

# Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®   
*Learn and Live*<sup>SM</sup>

## Trafficking of G Protein–Coupled Receptors

Matthew T. Drake, Sudha K. Shenoy and Robert J. Lefkowitz

*Circ. Res.* 2006;99;570-582

DOI: 10.1161/01.RES.0000242563.47507.ce

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2006 American Heart Association. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/cgi/content/full/99/6/570>

Subscriptions: Information about subscribing to Circulation Research is online at  
<http://circres.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore, MD 21202-2436. Phone 410-5280-4050. Fax: 410-528-8550. Email:  
[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at  
<http://www.lww.com/static/html/reprints.html>

This Review is part of a thematic series on **Microdomains in Cardiovascular Signaling**, which includes the following articles:

Caveolae and Caveolins in the Cardiovascular System

Focal Adhesion: Paradigm for a Signaling Nexus

Vesicular Trafficking of Tyrosine Kinase Receptors and Associated Proteins in the Regulation of Signaling and Vascular Function

Compartmentation of Cyclic Nucleotide Signaling in the Heart: The Role of A-Kinase Anchoring Proteins

Trafficking of G Protein–Coupled Receptors

Compartmentation of Cyclic Nucleotide Signaling in the Heart: The Role of Cyclic Nucleotide Phosphodiesterases

*Kathy K. Griendling and David A. Kass, Editors*

## Trafficking of G Protein–Coupled Receptors

Matthew T. Drake, Sudha K. Shenoy, Robert J. Lefkowitz

**Abstract**—G protein–coupled receptors (GPCRs) play an integral role in the signal transduction of an enormous array of biological phenomena, thereby serving to modulate at a molecular level almost all components of human biology. This role is nowhere more evident than in cardiovascular biology, where GPCRs regulate such core measures of cardiovascular function as heart rate, contractility, and vascular tone. GPCR/ligand interaction initiates signal transduction cascades, and requires the presence of the receptor at the plasma membrane. Plasma membrane localization is in turn a function of the delivery of a receptor to and removal from the cell surface, a concept defined most broadly as receptor trafficking. This review illuminates our current view of GPCR trafficking, particularly within the cardiovascular system, as well as highlights the recent and provocative finding that components of the GPCR trafficking machinery can facilitate GPCR signaling independent of G protein activation. (*Circ Res.* 2006;99:570-582.)

**Key Words:** GPCR ■ trafficking ■ GPCR kinase (GRK) ■  $\beta$ -arrestin ■ 7-transmembrane receptors

G protein–coupled receptors (GPCRs) are central mediators of nearly all aspects of cardiovascular biology. GPCRs were originally identified as receptors capable of coupling to specific guanine nucleotide-binding proteins (G proteins), thereby transducing an extracellular signal to an intracellular effector, although more recently, several GPCRs have been demonstrated to signal via G protein–independent mechanisms both in vitro and in vivo.<sup>1</sup> As a family of proteins, GPCRs share common structural features, including seven membrane-spanning domains, and thus are alternatively referred to as 7-transmembrane receptors. GPCRs are the largest superfamily of cell-surface receptors, accounting for approximately 2% of the human genome.<sup>2</sup> Further, ligands directed at GPCRs (primarily agonists and antagonists) represent the largest family of pharmacological agents, ac-

counting for nearly 30% of current clinical pharmaceutical agents available.<sup>3</sup> Both hormones and neurotransmitters exert their effects on the cardiovascular system via GPCRs. Examples of GPCRs with well-ascribed roles in cardiovascular biology include the  $\beta_1$ - and  $\beta_2$ -adrenergic receptors (ARs), the  $\alpha_1$ - and  $\alpha_2$ -ARs, the  $M_2$ - and  $M_3$ -muscarinic acetylcholine receptors, the angiotensin II (Ang II) receptors, the endothelin receptors, the adenosine receptor, the thrombin receptor, and the vasopressin receptor.

Over the past nearly 3 decades, a wealth of information has revealed much about the signaling properties of this family of seven membrane-spanning receptors. Much work has focused on revealing the ways in which the GPCRs regulate discrete effector molecules including adenylyl cyclase, phospholipases, and ion channels. Still further work has shed light on

Original received June 30, 2006; revision received August 10, 2006; accepted August 11, 2006.

From the Howard Hughes Medical Institute and Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC.

Correspondence to Robert Lefkowitz, Box 3821, Duke University Medical Center, Durham, NC 27710. E-mail lefko001@receptor-biol.duke.edu

© 2006 American Heart Association, Inc.

*Circulation Research* is available at <http://circres.ahajournals.org>

DOI: 10.1161/01.RES.0000242563.47507.ce

mechanisms by which GPCR signaling is regulated and has led to the discovery of additional proteins including the GPCR kinases (GRKs)<sup>4,5</sup> and  $\beta$ -arrestin proteins,<sup>6,7</sup> which respectively phosphorylate agonist-activated GPCRs and bind phosphorylated GPCRs to physically disrupt the receptor/G protein interaction, thereby leading to desensitization of receptor-mediated G protein activation. In addition to its role in GPCR desensitization,  $\beta$ -arrestin binding also promotes the cytosol to cell surface translocation of components of the endocytic machinery, namely adaptor protein-2 (AP-2)<sup>8</sup> and clathrin,<sup>9</sup> thereby facilitating receptor removal from the plasma membrane.

Although still substantial, comparatively less work has focused on GPCR trafficking, much of it related to mechanisms regulating endocytosis of GPCRs from the cellular surface, including the role of  $\beta$ -arrestin in facilitating GPCR endocytosis as above. Indeed, the appropriate delivery of GPCRs to the cell surface to permit receptor/ligand interactions, and their subsequent retrieval from the plasma membrane, are of fundamental importance for the regulation of GPCR activity. This review highlights our current understanding of GPCR movement from synthesis onward, with special emphasis on studies of GPCRs from the cardiovascular system. Lastly, we discuss the importance of the newly recognized role that GPCR trafficking itself can have on cellular signaling, including the recently recognized and expanding role for  $\beta$ -arrestins in GPCR signaling independent of G proteins.

