1 (blue). [Adapted from John et al. (433).]

forms (433) (Fig. 29). This model predicts that 1) binding of albumin and its uptake exhibit a saturable and a fluid-phase component, with the latter accounting for most of the albumin internalized in caveolae and 2) endothelial uptake of albumin is submaximal at normal plasma albumin concentration, implying that it could be augmented in response to signals induced by gp60 activation. Thus this is a useful model because it describes testable hypotheses. As the identity of gp60 is not yet known, the precise role and mode of action of gp60 as a receptor for albumin transcytosis remains enigmatic.

### D. Membrane Dynamics

As mentioned above (discussed in sect. xA), caveolae (the vesicle carriers involved in transcytosis) are abundant in vascular endothelial cells such that their number effectively doubles the available surface area of the luminal membrane. The plasma membrane of endothelial cells is constantly undergoing remodeling on the basis of vesicle trafficking. Caveolae biogenesis (discussed in sect. viiC) occurs through reorganization of plasmalemmal lipids into specialized membrane microdomains rich in cholesterol and sphingolipids (reviewed in Ref. 274). Formation of flask-shaped membrane lipid microdomains characteristic of caveolae is highly dependent on plasma membrane proteins (such as caveolin-1) having high-affinity binding sites for specific membrane lipids, e.g., cholesterol and sphingolipids (see Ref. 377). The essential

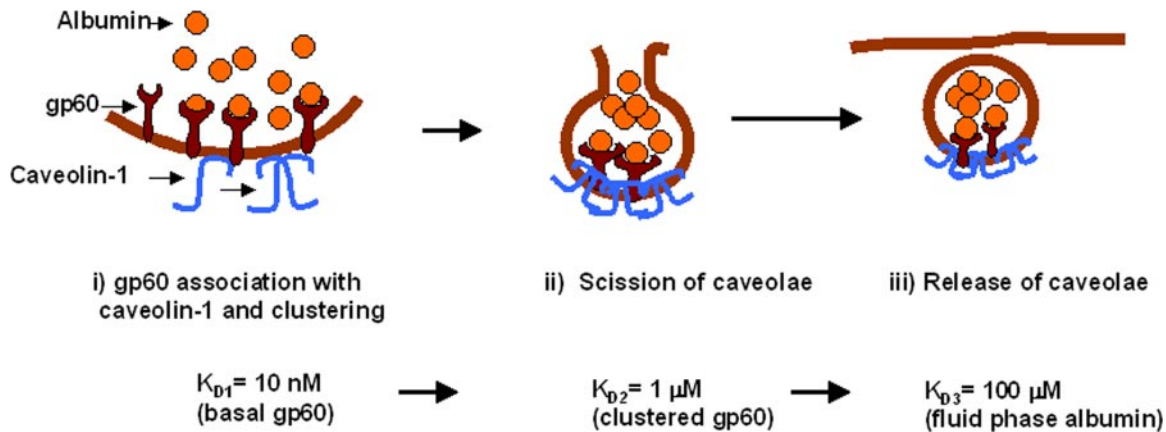


FIG. 29. Model of gp60-mediated albumin internalization in endothelial cells. Under albumin-free conditions, gp60 exists as a high-affinity ( $K_{D1} = 10 \text{ nM}$ ) cell surface albumin-binding protein; on activation with albumin gp60 clusters, resulting in a 100-fold decrease in albumin affinity ( $K_{D2} = 1 \mu\text{M}$ ; *i*); fluid-phase component of albumin vesicular association has 100-fold lower affinity ( $K_{D3} = 100 \mu\text{M}$ ) than gp60-bound albumin (*ii*). Release of caveolae from the plasma membrane traps fluid-phase albumin and other plasma constituents within the vesicle. In this model fluid phase accounts for 98% of the albumin internalized (*iii*). [Adapted from John et al. (433).]

role of caveolin-1 in mediating caveolar structure and function seems to be clear in that caveolae are not seen in *Cav-1*<sup>-/-</sup> mice (223, 740, 821; see also Refs. 584, 721). Caveolin-1 exists as an oligomeric complex of 14–16 monomers (complex molecular mass of 350–400 kDa) in caveolae (614, 784). Caveolin-1 binds with unusually high affinity to cholesterol, sphingolipids, sphingomyelin, and glycosphingolipids, both in vitro and in cells (273, 631). Studies showed that absolute plasma membrane levels of cholesterol need to rise 50% above a threshold level before caveolae form (346, 348, 835). Depletion of membrane cholesterol with cholesterol-sequestering agents such as nystatin, cyclodextrin, filipin, or simvastatin result in disassembly of caveolae (346, 767, 816). Perturbations in cholesterol levels were also shown to influence caveolin-1 expression (94, 265, 346), indicating that cholesterol plays an important role in caveolae biogenesis by transcriptionally regulating expression of caveolin-1.

The oligomerization of caveolin-1 is another crucial step for formation of plasmalemmal caveolae (614, 784). Caveolin-1 self-associates to form end-on-end oligomeric complexes (801, 887). In forming such complexes, caveolin-oligomers interact with each other by their COOH-terminal domains (residues 135–178). Oligomers thus formed are the presumptive structural units of caveolae. Interestingly, the self-assembly of the caveolin-1 oligomers from monomers begins in the endoplasmic reticulum immediately after synthesis of caveolin-1 (614). Supramolecular complexes of caveolin oligomers also are found in the *trans*-Golgi apparatus (229, 264). These oligomers are targeted to the plasma membrane where they interact with membrane cholesterol and sphingolipids to drive the process of membrane invagination, resulting in the formation of caveolae.

## XI. SIGNALING MECHANISMS REGULATING TRANSCYTOSIS

Transcytosis in endothelial cells is a multistep process involving a sequential series of events: budding, fission, translocation, docking, and fusion of caveolae with the abluminal membrane. A number of caveolae-associated proteins have a functional role in regulating transcytosis. Predescu et al. (722), on the basis of biochemical and morphological studies, identified a “endothelial macromolecular transcytotic complex (EMTC) consisting of proteins involved in fission, targeting, docking, and fusion of caveolae with the endothelial plasma membrane. These proteins representing the essential elements of the signaling complex include caveolin-1, dynamin, Rab5, NSF, and syntaxin. The following sections discuss the current state of understanding concerning the signaling intermediates regulating transcytosis in endothelial cells.

### A. Role of Src Kinase

Studies have pointed to the Src family kinases as the primary tyrosine kinases regulating caveolae-mediated endocytosis, the initial step in albumin transcytosis (324, 521, 603, 640, 683, 830, 955). The Src kinase inhibitors herbimycin (955) and PP2 (830) prevented albumin internalization and transcellular albumin transport and reduced the number of transport vesicles in endothelial cells (830, 955). Coimmunoprecipitation studies showed that caveolin-1 and dynamin coassembled presumably as a multimeric complex secondary to phosphorylation induced by Src (603, 830). The assembly of this complex was a requisite for the initiation of caveolar endocytosis (see review in Ref. 602). The proline-rich binding SH3



domains in Src are crucial in facilitating protein-protein interactions (see review in Ref. 120); thus Src not only mediates phosphorylation of specific proteins of the EMTC, i.e., caveolin-1 and dynamin, required for caveolar endocytosis, but also may signal other steps for transcytosis of caveolae such as docking and fusion to the basal membrane. Src kinase-induced phosphorylation of both dynamin and caveolin-1 in the presence of albumin occurred within minutes of exposure (831), consistent with the phosphorylation of these caveolae-associated proteins as a key "switch" required for signaling endocytosis. Dynamin Y597 was identified as the crucial residue phosphorylated by Src kinase responsible for caveolae-mediated endocytosis (830) (Fig. 25B). An important function of Src phosphorylation of dynamin may be to regulate its GTPase activity (discussed in sect. xB1), which is essential for caveolar fission (656, 830). Src-induced caveolin-1 and dynamin phosphorylation was also significantly increased in the presence of sodium orthovanadate (830), a broad-spectrum inhibitor of tyrosine phosphatases. As sodium orthovanadate treatment augmented albumin transcytosis in these studies (830), it is possible that a balance between tyrosine phosphorylation and dephosphorylation of caveolin-1 and dynamin is a determinant of caveolae-mediated endocytosis. In this regard, identification of the specific tyrosine phosphatases involved will be important in helping to explain the regulation of dynamin and caveolin-1 phosphorylation, and its functional significance in the mechanism of caveolae-mediated endocytosis.