### GPCR Trafficking: Posttranslation

Both during and subsequent to synthesis, membrane proteins including GPCRs undergo a continual process of maturation before reaching residence at the plasma membrane. They must be properly inserted into the membrane (a process believed to occur cotranslationally for most membrane proteins), achieve proper folding while still resident in the endoplasmic reticulum, traverse from the *cis*- to the *trans*-Golgi while undergoing modification, and finally be targeted to the plasma membrane where they attain residence as mature proteins. This section will discuss various aspects of this maturational process that have been determined for GPCRs (Figure, A).

### Folding and Chaperones

Strict quality-control mechanisms within cells ensure that improperly or incompletely folded proteins are targeted for degradation, usually via the proteasome pathway. For most nascent proteins studied, folding into a proper/functional conformation requires the presence of endogenous accessory chaperone proteins.<sup>10</sup> GPCRs are no exception to this quality-control process. As example, the human DnaJ protein HSP11b, a member of the heat shock protein (HSP) family of cytoplasmic cochaperones, regulates trafficking of rhodopsin from the endoplasmic reticulum (ER) to the cell surface.<sup>11</sup> Alternatively, single-membrane-spanning chaperone proteins can facilitate GPCR exit from the ER, as recently demonstrated for the calcitonin receptor-like receptor, which must form a heterodimeric complex with the receptor activity modifying protein (RAMP) before ER egress (reviewed by Tan et al<sup>12</sup>). Whether these or similar endogenous chaperone

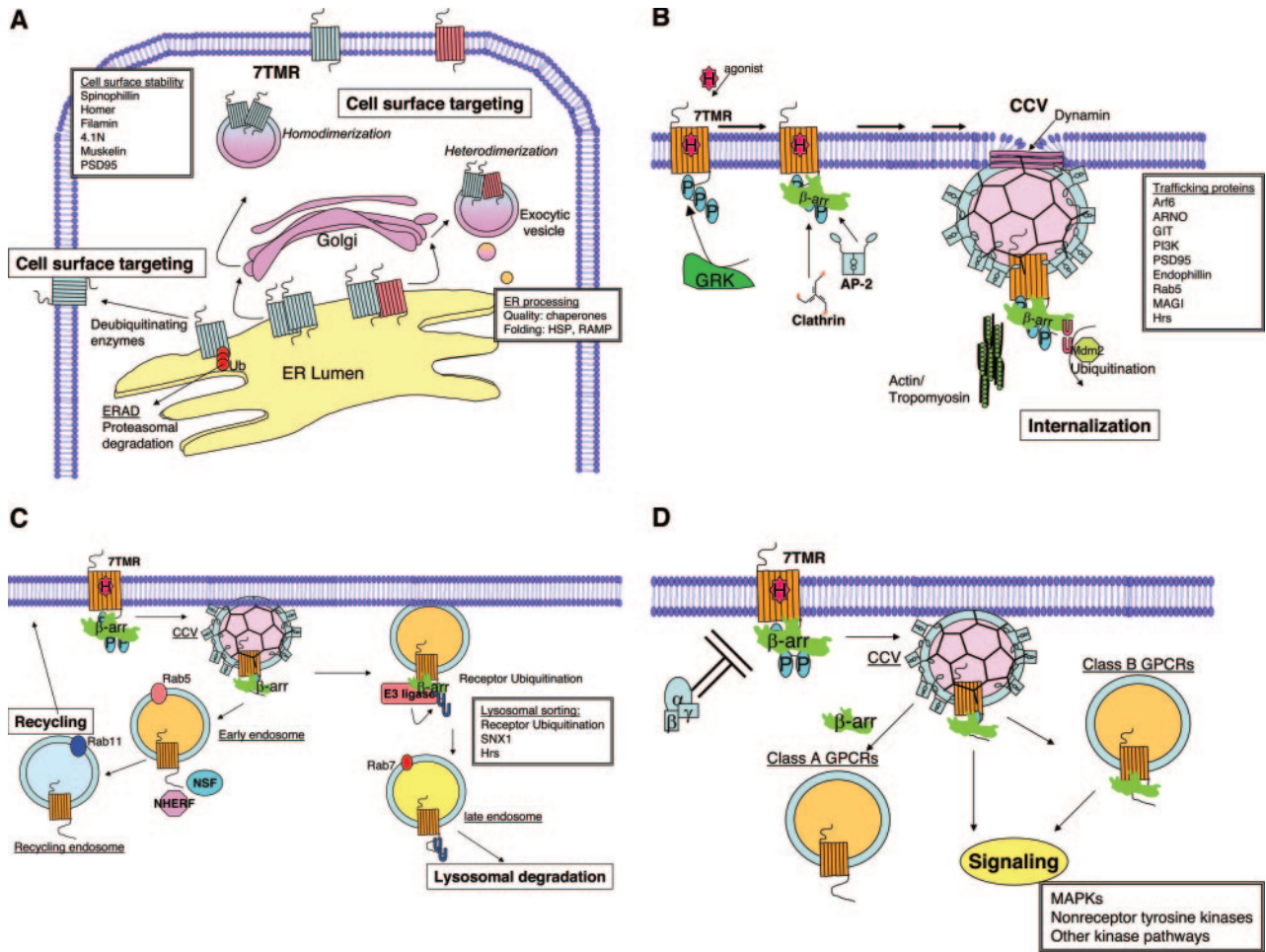
proteins regulate the folding of GPCRs important to some aspects of cardiovascular biology is currently unknown, but certainly possible given the structural conservation across this large protein family.

Despite such checks to ensure proper protein folding errors do, however, occur. As such, a variety of human diseases have been identified in which naturally occurring mutations result in the misfolding and/or mistargeting of a mutant protein. Such “protein conformational” diseases are thus considered to result from mutations that do not affect the functional domain(s) of the mutant protein, but rather interfere with the normal cellular trafficking of the protein (reviewed by Bernier et al<sup>13</sup>). Such misfolded proteins typically either form aggregates that are deleterious to the cell or are recognized as improperly folded and therefore targeted for degradation by the cellular quality-control mechanisms noted above (reviewed by Sitia and Braakman<sup>14</sup>). Interestingly, recent work has demonstrated that in some instances, chemical or pharmacological manipulation can rescue misfolded proteins and lead to their proper translocation to the plasma membrane where the proteins are functionally active.