### B. Role of PKC Isoforms

The PKC family of proteins consists of isoforms with different  $\text{Ca}^{2+}$  and phorbol ester requirements for activation: conventional ( $\alpha$ ,  $\beta$ ,  $\delta$ ), novel ( $\epsilon$ ,  $\gamma$ ), and atypical ( $\zeta$ ,  $\nu$ ) (34). In particular, the conventional isoforms of PKC- $\alpha$ , - $\beta$ , and - $\gamma$  were found to be concentrated in caveolae (878, 929); however, their role in caveolae-mediated endocytosis has not been extensively studied. Activation of conventional and novel PKCs by phorbol 12-myristate 13-acetate (PMA) inhibited caveolae internalization (878). Analysis of the amino acid sequence of caveolin-1 revealed a consensus site for PKC phosphorylation (929), but the mutants have not been studied to assess the role of PKC in regulating caveolae-mediated endocytosis. In nonendothelial cells, inhibition of PKC- $\alpha$  activation using pharmacological agents or dominant negative mutants prevented endocytosis (835), raising the possibility that PKC could phosphorylate caveolin-1, and thereby contribute to signaling endocytosis, but the role of PKC-regulated phosphorylation remains an important question that is so far unanswered.

### C. Role of PI 3-Kinase

Of the several mammalian PI 3-kinases identified, class I PI 3-kinase generating phosphatidylinositol 3,4,5-trisphosphate is the best described. Class I enzymes are heterodimers comprising separate catalytic and regulatory subunits (see Refs. 276 and 981a). The class IA enzyme (p85/p110) is activated by receptor tyrosine kinases (RTK) upon binding of p85 regulatory subunit to Y-P sites in the activated kinases or by tyrosine phosphorylated substrates of RTKs (45, 144). Class IB enzyme (PI 3-kinase  $\gamma$ ) is activated by the  $\beta\gamma$ -subunits of heterotrimeric G proteins (899). PI 3-kinase activity was found to be crucial for endocytosis (see Ref. 145); however, little is known about how and what classes of PI 3-kinases regulate endocytosis and vesicle trafficking in endothelial cells. Inhibition of PI 3-kinase activity with wortmannin (at nanomolar concentrations) blocked endocytosis in endothelial cells (640) and the formation of early endosomes (518). There have been no studies addressing the specific role of class IA or IB enzymes in the mechanism of endocytosis in endothelial cells. A potentially valuable strategy may be to use the inhibitory p85 peptide conjugated to the TAT sequence (632), which can enter endothelial cells and can selectively inhibit the class IA enzyme, to assess the role of PI 3-kinase in regulating caveolae-mediated endocytosis.

### D. Role of $\text{Ca}^{2+}$ Signaling

Caveolae contain the key elements of the  $\text{Ca}^{2+}$ -signaling machinery:  $\text{IP}_3$  receptor (which mediates intracellular  $\text{Ca}^{2+}$  release from ER stores) (280, 543), store-operated  $\text{Ca}^{2+}$  channels (SOCs) transient receptor potential channel-1 (TRPC1) and TRPC3 (543, 873), and  $\text{Ca}^{2+}$ -ATPase (278). However, whether  $\text{Ca}^{2+}$  signaling contributes to the mechanism of caveolae-mediated endocytosis and transcytosis of albumin remains uncertain. In secretory cells and nerve endings, localized increases in intracellular  $\text{Ca}^{2+}$  act as triggers for the delivery of vesicles containing neurotransmitters to sites of exocytosis (reviewed in Ref. 36). In endothelial cells, the increase in intracellular  $\text{Ca}^{2+}$  was shown to induce exocytosis of Weibel-Palade bodies (specialized secretory vesicles containing preformed proteins such as P-selectin which are released upon cell stimulation) (84, 277, 464). Therefore, it is possible that caveolar transcytosis of albumin requires a similar exocytic stimulus, but it is unknown whether  $\text{Ca}^{2+}$  signaling regulates this step.

### E. Role of 60-kDa Glycoprotein (gp60)

The albumin binding protein, gp60 mentioned above, was initially characterized by its affinity for galactose-

binding lectins, *Limax flavus* agglutinin and *Ricinus communis* agglutinin, which in competition experiments inhibited albumin binding to fat tissue microvascular endothelial cells from rats (316, 812). Siflinger-Birnboim et al. (859) showed that *Ricinus communis* agglutinin precipitated gp60 from bovine lung endothelial cell membranes and that these lectins also inhibited transendothelial albumin transport by ~50%. These findings suggest an important function for gp60 in regulating albumin permeability. Albumin was also colocalized with gp60 in luminal plasmalemma vesicles of pulmonary microvessel endothelial cells (Fig. 28), and activation of gp60 increased transcellular vesicle trafficking as monitored by styryl pyridinium dyes that fluoresce at lipid-water interfaces (433, 603). Vesicle trafficking in endothelial cells was also found to be sensitive to the cholesterol-sequestering agent filipin (816, 1010, 1012). Decreased cell surface expression of gp60, induced by downregulation of the protein with antibodies, blocked both albumin binding and transport in cultured rat lung microvascular beds (433, 602, 603, 815). In contrast, activation of gp60 by antibody-induced cross-linking of gp60 promoted clustering of gp60 and stimulated albumin transcytosis in both endothelial monolayers (433, 955) and lung microvessels (432, 1010, 1012). Studies in vessels also showed, surprisingly, that gp60 cross-linking did not increase  $K_{f,c}$  or induce tissue edema at a time when tracer albumin transport increased twofold (1012) (Fig. 8). Thus the permeability pathways for liquid and protein appear to be distinct and independently regulated; that is, transcellular albumin permeability after gp60 activation occurred via a nonhydraulic pathway compared with the transport of liquid. Also, the activation of gp60 had no effect on endothelial TER (955). Together, these studies show that binding of albumin to cell surface gp60 is a requisite for the activation of albumin transcytosis (as described in sect. xC).

Studies to some extent have addressed the signaling pathways by which gp60 induces transcytosis. Activation of gp60 by either albumin (the gp60 ligand) or a gp60 antibody cross-linking (as described above) induced phosphorylation of gp60, caveolin-1 (603, 955), and dynamin (830) on tyrosine residues (described in sect. x1A1) as well as phosphorylation of two members of Src family kinases, pp60Src and Fyn (955). In addition, gp60 upon activation was associated with caveolin-1, pp60Src, pp59Fyn (603, 955), and dynamin (830), indicating the formation of a multiprotein complex. Inhibition of tyrosine kinases by herbimycin A, genistein (955), or PP2 (a Src-specific inhibitor) (830) as well as expression of dominant-negative Src (603) prevented the gp60-activated vesicle formation and endocytosis of albumin (603, 830, 955). These results point to the key role of a Src pathway in gp60-induced albumin endocytosis (discussed in sect. xC). Inhibition of the heterotrimeric G protein,  $G_i$ , either

by pertussis toxin, expression of the selective peptide antagonist (11-amino acid COOH-terminal peptide of  $G_i$ ) (603), or sequestration of  $\beta\gamma$ -subunit of  $G_i$  by COOH-terminal  $\beta$ -adrenergic receptor kinase (ct-BARK) (831) also prevented gp60-induced albumin endocytosis. On the basis of these data, the current model (see sect. ixA) is that gp60 stimulates Src through activation of the  $\beta\gamma$ -subunit of  $G_i$ , and thereby signals albumin endocytosis (Fig. 30). Other studies have found an association of  $G_q\alpha$  with caveolin-1 in endothelial cells (657); however, whether  $G_q\alpha$  is also required for activation of albumin endocytosis remains unclear.