Nephrogenic diabetes insipidus (NDI) is an X-linked disorder<sup>15</sup> with an incidence in the population of approximately 1 per 250 000. NDI is characterized by renal resistance to the posterior pituitary-derived antidiuretic hormone (also called arginine vasopressin), an octapeptide which normally acts at the vasopressin 2 receptor ( $V_2R$ ) present on renal epithelial cells to allow for normal urinary concentration.<sup>15</sup> In patients with NDI and the complete absence of renal epithelial  $V_2R$  cell surface expression, daily urinary volume can exceed 15 L and lead to rapid death. More than 150 different mutations in the  $V_2R$  have been described, the majority of which ( $\approx 70\%$ ) impair  $V_2R$  cell surface trafficking (reviewed by Bernier et al<sup>13</sup>). The addition of a cell-permeable  $V_2R$  antagonist to a subset of mutant  $V_2Rs$  previously shown to accumulate in the ER resulted in the proper folding, ER exit, correct targeting to the cell surface, and functional rescue of receptor activity of the mutant proteins.<sup>16</sup> This is presumably attributable to the ability of the small molecule ligand to stabilize the native state of the protein, thereby facilitating the proper trafficking of the receptor. Additional mutant 7-transmembrane receptors known to have altered trafficking properties that result in human disease, and for which molecular chaperones have been identified, include rhodopsin, the sulfonyleurea receptor 1 (SUR1), smoothened, and the gonadotropin-releasing hormone receptor (reviewed by Bernier et al<sup>13</sup>). It remains to be seen, however, whether pharmacological treatment of the mistrafficked receptor targets identified above in cellular systems will translate to amelioration of human disease.

### Oligomerization

Early studies using rhodopsin,<sup>17,18</sup> muscarinic,<sup>19</sup> and  $\beta$ -adrenergic<sup>20</sup> receptors as model GPCRs suggested that GPCRs exist primarily as monomers, although modification of the detergent extraction systems used for protein purification led early investigators to suggest that a varying fraction of GPCRs may also be present in oligomeric form.<sup>19,20</sup> Much recent work using coimmunoprecipitation and resonance energy-transfer techniques have convincingly demonstrated



General model of GPCR trafficking. A, Following synthesis, GPCRs initially reside in the ER, where they undergo processing and folding guided by chaperone and quality-control proteins. Within the ER, many GPCRs likely form either homo- or heterodimeric structures. Following ER exit, GPCRs transit through the Golgi apparatus, where they may undergo additional modifications such as oligosaccharide processing. On the distal edge of the Golgi, GPCRs are packaged in exocytic transport vesicles and enter the endosomal system, where they are subsequently targeted to the plasma membrane. Multiple proteins, as listed, have been identified that affect GPCR stability at the cell surface. ERAD indicates ER-associated degradation. B, Although variations have been described, GPCR endocytosis from the plasma membrane most commonly occurs in a GRK- and  $\beta$ -arrestin-dependent manner. Ligand binding promotes GRK-mediated phosphorylation of the cytoplasmic surface of GPCR and subsequent  $\beta$ -arrestin translocation and binding to the receptor.  $\beta$ -Arrestin binding, in turn, facilitates the subsequent recruitment of AP-2 and clathrin and GPCR inclusion in CCVs before endocytosis via CCVs. C, Following endocytosis, GPCRs may be either recycled to the plasma membrane or sorted for lysosomal degradation. The Rab family of small GTPases is integral in determining the fate of a GPCR, whereas SNX1 has more recently been shown to play a role in endosomal to lysosomal GPCR sorting. Receptor ubiquitination also plays a role in receptor degradation via lysosomes. D, More recently, several GPCRs have been shown to be capable of signaling via  $\beta$ -arrestin-dependent pathways. Well-characterized  $\beta$ -arrestin-dependent signaling cascades that have been described include agonist-dependent nonreceptor tyrosine kinase activation as well as activation of the MAPK signaling pathway.

that dimeric GPCR structures are present at the plasma membrane and are the topic of several recent reviews.<sup>21–24</sup> Indeed, it has been postulated that homo- and heterodimeric GPCRs may represent the basic functional unit necessary for most, if not all, GPCR signaling (reviewed by Park et al<sup>25</sup>). Whether higher-order oligomeric complexes can form at the plasma membrane has not, however, been clearly demonstrated, although the M<sub>2</sub>-muscarinic receptor has been suggested to be capable of forming a trimer.<sup>26</sup>

Not well appreciated nor understood, however, is the likely important role that oligomerization of GPCRs plays in the biosynthesis and trafficking of nascent GPCRs to the cellular surface. Indeed, multiple GPCRs including the  $\beta_2$ -AR<sup>27</sup> and vasopressin receptors<sup>28</sup> undergo constitutive homodimeriza-

tion early in the biosynthetic pathway, likely occurring in the ER. Expression of mutant  $\beta_2$ -ARs constructed to either lack an ER-export motif or to contain a heterologous ER-retention signal led to entrapment of wild-type  $\beta_2$ -AR in the ER, likely because of receptor dimerization.<sup>27</sup> Importantly, addition of a peptide corresponding to the putative glycoporphin-like dimerization motif in the sixth-transmembrane domain of the  $\beta_2$ -AR inhibited both receptor dimerization and transit to the cell surface.<sup>29</sup> Similar results in which mutants of the V<sub>2</sub>R<sup>30</sup> or the D<sub>2</sub> dopamine receptor<sup>31</sup> act as dominant negatives for plasma membrane expression of their respective wild-type receptors suggest that receptor oligomerization before cell surface delivery may be a general mechanism by which multiple members of the GPCR family are regulated.