## XII. MEDIATORS OF INCREASED ENDOTHELIAL PERMEABILITY

The ultimate effect of permeability-increasing mediators is to form IEJ gaps between contiguous endothelial cells, and it has been found that various mediators utilize a number of different signaling pathways to arrive at the same end point, junctional gaps between endothelial cells (Fig. 31). Barrier disruption induced by these mediators varies greatly depending on location of the endothelial cells along the vascular tree and the species of origin. Some mediators (such as S1P and angiopoietin-1) demonstrate the ability to restore endothelial barrier integrity. The signaling pathways involved in barrier restoration are currently under intense investigation, since their understanding will shed new light on the specific signals utilized to suppress the potentially pathological increased permeability response (Fig. 31). In the following sections, we provide an overview of these mediators and the signaling pathways that regulate endothelial barrier function in response to these specific mediators.

### A. Mediators of Increased Endothelial Permeability

#### 1. Thrombin

Thrombin, a procoagulant serine protease, is well known to increase endothelial permeability (reviewed in Refs. 190, 225, 552, 553, 952). In addition, thrombin has been shown to regulate cellular processes such as hemostasis, mitogenesis, and smooth muscle contraction (190). Thrombin is generated by the proteolytic cleavage of the inactive proenzyme prothrombin by factors V and X with the aid of the cofactors  $Ca^{2+}$  and membrane phospholipids (see review in Ref. 338). Thrombin is typically short-lived in the circulation ( $t_{1/2} = 5$  min) and removed by activated protein C (reviewed in Ref. 245); thus thrombin can only induce a localized increase in vascular permeability after it is released into blood. Interestingly, thrombin can be concentrated 1,000-fold in fibrin clots and may

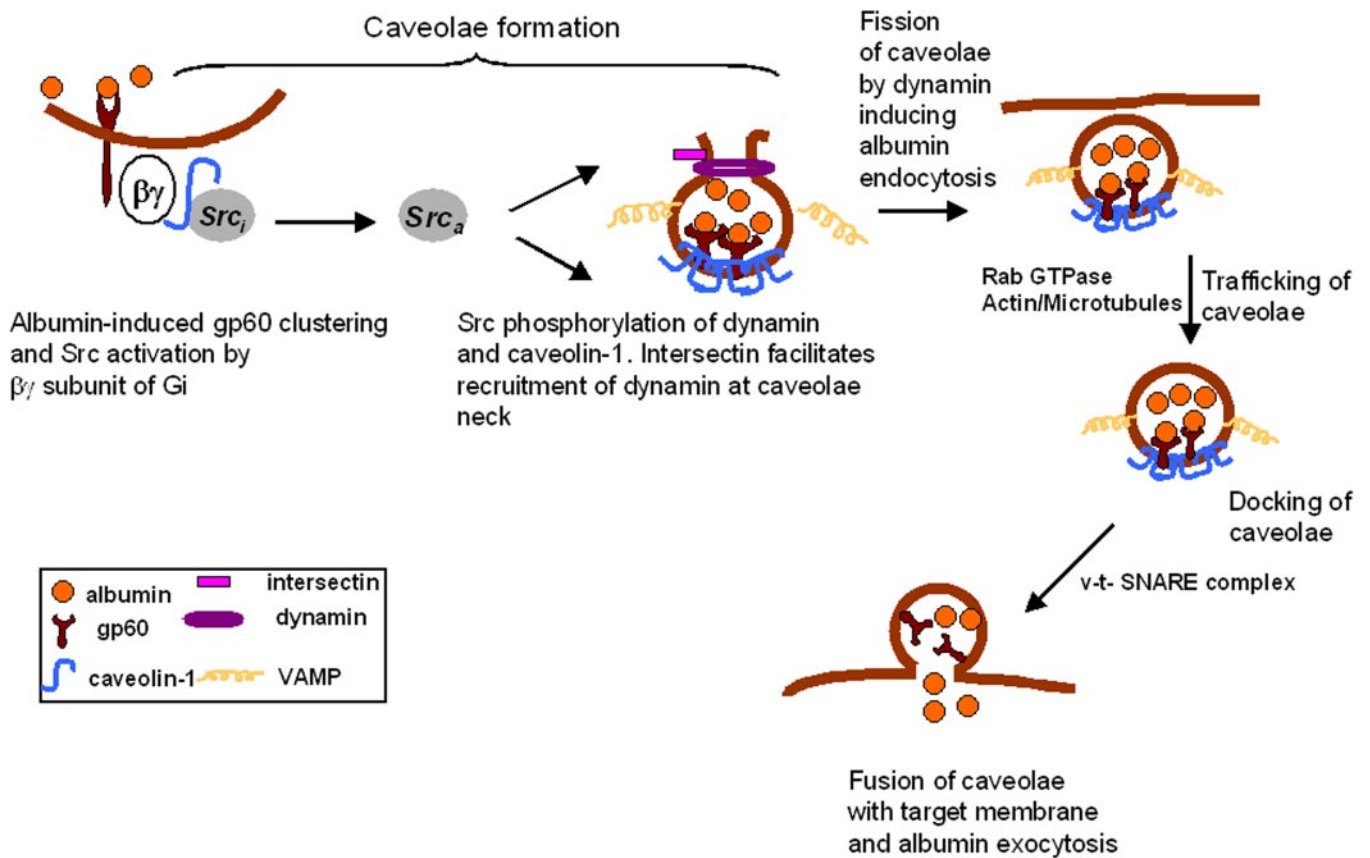


FIG. 30. Summary of signaling mechanisms regulating albumin transcytosis. Albumin binding to the albumin-binding protein gp60 activates G $\beta\gamma$ , which in turn induces activation of Src (Src $\alpha$ ). Activated Src phosphorylates dynamin and caveolin-1. Intersectin in association with phosphorylated dynamin induces fission of caveolae. The specific role of caveolin-1 phosphorylation is not clear, but it is known to regulate fission of caveolae. Free caveolae then move to a target membrane in a Rab GTPases-dependent manner, possibly mediated by actin/microtubules where v-SNAREs on caveolae interact with the t-SNAREs on target membrane to induce the fusion of the caveolae with the membrane and albumin exocytosis. [Modified from Minshall et al. (602).]

be gradually released into the microcirculation during fibrinolysis (see Ref. 245); thus the thrombin-induced increase in vessel wall permeability is associated with intravascular thrombosis coupled to fibrinolysis of clots. Thrombin's effects on endothelial permeability have been assessed in cultured cells and microvessels, and many aspects of the signaling pathways leading to the disruption of endothelial barrier have been delineated. Thrombin mediates increased endothelial permeability by binding to its receptors, PAR-1, on endothelial cells (reviewed in Refs. 190, 225, 952). PAR-1 is activated by its ligation, which induces thrombin-dependent proteolysis of the PAR-1 extracellular extension (between Arg-41 and Ser-42) (312, 1018). This tethered ligand binds to the second extracellular loop of heptahelical PAR-1 and initiates downstream signaling events (312, 738, 1018). The synthetic 14-amino acid peptide SFLLRNPNDKYEPF (TRP-14) can substitute for this tethered ligand (1018). Although PAR-1 is expressed in endothelial cells of capillaries and venules (385) and is crucial for the mechanism of permeability increase (1011; see also Ref. 952), three

other isoforms of PARs have been identified in endothelial cells of which only two are activated by thrombin (415, 440, 806, 1018). PAR-2 is trypsin activated (606, 654). Human umbilical vein endothelial cells were shown to contain two categories of binding sites, a small population of  $\sim 3,000$  binding sites/cell with dissociation constant ( $K_d$ ) of  $10^{-10}$  M (43) and a larger population of  $\sim 0.05 \times 10^6$  binding sites/cell with  $K_d$  greater than  $10^{-8}$  M (43, 950), but this can vary in endothelial cells obtained from different vascular components (950). Thus the magnitude of the permeability response is dependent on both PAR-1 number and affinity. The role of PAR-3 and PAR-4, the other thrombin receptors, in mediating an increase in endothelial permeability has not been established (448).