In addition to homodimerization, early heterodimerization is also likely to play an important role in the proper targeting of some GPCRs, as recently demonstrated for the  $\alpha_{1D}$ -AR, which required heterodimerization with the closely related  $\alpha_{1B}$ -AR for cell surface expression.<sup>32</sup> Immunoprecipitation studies in which epitope-tagged  $\beta_2$ -AR and Ang II type 1 (AT<sub>1</sub>) receptors (AT<sub>1</sub>Rs) were coexpressed suggested that the receptors are able to form oligomers before their localization on the plasma membrane, as the amount of immunoprecipitated receptor complex was unaffected by exposure to either agonist or antagonist.<sup>33</sup> Further studies have demonstrated the unanticipated finding that heterodimerization between the  $\beta_2$ -AR and either an olfactory receptor<sup>34</sup> or the  $\alpha_{1D}$ -AR<sup>35</sup> facilitates receptor ER export and cell surface expression. Finally, expression of the  $\beta_2$ -AR along with the  $\delta$  and  $\kappa$  opioid receptors in cultured cells leads to heterooligomerization of the  $\beta_2$ -AR with either the  $\delta$  or  $\kappa$  opioid receptor at the plasma membrane.<sup>36</sup> Interestingly, this association did not affect ligand binding or functional properties of the receptors but did alter the trafficking properties. In the  $\delta$ - $\beta_2$  cells,  $\delta$  receptors underwent  $\beta_2$ -AR agonist-stimulated internalization and  $\beta_2$ -AR underwent opioid-mediated endocytosis, whereas in  $\kappa$ - $\beta_2$ -AR cells, the  $\beta_2$ -AR did not internalize in response to either  $\beta_2$ -AR agonist or opioid.<sup>36</sup> Although this is a single example, such results suggest that GPCR heterooligomerization may be an important way of modulating GPCR trafficking and signaling. It is important to note, however, that in each oligomerization study described above, overexpression of the receptor(s) of interest was performed and that such alterations in cellular receptor content may modify the endogenous molecular interactions that occur in the absence of receptor overexpression.

Thus, in addition to the important role that endogenous molecular chaperones such as the HSP and RAMP family proteins play in protein folding and ER export of GPCRs, homo- and heteroreceptor oligomerization also likely play a critical step in the pathway used by at least some GPCRs for cellular trafficking, although it is at present unclear whether oligomerization following protein synthesis is a general pathway used by all GPCRs.

### Cell Surface Stability

In order for a GPCR to transduce an extracellular signal, it must both traffic correctly to and be retained at the cellular surface to allow for receptor/ligand interaction. Multiple proteins not directly involved in the signal transduction cascade have been identified which stabilize receptor surface expression. These include spinophilin, Homer, actin-binding protein 280/filamin A, protein 4.1N, muskellin, and postsynaptic density-95 (PSD-95) (reviewed by Tan et al<sup>12</sup>). Of these, PSD-95, a multiple PDZ domain-containing scaffolding protein, has been most conclusively shown to specifically interact with a GPCR fundamental to cardiovascular biology, namely the  $\beta_1$ -AR. This interaction occurs via the third PDZ domain of PSD-95, which interacts with the carboxyl terminus of the  $\beta_1$ -AR. Interestingly, overexpression of PSD-95 decreased  $\beta_1$ -AR internalization but did not affect agonist-stimulated cAMP production or receptor desensitization,

suggesting a role for PSD-95 in maintaining the  $\beta_1$ -AR at the cellular surface.<sup>37</sup>

### GPCR Trafficking: Endocytosis

Much work over the past several decades has illuminated our current understanding of the molecular mechanisms underlying GPCR removal from the cell surface. Fundamental to this process are 2 families of proteins, the GPCR kinases (GRKs) and the  $\beta$ -arrestins, both of which were initially identified in studies of GPCR desensitization and which are involved in removal of ligand-activated GPCRs from the plasma membrane. Additional work has identified an ensemble of accessory proteins, which interact with the GRK and  $\beta$ -arrestin classes of proteins, and much recent effort has been devoted to delineating the details of these multiple interactions that are inherent to the process of GPCR endocytosis. Furthermore, as will be described below, the agonist-induced post-translational ubiquitination of both receptor and  $\beta$ -arrestin play definitive and discrete roles in regulating the life cycle of GPCRs (Figure, B).

### Role of the Lipid Microenvironment in GPCR Trafficking

Understanding of the importance of the membrane lipid microenvironment for GPCR signaling and trafficking is rapidly evolving. As example, it has recently been demonstrated that following translation, the AT<sub>1</sub>R requires caveolin as an intracellular molecular chaperone for trafficking to the plasma membrane.<sup>38</sup> Moreover, once at the cell surface, it is clear that some subsets of GPCRs are preferentially segregated to discrete regions of the membrane defined as lipid rafts.<sup>39–41</sup> GPCRs of fundamental importance to cardiovascular biology that have been localized to lipid rafts and/or caveolae include the adenosine A<sub>1</sub>,  $\alpha_1$ -AR,  $\beta_1$ -AR,  $\beta_2$ -AR, AT<sub>1</sub>R, the endothelin (ETA-A and ET-B) receptors, and the M<sub>2</sub>-muscarinic receptors.<sup>42</sup>

Caveolae, a specific type of lipid microdomain, represent for some GPCRs the preferred microenvironment for certain events such as signaling. However, a receptor's maintenance within a specific microenvironment may be subject to dynamic regulation. Indeed, reversible GPCR modifications have been described, including both covalent attachment of a lipid to the GPCR or GPCR phosphorylation, which can shift the GPCR between different membrane milieus. For example, a reversible lipid modification (eg, palmitoylation/depalmitoylation of cysteine residues) has been demonstrated to target GPCRs such as the 5-HT<sub>1a</sub> receptor to lipid rafts.<sup>43</sup> Interestingly, agonist-induced endocytosis of the  $\beta_1$ -AR via clathrin-coated pits (CCPs) in human embryonic kidney (HEK) 293 cells requires GRK phosphorylation of the receptor, whereas endocytosis of the  $\beta_1$ -AR in lipid rafts/caveolae is dependent on the receptor undergoing protein kinase A phosphorylation.<sup>44</sup> Further evidence supporting the importance of GPCR membrane microdomain restriction is that confinement of the  $\beta_2$ -AR to caveolae has been reported to be of critical importance for regulation of the intrinsic contraction rate in neonatal cardiac myocyte membrane preparations.<sup>45</sup> The importance of the lipid microenvironment for the assemblage of signaling scaffolds beneath the GPCR/mem-

brane interface is also an area of active investigation and may play a role in multiple aspects of GPCR trafficking but is beyond the scope of this review. For a more complete review of the role of the lipid microenvironment on both GPCR signaling and trafficking, refer to several recent excellent reviews.<sup>39,42</sup>