Data from endothelial cells show that thrombin increases transendothelial permeability (as determined using tracer albumin and transendothelial resistance) within minutes, and reversal of the response occurred within 2 h after removal of the agonist (303, 549, 587, 620, 622, 951). In the continuous presence of thrombin, recovery also occurred within 2 h (587, 620, 622, 951), indicating either



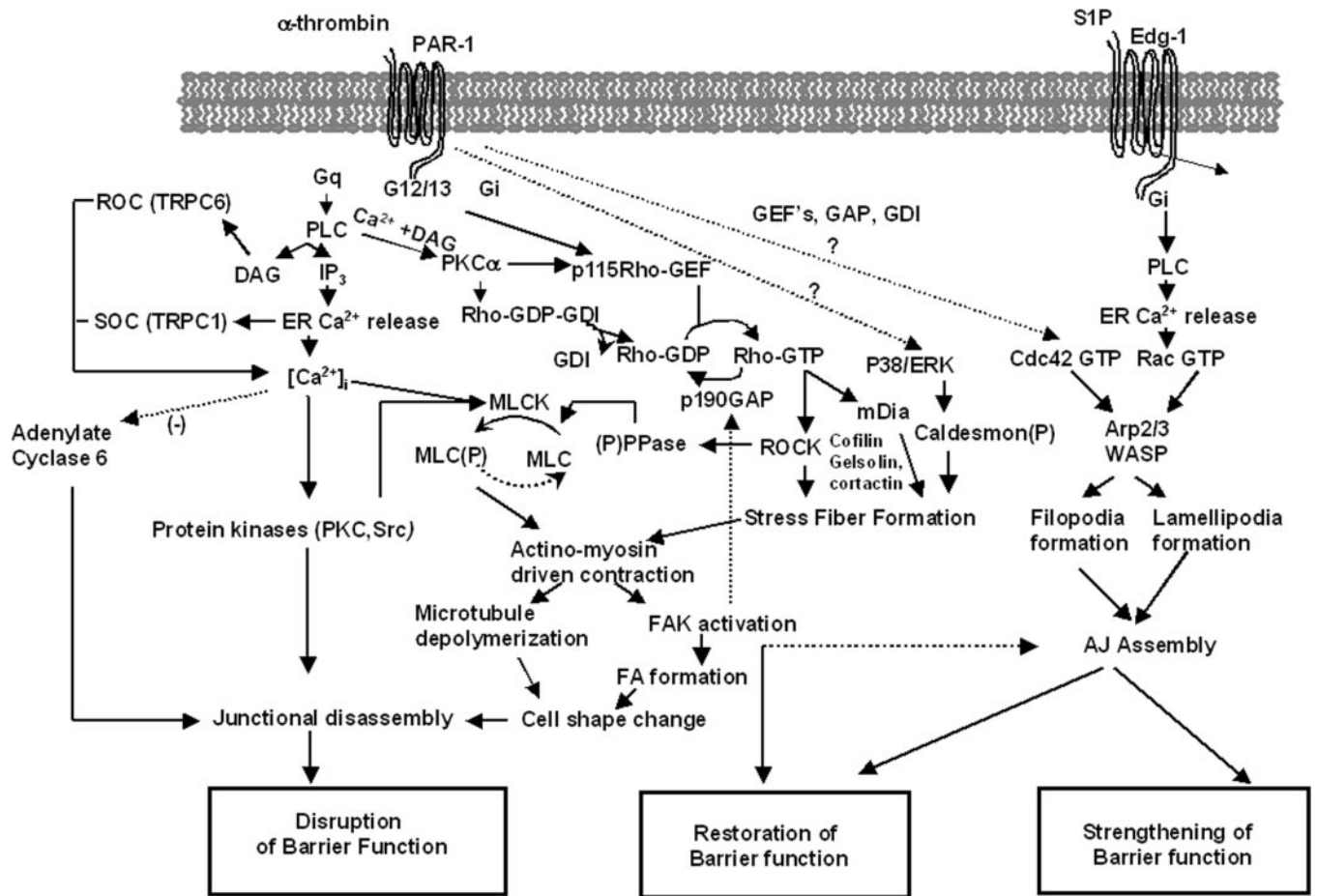


FIG. 31. Signaling mechanisms that mediate increased endothelial permeability and subsequent barrier restoration via the paracellular pathway. Agonists such as thrombin induce increased intracellular  $\text{Ca}^{2+}$ , which increases the activity of EC MLCK and the phosphorylation level of MLC (MLC-P). Increased intracellular  $\text{Ca}^{2+}$  also activates protein kinases such as PKC- $\alpha$  (also requires DAG) or Src but inhibits adenylate cyclase-6 ( $\text{AC}_6$ ). Permeability-increasing mediators via PKC- $\alpha$  phosphorylate GDI-1 and p115RhoGEF, which in turn increase the activity of RhoA GTPase. RhoA activates ROCK and mDia, which regulate actin binding proteins (gelsolin, cofilin) and thus the state of actin polymerization. ROCK inhibits MLC phosphatase (Ppase) by phosphorylation and therefore increases MLC-P. Together, these events lead to increased actin-myosin cross-bridging, resulting in increased endothelial contraction. Thrombin also activates p38/ERK MAP kinases, which phosphorylate caldesmon. Caldesmon increases stress fiber formation and induces contraction in the absence of MLC phosphorylation. Contraction of endothelial cells in turn leads to disassembly of adherens junctions, depolymerization of microtubules, and FAK activation. FAK increases focal adhesion formation, promoting the interaction of endothelial cells with ECM. FAK may also suppress RhoA activity by activating p190RhoGAP and may also trigger IJEs assembly. Disassembly of AJs induces activation of Cdc42, which activates filopodia formation involved in establishing new cell-cell contacts and reannealing of the endothelium. Thus FAK and Cdc42 acting serially or in parallel restore barrier function. S1P by binding to its Edg-1 receptor strengthens barrier function in a Gi-Rac-dependent formation of lamellipodia and AJ assembly.

desensitization of PAR-1 or activation of the signaling pathways opposing endothelial retraction but promoting intercellular and cell-ECM adhesion, and ultimately leading to barrier recovery (see sect. IXB). There is also the untested possibility that thrombin activates enzymes such as sphingosine kinase that by synthesis of the barrier-protective phospholipid S1P can reverse the increase in endothelial permeability in an autocrine/paracrine manner (113).

With the use of an intact lung model, thrombin was shown to increase pulmonary vascular resistance in conjunction with a rise in the  $K_{f,c}$  and lung water content (383, 1011). Development of edema in isolated lung prep-

aration was attributed both to thrombin's pressor effect and an increase in vascular endothelial permeability (383, 1037). However, with sufficiently low concentration of either thrombin or PAR-1 agonist peptide (TFLLRNPNDK-NH<sub>2</sub>), Vogel et al. (1011) were able to dissociate the increased lung wet weight secondary to the rise in pulmonary capillary hydrostatic pressure from that induced by increased vascular permeability. These findings indicate the direct effect of thrombin on vessel wall permeability. The permeability-increasing effect of thrombin on lung microvessels was primarily attributed to the activation of PAR-1, since increases in the  $K_{f,c}$ , pulmonary capillary hydrostatic pressure, and lung wet weight were

absent in mice deficient in the PAR-1 gene (*PAR-1*<sup>-/-</sup>) (1011). Increased microvessel permeability induced by PAR-1 presumably required endothelial contraction because thrombin failed to induce MLC phosphorylation in *PAR-1*<sup>-/-</sup> mice (1011). Furthermore, EC MLCK was required because thrombin failed to increase lung microvessel permeability in *EC MLCK*<sup>-/-</sup> mice (S. Vogel, unpublished observations). Another study showed that PAR-4 in conjunction with PAR-1 could also contribute to signaling of the permeability increase in endothelial cells (448); however, these data are difficult to reconcile with the observation that the thrombin-induced increase in endothelial permeability was fully abrogated in *PAR-1*<sup>-/-</sup> mice (1011).

Studies in the mesenteric vascular bed of rats showed that thrombin failed to increase  $L_p$  (198); however, when the vascular bed was challenged with LPS, thrombin had a marked effect in increasing the leakiness of the barrier. This lack of response in the unperturbed microcirculation may be the result of a low level of expression of PAR-1 in endothelial cells and PAR-1 affinity for thrombin; thus it may be that LPS-induced expression of PAR-1 is able to unmask the response to thrombin.