### Agonist-Dependent Versus Agonist-Independent GPCR Internalization

Receptor internalization following agonist exposure is a well-documented response for a wide variety of GPCRs important for cardiovascular biology. As example, the prototypic GPCR, the  $\beta_2$ -AR, was initially shown to internalize following exposure to agonist, as demonstrated by loss of surface binding of a nonpermeable membrane ligand.<sup>46</sup> Use of a membrane permeant ligand, however, demonstrated that the  $\beta_2$ -AR was still ligand accessible, suggesting that the receptor was sequestered in an intracellular compartment following agonist treatment.<sup>46</sup> Alternatively, whereas exposure of the  $AT_1R$  to Ang II leads to receptor internalization and endosomal sequestration, the Ang II type 2 receptor ( $AT_2R$ ) does not undergo endocytosis with Ang II addition, demonstrating that subtype-specific receptor sorting and internalization can occur within the cardiovascular GPCR system.<sup>47</sup>

Internalization for most GPCRs occurs on the order of minutes and correlates with receptor phosphorylation by the GRKs and subsequent  $\beta$ -arrestin translocation, as will be discussed below. Indeed, agonist-induced  $\beta_2$ -AR receptor internalization can be inhibited either by mutations of the  $\beta_2$ -AR, which inhibit agonist-induced GRK phosphorylation<sup>48</sup> or by mutations in the  $\beta$ -arrestin proteins.<sup>49</sup> Following internalization, receptors may be either recycled to the cell surface or targeted for lysosomal degradation (reviewed by Bohm et al<sup>50</sup>).

Internalization of GPCRs in the absence of agonist has also been examined. Although mean rates of internalization vary between receptors assayed, rates are in general substantially slowed in the absence relative to the presence of the cognate ligand of a GPCR. The  $\beta_2$ -AR, as example, undergoes sequestration from the cell surface with a half-life of approximately 10 minutes in the presence of agonist but remains on the cell surface for greater than 1 hour in the absence of agonist.<sup>51</sup>

### The Role of Accessory Proteins in the Endocytosis of GPCRs

Endocytosis of GPCRs can occur via caveolae, clathrin-coated vesicles, or uncoated vesicles.<sup>52</sup> Although short linear amino acid stretches in the cytoplasmic domains of GPCRs likely play a role in their endocytosis, the majority of work to date has demonstrated that much of GPCR endocytosis is primarily regulated by GRK and  $\beta$ -arrestin-dependent processes involving clathrin-coated pits.

### GPCR Kinases

As shown for multiple GPCRs, the serine/threonine-specific GPCR kinases (GRKs) are recruited following agonist bind-

ing to the cytoplasmic surface of the activated receptor, leading to receptor phosphorylation. The phosphorylated surface of the GPCR is then competent to serve as a platform for the cytosol to membrane translocation of the  $\beta$ -arrestin proteins (reviewed by Shenoy and Lefkowitz<sup>53</sup>).

The GRK family of kinases is composed of 7 members that share significant amino acid and structural homology (reviewed previously<sup>54,55</sup>). Within this family, 4 kinases (GRKs 2, 3, 5, and 6) are expressed broadly and are believed to play a role in GPCR phosphorylation within the cardiovascular system. GRK2 and GRK3 reside in the cytosol in the absence of agonist and translocate to the membrane following GPCR stimulation. GRK2/3 translocation and membrane localization are mediated in part by their binding to heterotrimeric G protein  $\beta\gamma$  subunits.<sup>56</sup> GRK5 and GRK6, on the other hand, are constitutively localized to the plasma membrane. Whereas GRK6 palmitoylation is essential for membrane association,<sup>57</sup> localization of GRK5 to the plasma membrane is believed to be attributable to an electrostatic interaction between the highly basic carboxyl terminus of GRK5 and phospholipids at the plasma membrane.<sup>58,59</sup>

Although GRK-specific phosphorylation of the cytoplasmic surface of agonist-occupied GPCRs mediates  $\beta$ -arrestin recruitment, the structural features common to activated receptors that are recognized by the GRKs remain largely unknown. Indeed, it is a question of fundamental importance as to how members of this limited group of broadly expressed GRKs are able to phosphorylate such a diverse array of activated GPCRs and thereby lead to  $\beta$ -arrestin recruitment.

Functionally, 2 classes of GPCRs, denoted "class A" and "class B," can be defined, based on the relative stability of the GPCR/ $\beta$ -arrestin interaction. For the  $\beta_2$ -AR (a class A receptor) and the vasopressin receptor (a class B receptor), these determinants appear to be present within the carboxyl termini of the receptors, as the stability of their interaction with  $\beta$ -arrestin, as well as their ability to be dephosphorylated, recycled, and resensitized was completely reversed in mutant receptors in which their carboxyl-terminal tails were switched.<sup>60</sup>

Interestingly, whereas in vitro studies have localized GRK2- and GRK5-mediated phosphorylation sites of the  $\beta_2$ -AR to distal portion of the cytoplasmic tail of the receptor,<sup>61</sup> more recent studies in intact cells have suggested that agonist induced  $\beta_2$ -AR phosphorylation occurs in the proximal portion of the carboxyl terminus of the receptor.<sup>62</sup> Although the observed  $\beta_2$ -AR proximal tail phosphorylation was believed to be mediated by GRK rather than protein kinase A, this was not confirmed. Thus although GRK-mediated phosphorylation of agonist-stimulated GPCRs underlies  $\beta$ -arrestin recruitment and thereby initiates GPCR endocytosis in CCPs, many of the molecular details remain to be determined. Notably, a recent study using high-throughput RNA interference implicated GRKs as playing a more general role in the process of clathrin-mediated endocytosis itself.<sup>63</sup>