PAR-1 function is controlled by receptor proteolysis, phosphorylation, internalization, and degradation (114, 238, 833, 1081). Internalized PAR-1 is sequestered in endosomes via clathrin-coated pits that fuse with lysosomes (387). Endothelial cells also possess significant intracellular preformed pool of PAR-1, probably associated with the Golgi apparatus (385). Degradation can result in subsequent resensitization of PAR-1 within 60–90 min (238, 903). When the intracellular receptor pool was depleted, there was a further delay in recovery of thrombin responsiveness probably due to the requirement for de novo synthesis of PAR-1 (238, 903).

PAR-1 has the characteristic seven membrane-spanning domains typical of all G protein-coupled receptors. Multiple heterotrimeric G proteins,  $G_q$ ,  $G_{12/13}$ , and  $G_{i/o}$ , couple to PAR-1 (52, 189, 393, 655, 737, 981b); however, the affinity of PAR-1 for each of these G proteins is unclear. An additional level of complexity is added by regulators of G protein signaling (RGS) because they are able to "switch-off" G protein activity by their GTPase activity (see Ref. 480). Given the finding that PAR-1 activation induced a reversible increase in endothelial permeability, studies investigating the role of PAR-1 interaction with different G proteins and the role of RGS proteins in regulating these interactions are important areas of investigation. These studies will fill in the gap in our understanding of how PAR-1 interaction with heterotrimeric G proteins can be switched off, thereby restoring barrier function.

Downstream of PAR-1, the  $G\alpha_q$  subunit of holoprotein,  $G_q$ , activated the enzyme phospholipase C (PLC),

which catalyzes production of  $IP_3$  and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) (see Refs. 85 and 952).  $IP_3$  in turn activates  $IP_3$  receptor in ER to cause the rapid release of sequestered  $Ca^{2+}$  into the cytosol. Depletion of ER stores elicits  $Ca^{2+}$  entry from the extracellular milieu via SOCs.  $Ca^{2+}$  entry makes an important contribution to the endothelial permeability response to thrombin (discussed in section *xAIC*). In contrast to  $G_q$ , the roles of  $G_{12}$  and  $G_{13}$  in regulating endothelial permeability downstream of active PAR-1 receptors are only beginning to emerge. Studies in various cell types indicate that overexpression of dominant-active  $G_{12/13}$  mutants induce RhoA activation through their effector, p115RhoGEF (81, 351, 479, 1046). PAR-1 activation was shown to induce the phosphorylation of GDI-1 (586) and p115RhoGEF (381). Inhibition of PKC- $\alpha$  under these conditions suppressed the phosphorylation of GDI-1 (586) and p115RhoGEF (381), preventing RhoA activation (381, 586). Thus RhoA activation via the  $G_{12/13}$ -coupled PAR-1 evidently requires PKC- $\alpha$ -dependent phosphorylation of p115RhoGEF. These studies are intriguing as they suggest that RhoA is the common downstream effector for both  $G_q$ - and  $G_{12/13}$ -coupled receptors, and thus its position at a nodal point is likely to be crucial in the mechanism of thrombin-induced increase in endothelial permeability.

PAR-1 activation was shown to cause a delayed activation of Cdc42, coinciding with the recovery of endothelial barrier function from thrombin's permeability-enhancing effects (475). However, the identity of G proteins and GEFs/GDIs specific to Cdc42 in this response is not clear. There is also evidence that the  $G_{i/o}$  family of heterotrimeric G proteins is coupled to PAR-1 receptor and negatively regulates adenylate cyclase activity (564). For example, thrombin inhibited isoproterenol- or 3-isobutyl-1-methylxanthine (IBMX)-stimulated cAMP production in rat lung endothelial cells, whereas inhibition of  $G_i$  by pertussis toxin prevented this response (564). Inhibition of  $G_i$  by pertussis toxin also prevented the thrombin-induced increase in endothelial permeability (981b). These findings indicate that thrombin may additionally signal barrier disruption by a  $G_i$ -coupled pathway. However, pertussis toxin alone was shown to induce a leaky endothelial barrier (690), indicating that the basal activity of  $G_i$  may itself be required for barrier function. In fact,  $G_i$  activation was required to strengthen barrier function in response to S1P (240, 300, 466, 585, 780).  $G_i$  also contributes to caveolae-mediated albumin transcytosis (602, 603), but its role in the mechanism of thrombin-induced increase in endothelial permeability is uncertain. Thus the challenge in understanding the role of specific heterotrimeric G proteins in PAR-1 signaling is to investigate the specific effectors through which these G proteins integrate signals, and thereby regulate endothelial permeability.

## 2. Bradykinin

Bradykinin is a potent inflammatory and vasoactive nonapeptide generated by a family of serine proteases, the kallikreins, at sites of tissue injury (see Ref. 744). Bradykinin disrupts endothelial barrier in a cultured cell monolayer as well as in intact microvessels (8, 20, 35, 254, 357, 789, 848, 908, 914, 915, 1020, 1091). Bradykinin is rapidly degraded (with  $t_{1/2} = \sim 27$  s) by the peptidases angiotensin converting enzyme and dipeptidyl carboxypeptidase kinase II localized on the endothelial cell surface (1091; see Ref. 875 for review). The biological effects of bradykinin are mediated through the activation of three receptor subtypes, classified as B1, B2, and B3 (255, 744). These receptors differ in their affinity for bradykinin and relative potencies for antagonists of bradykinin receptors (255, 744). B2 receptors have greater affinity for bradykinin than for kininase I metabolites such as des-Arg<sup>9</sup>-bradykinin, whereas B1 receptors have greater affinity for des-Arg<sup>9</sup>-bradykinin than bradykinin (82, 744). Endothelial cells have both B1 and B2 receptors; however, several differences were found between the two receptors (361, 611, 908). Whereas stimulation of the B1 receptor resulted in a prolonged response because this receptor is resistant to desensitization and internalization, B2 receptor activation is short-lived as the receptor is rapidly sequestered and desensitized (40, 220b, 566; see Ref. 514 for review). B2 receptors are constitutively expressed, and B1 receptor expression is only seen following injury or inflammation (reviewed in Ref. 566). An additional level of complexity arises from the fact that the response of the endothelium to bradykinin *in vitro* depends on the source of cultured endothelial cells. Bovine pulmonary arterial endothelial cells (BPAEC) or bovine microvessel endothelial cells (BMVEC) responded with increased transendothelial permeability when stimulated with 0.1 or 1.0  $\mu$ M bradykinin (16, 611). However, HUVECs were unresponsive to bradykinin even in the presence of captopril, the kininase II inhibitor (152), perhaps because the receptor expression is low in these cells. Activation of B1 or B2 receptors via G<sub>q</sub>/G<sub>i</sub> signaled increased intracellular [Ca<sup>2+</sup>], accelerated PI turnover, and production of prostaglandins and NO in endothelial cells (82, 181, 618, 783, 879, 937). Unlike thrombin, the bradykinin-induced increase in endothelial permeability was surprisingly not affected by inhibition of MLCK or Rho (5, 8). Thus, despite G<sub>q</sub> involvement, the bradykinin-induced endothelial permeability increase occurred independently of MLCK or the Rho pathway. An important unanswered question is whether bradykinin disrupts AJs through increases in cytosolic NO and the nitration of effector proteins. This mechanism of increased endothelial permeability may be important since nitration of albumin has been shown to increase IEJ permeability, implying that nitration creates

an albumin form capable of directly increasing permeability by disruption of the junctions (720).

## 3. Histamine

Another classical mediator of increased endothelial permeability, histamine, is the biogenic amine stored in cytoplasmic granules of mast cells. Histamine acts locally to activate endothelial cells, fibroblasts, and smooth muscle cells following its release from mast cells (reviewed in Ref. 366). Histamine mediates its effect by binding to one of three receptor subtypes, H1, H2, and H3 (366). Both H1 and H2 receptors are present in large-vessel and microvessel endothelial cells (139, 358, 366); however, the histamine effect on vascular permeability occurs through the activation of H1 receptors (148, 768; see Ref. 981). Evidence showed that histamine increased endothelial permeability as the result of the transient development of IEJ gaps (560). This finding has been reproduced in animal models and cultured cells (147, 148, 349, 461, 574, 620, 622, 623, 768, 1068). Histamine increased the  $K_{f,c}$  in perfused rat hindquarter (349) and coronary venules (1067), increased the extravasation of dextran (a measure of endothelial macromolecule permeability) in the hamster cheek pouch preparation (574), increased permeability to  $\alpha$ -lactalbumin in rat mesenteric venules (1068), and caused interstitial edema from leaky bronchial venules (but not pulmonary venules) in dog lungs (710). McDonald et al. (575) have reported that the size of IEJ gaps formed after histamine treatment range from 100 to 400 nm and had a half-life of  $\sim 1.9$  min. Histamine also induced junctional gap formations by increasing intracellular Ca<sup>2+</sup> and MLC phosphorylation in endothelial cells, albeit to a lesser extent than that reported for thrombin (236, 389, 391, 622, 984), which may account for the short-lived histamine-induced permeability responses. Histamine also disrupts IEJs by activating the phosphorylation and recruitment of AJ and TJ components (307, 372, 739, 837, 1057). In addition, histamine induced actin stress fiber formation in a RhoA- and Rac-dependent manner (1057).

## 4. Oxidants

Reactive oxygen species (ROS) generated within the microcirculation during inflammation and infection are a general class of mediators of increased endothelial permeability (235, 409, 453, 460, 478, 515, 550, 1105; see also Refs. 555, 857). ROS generation is a well-recognized feature of acute lung injury and ischemia/reperfusion-induced tissue injury. Many reports have shown that the oxidants, H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, and hydroxyl radical ( $\cdot$ OH) are capable of injuring endothelial cells at levels produced by activated neutrophils adherent to the vascular endothelium (62, 164, 437, 513, 838, 992). Cultured endothelial cell monolayers exposed to H<sub>2</sub>O<sub>2</sub> (20, 858), hyperoxia (705), or treated with oxidant-generating system xanthine/xan-



thine oxidase (836) demonstrated increased transendothelial permeability of albumin. Perfusion of isolated-perfused guinea pig lungs with  $H_2O_2$  (434), rabbit lungs with xanthine-xanthine oxidase (50), and rabbit lungs with activated neutrophils (930) resulted in increased  $K_{f,c}$  and edema formation. Endothelial cells exposed to hypoxia followed by reoxygenation increased  $H_2O_2$  release (550, 797) and endothelial permeability to [ $^{14}C$ ]inulin (409) and [ $^{125}I$ ]albumin (550).

Additionally vascular endothelial cells can themselves generate oxidants contributing to increased endothelial permeability (550, 1112). The endothelial NADPH oxidase complex, an enzyme system similar to the well-characterized bactericidal NADPH oxidase of phagocytes, is a major producer of vascular endothelial  $O_2^-$  signaling (see Ref. 503). The NADPH oxidase complex is comprised of several *phox* proteins: membrane-bound gp91<sup>phox</sup> and p22<sup>phox</sup> and cytosolic p40<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup>. Apart from the above core components, Rac, in its GTP-bound state, is required for the assembly and activation of NADPH oxidase in amphiphile-activated cell-free system (467). Rac activation by Rac-GEFs requires Rac dissociation from GDI (101), indicating that upstream events mediating Rac dissociation from GDI and its activation by GEFs are important in regulating  $O_2^-$  generation from NADPH. However, this possibility and the consequences of endothelium-derived ROS production on barrier function remain to be addressed in endothelial cells.

The mechanism of oxidant-induced endothelial barrier dysfunction involves the interaction of oxidants with the plasma membrane. Oxidants have been reported to reduce plasma membrane fluidity (97) and induce lipid peroxidation, leading to leakiness of membranes to ions and water. Oxidants were also shown to cause DNA strand breakage (820, 891), endothelial contraction by activating RhoA and MLCK (305, 367, 692, 974), an increase in intracellular  $Ca^{2+}$  concentration (692, 974), and activation of phospholipase  $A_2$  (154), phospholipase D (636), PLC (840), PKC (856; see also Ref. 857), and MAP and Src kinases (975). Many of these pathways are known to influence IEJs and cell-ECM attachments (as discussed earlier in review), and thus they could signal in a complex manner the ROS-induced increase in endothelial permeability (reviewed in Ref. 555).

## 5. VEGF

VEGF [originally vascular permeability factor (VPF)] is a mediator of increased vascular permeability as well as an endothelial cell mitogen (reviewed in Ref. 260). Expression of VEGF has been localized to perivascular cells in many organs (3, 167, 563, 612). VEGF expression is critical for normal vascular development (261, 313, 1100). The physiological effects of VEGF are mediated by at least two endothelial phosphotyrosine-containing trans-

membrane receptors: fms-like tyrosine kinase (Flt-1) and kinase insert domain-containing receptor (KDR), the mouse homolog of which is fetal liver kinase (Flk-1) (reviewed in Ref. 260). These receptors contain an extracellular domain consisting of seven immunoglobulin-like motifs, transmembrane domain, juxtamembrane domain, and a tyrosine kinase that is split by a kinase insert region, and cytoplasmic COOH-terminal tail. Because of the demonstrated weak kinase activity of Flt-1, it has been suggested to serve as a decoy rather than as a true tyrosine kinase (374). Endothelial cell studies have implicated the activation of KDR/Flk-1, but not Flt-1, in VEGF-induced increase in endothelial permeability (246). In humans, VEGF mRNA exists as four transcripts, derived by alternative splicing of a single precursor mRNA, which encodes proteins of 121, 165, 189, and 206 amino acids (956). Whereas VEGF<sub>121</sub> and VEGF<sub>165</sub> isoforms are secreted as homodimeric glycoproteins, VEGF<sub>189</sub> and VEGF<sub>206</sub> isoforms are bound to ECM (reviewed in Ref. 232).

VEGF increased permeability in intact vessels (53, 54, 639, 1063) as well as cultured endothelial cell monolayers (61, 370), implicating VEGF as an important mediator regulating endothelial permeability. VEGF upon binding to its receptor activates tyrosine kinase-linked-PLC $\gamma$ , which in turn activates DAG and IP<sub>3</sub> production (see Ref. 56). Generation of these second messengers increases intracellular  $Ca^{2+}$  by increasing  $Ca^{2+}$  release from intracellular stores as well as  $Ca^{2+}$  influx through store-dependent and store-independent channels, including TRP channels (55, 428, 712). Jho et al. (428) demonstrated that TRPC1-induced  $Ca^{2+}$  entry is involved in the VEGF permeability response by activating the coupling of IP<sub>3</sub>R to TRPC1 channels. This interaction occurred independent of PLC activity. The increase in [ $Ca^{2+}$ ]<sub>i</sub> may result in increased paracellular permeability via activation of actin-myosin machinery and increased transcellular permeability via increased vesicle fusion leading to VVOs, fenestrations, and transcellular channels (reviewed in Ref. 56). Studies showed that VEGF can also stimulate endothelial cell signaling through the heterotrimeric G protein G<sub>q/11</sub>- and G<sub>q</sub>-mediated RhoA (1098), indicating the possibility of cross-talk between VEGF and G protein-coupled receptors, but the role of this interaction in VEGF-induced permeability increase is not clear.