### $\beta$ -Arrestin As an Endocytic Adaptor Protein

Within humans, there exist 2 isoforms of the nonvisual  $\beta$ -arrestin proteins, namely  $\beta$ -arrestin1 and  $\beta$ -arrestin2, both

of which show ubiquitous tissue distribution. In addition to their well-described role in limiting receptor-G protein interaction, the  $\beta$ -arrestin proteins also serve to both recruit and physically bridge the receptor to the endocytic machinery. Experimentally, receptor mutations that impair agonist-induced GPCR phosphorylation limit  $\beta$ -arrestin recruitment and lead to poor receptor internalization, as demonstrated for a  $\beta_2$ -AR in which all of the GRK phosphorylation sites had been altered.<sup>48</sup> Further, expression of “dominant-negative” mutant  $\beta$ -arrestin proteins (such as  $\beta$ -arrestin1 V53D or  $\beta$ -arrestin2 V54D) inhibit  $\beta_2$ -AR internalization.<sup>64</sup> In addition, the  $\beta$ -arrestin proteins themselves are able to interact directly with the essential components of the clathrin-coated vesicle (CCV) coat machinery, namely the heterotetrameric AP-2 complex,<sup>8</sup> as well as clathrin,<sup>9</sup> and these interactions are critical both for recruitment of the  $\beta_2$ -AR into clathrin-coated pits as well as for receptor internalization. Studies with other GPCRs, including the  $\alpha_2$ -AR<sup>65</sup> and the  $A_{2B}$  adenosine receptor<sup>66</sup> have also shown important roles for the  $\beta$ -arrestins in receptor endocytosis.

Interestingly, although the 2  $\beta$ -arrestin isoforms exhibit nearly 80% amino acid identity,<sup>6</sup> they do not appear to perform redundant biologic roles and, indeed, exhibit differences in their regulation. Whereas  $\beta$ -arrestin1 is phosphorylated by extracellular signal regulated kinase (ERK) enzymes,<sup>67</sup>  $\beta$ -arrestin2 is phosphorylated by casein kinase II.<sup>68</sup> For both  $\beta$ -arrestin proteins, however, the phosphorylation/dephosphorylation status appears to regulate the ability of the  $\beta$ -arrestin protein to promote internalization of the  $\beta_2$ -AR via clathrin-coated vesicles.

As noted above, analysis of agonist-stimulated  $\beta$ -arrestin translocation for a variety of GPCRs suggests there exist 2 largely distinct classes receptors with which the  $\beta$ -arrestins associate, denoted class A and class B GPCRs. Following agonist exposure, class A receptors including the  $\beta_2$ -AR, endothelin A receptor, and  $\alpha_{1b}$ -AR preferentially recruit  $\beta$ -arrestin2.<sup>69</sup> In contrast, class B receptors, including the  $AT_{1a}R$  and  $V_2$  receptor, are able to bind both  $\beta$ -arrestin isoforms with nearly equal affinity.<sup>70</sup> Whereas the  $\beta$ -arrestin1/2–class A receptor interactions occur solely at the plasma membrane and are lost following GPCR internalization,  $\beta$ -arrestin1/2 interactions with class B receptors are much more stable and can be detected on endosomal structures following receptor endocytosis (reviewed by Pierce and Lefkowitz<sup>71</sup>). Further, class A receptors generally recycle to the plasma membrane rapidly, whereas class B GPCRs recycle more slowly. The role of ubiquitination in modulating GPCR/arrestin interaction is likely important and is discussed below. Mouse embryonic fibroblasts generated to lack both  $\beta$ -arrestin isoforms showed a marked impairment of agonist-stimulated internalization of either the class A  $\beta_2$ -AR or the class B  $AT_{1a}R$ , whereas only  $\beta_2$ -AR internalization was affected by the single deletion of the  $\beta$ -arrestin2 isoform.<sup>69</sup> Studies using RNA interference technology to selectively ablate the  $\beta$ -arrestin proteins have shown similar results with respect to internalization of the  $\beta_2$ -AR and  $AT_1$ -AR as model class A and B receptors, respectively.<sup>72</sup>

### Agonist-Induced GPCR Ubiquitination and Sorting

Posttranslational modification of substrate proteins by the covalent attachment of ubiquitin (ubiquitination), originally discovered in the context of cellular protein degradation, has recently been shown to play a noncanonical role in regulating the postendocytic sorting of several membrane proteins including GPCRs.<sup>73,74</sup> Protein ubiquitination is mediated by the concerted action of 3 enzymes. The first 2 enzymes (E1 and E2) are responsible, respectively, for activating ubiquitin and escorting the activated ubiquitin. The third enzyme, E3 ubiquitin ligase (E3), recognizes and modifies the substrate in a timely fashion.<sup>75</sup> For the  $\beta_2$ -AR, both GRK-mediated phosphorylation and  $\beta$ -arrestin binding are essential for receptor ubiquitination to occur.<sup>76</sup> Importantly, this agonist-stimulated  $\beta_2$ -AR ubiquitination modification is necessary for the receptor to undergo degradation in lysosomes. Further, ubiquitin-dependent lysosomal degradation is applicable to other GPCRs such as  $V_2R$  and the protease-activated receptor2 (PAR2).<sup>77,78</sup> For both the  $\beta_2$ -AR and  $V_2R$ , receptor ubiquitination requires the  $\beta$ -arrestin proteins. Although the nature of this requirement is not entirely clear, 1 supposition is that the  $\beta$ -arrestins may serve as adaptors to bring as yet unidentified E3 ligase(s) to the receptors in a stimulus dependent fashion. In the case of the PAR2 and the CXCR4 receptors, ubiquitination is mediated by the E3 ligases c-Cbl<sup>78</sup> and AIP4,<sup>79</sup> respectively, but no involvement of the  $\beta$ -arrestins has as yet been demonstrated.