## 6. TNF- $\alpha$

TNF- $\alpha$  is another mediator of increased endothelial permeability in vivo (379, 386, 960), isolated lungs (379), and cultured endothelial cells (108, 262, 263, 685). TNF- $\alpha$  is an inflammatory cytokine released from activated monocytes and macrophages sequestered in the microcirculation (379). Increased TNF- $\alpha$  levels have been reported in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome (401, 596, 912). TNF- $\alpha$  is

believed to contribute to disruption of lung vascular barrier dysfunction upon engaging TNF- $\alpha$  receptor-1, leading to increased permeability to protein (reviewed in Ref. 552). In addition, TNF- $\alpha$  results in the upregulation of endothelial adhesion molecules ICAM-1 and E-selectin (541, 735, 736, 961), thereby promoting neutrophil adhesion to endothelium and ROS generation (329, 417, 735), which in turn can increase endothelial permeability (see sect. XIIA4). Evidence indicated that a TNF- $\alpha$ -induced increase in endothelial permeability required PKC- $\alpha$  (262). As PKC- $\alpha$  is known to activate RhoA (381, 586), it is possible that PKC- $\alpha$  by inducing RhoA activity mediates the TNF- $\alpha$ -induced increase in endothelial permeability. Studies using dominant-negative mutants or inhibitors of RhoA or ROCK support this conclusion, since inhibition of RhoA prevented actin stress fiber formation, AJ disassembly, and the permeability-increase following stimulation of endothelial monolayers with TNF- $\alpha$  (1056). There is also evidence that the TNF- $\alpha$ -induced increase in endothelial permeability is dependent on microtubule rearrangement and AJ disassembly via p38 MAPK activation (459, 652, 704). In addition, TNF- $\alpha$  may contribute to increased endothelial permeability by activating the zinc-dependent MMP gelatinase (685), thereby altering integrin-mediated adhesion of endothelial cells to fibronectin matrix as well as disrupting fibronectin matrix itself (769). TNF- $\alpha$  exposure of endothelium for 2 h (100 U/ml) also potentiated thrombin-induced increase in endothelial permeability by increasing tyrosine phosphorylation of IEJ proteins, actin stress fiber formation, and Ca<sup>2+</sup> influx by a Src-dependent mechanism (953). Finally, TNF- $\alpha$  was directly shown to induce expression of TRPC1 (676), indicating during periods of increased TNF- $\alpha$  levels such as sepsis, TRPC1 expression may induce a leaky barrier by enhancing Ca<sup>2+</sup> influx. However, prolonged incubation with TNF- $\alpha$  decreased thrombin receptor expression and downstream signaling events such as release of intracellular Ca<sup>2+</sup> (1082). Thus TNF- $\alpha$  can affect endothelial permeability by multiple mechanisms, increasing permeability under specific instances and also modulating it by transcriptionally regulating the expression of receptors of proinflammatory agonist (e.g., PAR-1) and the Ca<sup>2+</sup> channel TRPC1.

## 7. LPS

LPS, also known as endotoxin, is a structural component of the outer membrane of Gram-negative bacteria and during sepsis triggers a systemic inflammatory response, which includes vasomotor dysfunction, endothelial cell apoptosis, and coagulation activation with fibrin deposition (see Ref. 175 for review). Administration of LPS in various models induced profound vascular leakage in vivo (297, 371, 699) and increased permeability of cultured endothelial cells (69, 220a, 328, 590). Studies

showed that in cultured endothelial cells, an LPS-induced increase in endothelial permeability occurred as the result of endothelial contraction caused by RhoA-dependent increase in MLC phosphorylation (249), reorganization of actin filaments (328), and protein tyrosine phosphorylation (49). In addition, LPS has been shown to induce HSP27 phosphorylation in association with endothelial barrier dysfunction in rats (371). LPS effects on the endothelium can also occur secondary to release of secondary mediators such as TNF- $\alpha$ , IL-1, and IL-8 (384, 418; see also Ref. 175). LPS activates the release of these cytokines by a transcriptional mechanism involving the activation of NF- $\kappa$ B (see Ref. 175). LPS binds with LPS-binding protein (LBP) (824), and this complex interacts with soluble opsonic receptor CD14 (sCD14). sCD14 is found in serum of healthy individuals, but its levels are increased during sepsis (500). LPS-LBP-sCD14 in turn associates with the Toll-like receptor 4 (TLR4), which requires MD-2 for inducing the activation of NF- $\kappa$ B, and the subsequent transcription of cytokines (see Ref. 175). Intriguingly, activation of TLR4 by LPS was shown to require a lipid raft/caveolae-mediated pathway (1023). Walton et al. (1023) showed that LPS induced the membrane translocation of TLR4 to the lipid raft/caveolar fraction in endothelial cells. Caveolin-1 overexpression also increased the LPS-induced IL-8 production (1023). Oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (ox-PAPC) inhibited the activation of TLR4 by preventing caveolae-mediated translocation of TLR4 (1023). Another phospholipid S1P also inhibits vascular leakage and inflammation in a murine model of LPS-mediated acute lung injury (698). Whether S1P protects LPS-induced vascular injury by a caveolae-mediated pathway or by annealing the IEJs remains unclear.

## B. Endothelial Barrier Stabilizing Mediators

A number of mediators (a growing list) have been identified that are capable of decreasing endothelial permeability (295, 300, 462, 689, 780), raising the possibility that endothelial barrier function alterations are the net result of permeability-increasing mediators and those opposing them. The endothelial barrier stabilizing mediators may be released in response to permeability-increasing mediators and could serve to restore endothelial barrier function. Their role has the potential for providing novel therapeutic strategies in inflammatory diseases that would reverse the defects in vessel wall permeability. van Nieuw Amerongen and van Hinsbergh (986) have summarized these strategies, and therefore, they are not discussed in detail here. We focus on the newly identified endothelial barrier protective mediators, S1P and Ang-1, and the signaling mechanisms of their action.

## 1. S1P

Several findings showed that proteins and phospholipids released from platelets enhance the integrity of the microvessel endothelium (320, 771) and the barrier properties of confluent cultured endothelial monolayers (17, 18, 354, 794). Among these phospholipid mediators, S1P has been shown to have a unique barrier-protective property in cultured endothelial cells (226, 300, 510, 780, 794) and intact microvessels (580, 698). Sphingosine kinase, phosphatase, and lyase regulate S1P plasma levels (890). Sphingosine kinase by phosphorylating sphingosine leads to the formation of S1P (890). Degradation of S1P is mediated by either sphingosine phosphatase or sphingosine lyase that catalyzes the phosphate-dependent lysis of S1P to phosphoethanolamine (see Ref. 890). Platelets lack sphingosine-1-lyase and thus are the primary storage sites of S1P (1087). S1P released from platelets binds to serum albumin. Therefore, endothelial cells may be continuously exposed to S1P from circulating platelets *in vivo*. Endothelial cells possess specific G protein-coupled receptors for S1P, the endothelial differentiation gene (EDG) receptors, Edg-1, -3, and -5 (reviewed in Refs. 169, 579). Ligand-receptor interactions activate endothelial programs for angiogenesis, cell motility, and proliferation (see Refs. 376, 673, 890). The endothelial barrier-enhancing effect of S1P is dose dependent and characterized by a rapid and sustained increase in transendothelial monolayer electrical resistance (226, 300, 794). S1P was shown to attenuate pulmonary and renal vascular leakage and inflammation in a murine model of LPS-mediated acute lung injury (698). These S1P effects depend on  $G_i$  receptor coupling to Edg-1 and Edg-3 (300, 510). A key effector of this pathway is activated Rac, which leads to endothelial cell cortical actin assembly and assembly of AJ (300, 585, 1017). There is also evidence that S1P induces AJ and FAC assembly (585, 780, 849, 850), which may contribute to its barrier promoting effect. The finding that  $G_i$  couples to Edg-1 and Edg-3 as well as albumin binding protein gp60 raises the possibility that S1P may also interfere with the transcellular permeability pathway. Interestingly, S1P at high concentrations (5  $\mu$ M) activated RhoA and actin stress fiber formation (850) possibly via  $G\alpha_q$  and  $G\alpha_{12}/G\alpha_{13}$  (853), thus endothelial barrier-promoting effect of S1P may be concentration dependent and occur in narrow range. However, as normal serum concentrations of S1P range from 250 to 500 nM (673), S1P may normally function to dampen endothelial permeability. S1P increased  $[Ca^{2+}]_i$  by mobilizing  $Ca^{2+}$  from intracellular stores and by activating  $Ca^{2+}$  entry through nonselective cation channels via a pertussis toxin-sensitive  $G_i$  protein (512, 585). However, inhibition of  $Ca^{2+}$  entry by S1P failed to prevent S1P-induced Rac translocation to junctions and AJ assembly, indicating S1P promotes endothelial junctional integrity by activating the release of ER- $Ca^{2+}$ ,

which induces Rac activation and promotes AJ annealing (585). Important issues remain to be addressed concerning the mechanism of S1P-induced strengthening of the endothelial barrier: 1) which GEF, GAP, or GDI regulates the Rac activation, 2) which actin filament polymerizing proteins are required for the Rac-induced strengthening of barrier, and 3) whether S1P effects on barrier function require the engagement of TJs and AJs. Interestingly, S1P reversed the thrombin-induced barrier dysfunction by accelerating recovery of endothelial retraction response (300, 794). Thus S1P may shift barrier function in the direction of barrier restoration through signaling pathways that activate Rac and favor resealing of IEJs. This raises the possibility that the activation of S1P receptors leads to functional antagonism of the PAR-1-mediated increase in endothelial permeability. It is also possible that activation of PAR-1 could stimulate synthesis and release of S1P that in turn is responsible for reannealing of IEJs and restoring endothelial permeability (113). Such a mechanism potentially could help to explain barrier recovery occurring in the continuous presence of inflammatory mediators.