For the above mammalian GPCRs, as well as for others such as the chemokine receptor CXCR4,<sup>80</sup> receptor ubiquitination is not required for internalization per se but is crucial for the sorting of ubiquitinated receptors to lysosomes. A recent study of the  $\beta_1$ -AR in a heterologous cellular system reported the resistance of the receptor protein to ubiquitination as well as agonist-mediated degradation, suggesting a strong relationship between this receptor modification and downregulation pathways.<sup>81</sup>

Whereas the degradation of GPCRs and other membrane proteins is known to occur in lysosomes, that of some membrane receptors such as the single-membrane spanning erythropoietin receptor involve both lysosomes and 26S proteasomes, the megaprotease complexes that degrade most cellular proteins.<sup>82</sup> Interestingly, however, a recent report suggests that ubiquitination and proteasomal degradation of newly synthesized intracellular  $A_{2A}$  adenosine receptors serves as a method of ER quality control (Figure, A). Importantly, this degradation could be overcome by the coexpression of USP4, a deubiquitinating enzyme belonging to a family of enzymes that catalyze removal of ubiquitin from the modified substrates.<sup>83</sup> USP4 expression led to more robust functional expression of the  $A_{2A}$  receptor at the plasma membrane, suggesting that deubiquitination can facilitate cell-surface targeting of membrane proteins (Figure, A).

On the other hand, GPCR internalization can be regulated by the agonist-dependent ubiquitination of  $\beta$ -arrestin by the E3 ligase Mdm2, as demonstrated for the  $\beta_2$ -AR.<sup>76</sup> Moreover, the stability of  $\beta$ -arrestin/GPCR binding that defines GPCRs as class A or B (as described above) also correlates with the ubiquitination status of the  $\beta$ -arrestin proteins. The separation

of  $\beta$ -arrestin from class A GPCRs results from rapid  $\beta$ -arrestin deubiquitination, whereas the more stable  $\beta$ -arrestin interaction with class B receptors is caused by the sustained ubiquitination of  $\beta$ -arrestin.<sup>84</sup> As will be described below, ubiquitination of  $\beta$ -arrestin appears to not only be capable of regulating GPCR trafficking properties but also likely plays an important role in directing downstream signaling events.

### Sorting Signals Used for GPCR Intracellular Trafficking and Endocytosis

The identification of short, linear amino acid signals present in the intracellular domains of transmembrane proteins responsible for mediating the intracellular sorting and endocytosis of a transmembrane protein from the plasma membrane has been the focus of much work over the past 2 decades. Such sequences are believed to act as recognition sites for components of the cellular adaptor protein machinery necessary for intracellular protein trafficking. The importance of such signals contained within the intracellular domains of GPCRs, however, is less well described than that for other transmembrane proteins. Albeit limited in number, exceptions to the general paradigm of GRK/ $\beta$ -arrestin-mediated endocytosis have been delineated for 7-membrane-spanning receptors important to the cardiovascular system. Interestingly, the best-described motifs are analogous to those used by non-GPCR transmembrane receptors and include di-leucine-based (LL or LXL) and tyrosine-based (NPXY or NPXXY, or YXXO) motifs.

As a prototypic GPCR, the  $\beta_2$ -AR contains a di-leucine motif within its carboxyl terminus, alteration of which does not affect the ability of the receptor to traffic correctly to the cell surface, bind agonist, or to activate adenylyl cyclase. Agonist addition, however, does not lead to internalization of this mutant  $\beta_2$ -AR,<sup>85</sup> demonstrating a role for di-leucine motif of the  $\beta_2$ -AR in agonist-induced receptor endocytosis. Similarly, mutations introduced into the di-leucine motif in the cytoplasmic tail of the vasopressin 1a receptor ( $V_{1a}R$ ) significantly impaired agonist-induced receptor internalization.<sup>86</sup> Interestingly, however, mutation of the analogous di-leucine motif in the  $V_2R$  resulted in a receptor that was unable to escape from the ER, suggesting a role for this motif in  $V_2R$  maturation.<sup>87</sup>

Tyrosine-based sorting signals have also been shown to be necessary in the trafficking of the  $\beta_2$ -AR. Mutation of Y326 in the human  $\beta_2$ -AR, located at the proposed junction of the seventh-transmembrane domain and the proximal portion of the carboxyl terminus and conserved in position across many members of the large superfamily of GPCRs, does not affect the ability of the receptor to traffic correctly to the cell surface, bind agonist, or to activate adenylyl cyclase when the receptor is overexpressed.<sup>88</sup> The mutation, however, did completely abolish  $\beta_2$ -AR agonist-induced internalization. Complicating this interpretation, however, was the finding that lower expression levels of mutant  $\beta_2$ -AR resulted in the loss of ligand binding and adenylyl cyclase coupling, likely because of intracellular retention of the mutant receptor.<sup>89</sup> More recently, a highly conserved tyrosine-based motif (YXXO) in the cytoplasmic tail of the protease-activated

receptor-1 (PAR1), a GPCR for thrombin, which has previously been shown to undergo  $\beta$ -arrestin-independent internalization, was shown to be necessary for agonist-mediated but not constitutive internalization.<sup>90</sup> Finally, more recent studies have shown a direct interaction between a stretch of 8 arginines contained in the carboxyl terminus of the  $\alpha_{1b}$ -AR and the AP-2  $\mu$  subunit.<sup>91</sup> Whether the alternative tyrosine-based motif found in PAR1 or the nonclassical arginine motif identified in the  $\alpha_{1b}$ -AR plays a role in the endocytosis and intracellular trafficking of other GPCRs classically identified as fundamentally important to the cardiovascular system remains to be determined.

### Role for Additional Proteins in the Endocytosis of GPCRs

In addition to the well-recognized roles of the GRK and  $\beta$ -arrestin proteins in GPCR internalization, multiple other proteins have been demonstrated to be important in the endocytic process. A partial list and description of the role these proteins play in GPCR endocytosis is discussed below.