## 2. Ang-1

Another endothelial barrier-stabilizing mediator, angiopoietin-1 (Ang-1), is an important regulator of embryonic and postnatal neovascularization like VEGF (293). Ang-1 activates the endothelial cell-specific tyrosine kinase receptor Tie-2 and cooperates with VEGF during later stages of embryonic angiogenesis in signaling the formation of the mature endothelial barrier (910). Ang-1 was shown to oppose the VEGF-induced increase in endothelial permeability as demonstrated in several *in vivo* and cell culture models (429, 942, 943). Ang-1 pretreatment of endothelial monolayers also blocked the permeability increase induced by thrombin in lungs and dermal microvascular endothelial cells (D. Jho, G. Ahmed, X.-P. Gao, D. Mehta, D. Minshall, and A. B. Malik, unpublished observations). Ang-1 also prevented the permeability-increasing effects of PAF, bradykinin, and histamine (711). The mechanisms by which Ang-1 inhibits increases in endothelial permeability are not yet fully understood. Ang-1 inhibition of the VEGF-induced permeability occurred through its ability to prevent VEGF-induced  $Ca^{2+}$  influx (at level of TRPC1) and inhibition of actin-myosin dependent cell contraction (428). Because both of these signaling events are known to be RhoA dependent, it is conceivable that Ang-1 ultimately opposes the VEGF-induced effects by inhibiting the RhoA pathway (321).

## XIII. CONCLUSIONS AND FUTURE DIRECTIONS

It is apparent that the study of endothelial cells has reached a high baroque period. The field of angiogenesis



for example has burgeoned, and it is now one of the most intensely studied areas of basic and clinical research; however, as our review has highlighted, the regulation of endothelial permeability is still incompletely understood, as many questions remain that have important physiological and pathophysiological consequences and critical relevance to other areas of research such as angiogenesis. We have emphasized in this review the role of signaling mechanisms in the homeostatic regulation of endothelial permeability by the paracellular and transcellular pathways. However, another perhaps overlapping set of signals is engaged in the response to humoral stimuli that are responsible for increasing endothelial barrier permeability; thus endothelial permeability needs to be understood as a constitutive process and as a property that can be upregulated and downregulated in response to specific stimuli. The increased permeability responses are in a sense physiological as they enable the extravasation in continuous endothelial of plasma proteins albumin, immunoglobulins, and others which can serve host-defense functions; but increased permeability if unchecked can also lead to a pathology characterized by excessive exudation of plasma proteins, protein-rich tissue edema, and inflammation. A distinction needs to be made between these two faces of endothelial barrier regulation. Figure 31 summarizes the signaling mechanisms mediating increased paracellular endothelial permeability about which considerably more is understood than the mechanisms regulating transcellular permeability via transcytosis. As emphasized in this review, a great deal of recent work has been carried out using mouse models in which genes such as EC MLCK or TRPC4 are deleted. These studies have for the first time provided clues concerning the *in vivo* role of the signaling pathways mediating increased endothelial permeability. The studies have established the *in vivo* relevance of the endothelial contractile apparatus, in particular the involvement of MLC phosphorylation, activated by increases in concentration of intracellular  $\text{Ca}^{2+}$  (induced by engagement of specific  $\text{Ca}^{2+}$  channels) in regulating microvessel endothelial permeability. Although there is an appreciation for the role of endothelial contractility, studies have also unmasked permeability regulation by MLC-independent mechanisms such as FAK and caldesmon. Both intact microvessel and cell studies have also shown the importance of the Rho GTPases in controlling endothelial barrier function in terms of their spatiotemporal role in increasing endothelial permeability as well as in the time-dependent restoration of endothelial barrier function. Rho GTPase and thus "on-and-off switches" are of fundamental importance, but their upstream regulators relevant to permeability, the GEFs, GDIs, and GAPs, are still not known. To date, 40 GEFs (reviewed in Ref. 243), 11 GAPs (reviewed in Ref. 70), and 3 GDIs (see Ref. 664) have been identified, but it is not known which are important and when and where in the endothelial cell

they are activated, and precisely how they control endothelial permeability. It is known that signals generated by certain G protein-coupled receptors such as PAR-1 are important in increasing endothelial permeability, but there are also many unknowns here as well: how these receptors activate TRPC channels, and how  $\text{Ca}^{2+}$  signaling induces the activation of MLCK and RhoA to increase the gaps in IEJs and uncouple focal adhesions from the ECM. A particularly promising area of research involves identifying the signals mediating endothelial "barrier stabilization." It is known that mediators such as SIP prevent the increase in endothelial permeability, but the intricacies of these signaling networks are not well understood. This area is important, since a more extensive understanding of signals preventing or reversing inappropriate increases in endothelial permeability may lead to identification of novel anti-inflammatory therapeutic targets that may be exploited to reduce inflammation.

The concept that the transport of albumin in a continuous endothelial cells occurs via transcytosis has emerged in the last several years. We have analyzed the role of caveolae-based transcellular mechanism in regulating endothelial permeability, and compared this mode of transport with the paracellular pathway. Approaches inhibiting caveolae function such as deletion of caveolin-1 protein have demonstrated that a highly active transcytosis pathway regulates endothelial barrier function with respect to albumin and other plasma proteins. Studies in *Cav-1*<sup>-/-</sup> mice show caveolae to be the essential carriers mediating albumin via the transcellular route. However, the importance of transcytosis as a mechanism of protein exudation and tissue inflammation remains unclear, that is, whether transcytosis can be activated in response to inflammatory mediators. Fundamental questions remain unanswered such as how the "transcytotic carriers" avoid lysosomes, how they are guided through the cytosol, and how they dock and fuse to the target membrane. Recent imaging studies (697) showed that there are at least two pools of caveolae, one of which is highly dynamic and apparently transport-competent, but it is not clear whether this is the pool attached to the plasma membrane and responsible for the transcytosis of albumin. An important concept that is evolving is that signals emanating from caveolae can affect IEJs and hence junctional permeability, that is, cross-talk may exist between the two pathways responsible for integrating endothelial permeability to liquid and protein. Studies in *Cav-1*<sup>-/-</sup> mice showed that IEJs in capillaries and venules are open and hyperpermeable (721, 822) and are thereby capable of transporting albumin (721, 822). This finding lends credence to the idea that caveolae may regulate junctional permeability. Recent evidence also shows that caveolin-1 and dynamin both have potentially important functions in trafficking IEJ components to junctions (547, 842). Caveolin-1 interacts with TRPC1 and eNOS such that these

interactions may regulate intracellular  $\text{Ca}^{2+}$  concentration and NO production (420, 822, 1107) and control junctional permeability. Dynamin also interacts with the actin-binding proteins cortactin, WASP, and profilin (667), and may have a role in regulating actin stress fiber formation, focal adhesion turnover, and thereby junctional permeability. Intersectin (long form) is a GEF for Cdc42 (395, 882) and may be involved in annealing of IEJs. Intersectin also binds WASP and dynamin (395, 576, 724, 885). Thus it is possible that the proteins caveolin-1, dynamin and intersectin via protein-protein interactions also regulate endothelial cell shape crucial for endothelial permeability. However, to address this and other questions outlined in this review, it will be necessary to go beyond the cell-based studies and use a combination of in vivo imaging and physiological approaches in relevant animal models. It is expected that these types of integrative studies will advance the understanding of the mechanisms regulating microvessel endothelial permeability in the normal state and during inflammation.

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