#### ADP-Ribosylation Factor 6

ADP-ribosylation factor 6 (ARF6) is 1 member of the ARF family of small GTP-binding proteins known to be key players in vesicular trafficking events. In addition to its role in binding AP-2 and clathrin,  $\beta$ -arrestin is also able to directly bind to ARF6 and modulate its activity. ARF6 activation requires the exchange of GTP for GDP, a reaction that is catalyzed by the ARF guanine nucleotide exchange factor (GEF) ARNO (ARF Nucleotide-binding site Openner). Importantly, ARNO is constitutively associated with  $\beta$ -arrestin2.<sup>92</sup> As shown for the  $\beta_2$ -AR, expression of mutant ARF6 proteins containing single amino acid substitutions rendering them deficient in their ability to either bind (T27N) or hydrolyze (Q67L) GTP inhibited agonist-induced  $\beta_2$ -AR internalization.<sup>92</sup> Overexpression of ARNO alone, however, increases  $\beta_2$ -AR internalization by stimulating GTP nucleotide exchange on ARF. Thus, agonist-promoted recruitment of  $\beta$ -arrestin to an activated receptor leads to the local regulation of endocytosis by  $\beta$ -arrestin attributable to its inherent ability to bind both ARNO and ARF6.

In addition to requiring a GEF protein, ARF proteins also require a GTPase-activating protein (GAP) to accelerate hydrolysis of bound GTP. Initially identified as GRK-interacting proteins (GITs), GIT1 and GIT2 are zinc finger-containing proteins that function as GAPs for ARF6.<sup>93,94</sup> GIT1 overexpression reduces the internalization of transmembrane receptors in CCPs and CCVs, including the  $\beta_1$ - and  $\beta_2$ -ARs, the adenosine 2B receptor, and the  $M_1$ -muscarinic receptor.<sup>95</sup> Importantly, ARF-GAP activity of the GIT proteins is stimulated by phosphatidylinositol 3,4,5-trisphosphate, whereas other ARF-GAPs, such as ARF-GAP1, are stimulated by phosphatidylinositol 4,5-bisphosphate and diacylglycerol.<sup>94</sup> This raises the interesting possibility that GIT regulation of ARF6 activity may be integrated through activation of the phosphatidylinositol 3-kinase signaling pathway.



### Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinases (PI3Ks) are a conserved family of kinases with both lipid and protein kinase activity which can be activated in response to GPCR stimulation. As a family, they have been shown to play important roles in an array of cellular functions as divergent as cell survival, cell motility, and receptor endocytosis. Within the cytosol, PI3K is constitutively complexed with GRK2.<sup>96</sup> As demonstrated for the  $\beta_2$ -AR, agonist binding induces translocation of the GRK/PI3K complex to the activated receptor, formation of phosphatidylinositol 3,4,5-trisphosphate, and subsequent recruitment of AP-2 and clathrin, leading to receptor endocytosis. Importantly, receptor internalization is blocked by overexpression of the portion of PI3K that mediates its interaction with GRK2, as well as by the specific 3,4,5 trisphosphate lipid phosphatase PTEN, demonstrating the importance of the lipid kinase activity of PI3K for the localized production of D-3 phosphoinositides in regulating ligand-induced endocytosis of the  $\beta_2$ -AR.<sup>97</sup>

As noted above, in addition to possessing lipid kinase activity, PI3K family members are also able to function as protein kinases, although the importance of this activity has remained largely obscure given the limited number of protein substrates recognized by PI3Ks.<sup>98</sup> Recently, however, the importance of PI3K protein kinase activity in the regulation of  $\beta_2$ -AR endocytosis has been illuminated. In an elegant study, Naga Prasad et al identified the cytoskeletal protein nonmuscle tropomyosin (an actin filament binding protein) as a substrate for the  $\gamma$  isoform of PI3K and further demonstrated that PI3K can selectively phosphorylate a single site (S61) within tropomyosin.<sup>99</sup> Alteration of this site within tropomyosin to mimic constitutive phosphorylation (S61D) leads to complementation of a protein kinase defective PI3K, whereas change to a phospho-deficient residue (S61A) blocked agonist-induced  $\beta_2$ -AR internalization. Thus, through both its lipid and kinase activity, PI3K plays a central role in the agonist-induced removal of the  $\beta_2$ -AR, the prototypic model cardiovascular GPCR, from the cell surface. Whether this paradigm will extend to other members of the cardiovascular GPCR family is at present unknown but seems likely, given that endocytosis via AP-2 containing CCVs is the predominant mechanism of internalization for most ligand-activated GPCRs.

### Intracellular Trafficking: Sorting/Recycling/Degradation

Once internalized from the cell surface, GPCRs can be sorted along multiple pathways (Figure, C). They may undergo dephosphorylation, resensitization, and be recycled back to the plasma membrane. Alternatively, GPCRs may be targeted for degradation via the endosomal/lysosomal pathway. Finally, multiple GPCRs have more recently been shown to initiate G protein–independent intracellular signaling pathways following endocytosis, as is discussed below. These multiple trafficking fates for internalized GPCRs are the subject of an excellent recent review.<sup>100</sup> We would like to highlight a few proteins important for these processes.

### Na<sup>+</sup>/H<sup>+</sup>-Exchanger Regulatory Factor

Following adrenergic receptor stimulation, it has long been recognized that G protein–dependent changes in cellular metabolism, excitability, and growth occur.<sup>101</sup> Likewise, cellular changes apparently independent of G protein activation have been demonstrated, including alterations in cellular pH via regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger. Recent work has established that agonist stimulation of the  $\beta_2$ -AR promotes direct association of the extreme carboxyl terminus of the receptor with the first PDZ domain within Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor-1 (NHERF1).<sup>102</sup> Similar associations have been shown to occur for other GPCRs containing sequences conforming to the consensus motif D-S/T-x-L, including the purinergic P2Y1 receptor and the CFTR,<sup>103</sup> whereas other GPCRs such as the parathyroid hormone receptor<sup>104</sup> have been shown to interact with both PDZ domains of both NHERF1 and NHERF2 via a slightly different (ETVM) PDZ consensus motif. For the  $\beta_2$ -AR, disruption of this interaction markedly impairs agonist-induced changes in intracellular pH.

NHERF is also of critical importance for proper intracellular sorting of the  $\beta_2$ -AR. In addition to its ability to bind the extreme carboxyl terminus of the  $\beta_2$ -AR via its PDZ domain, NHERF is able via its ezrin–radixin–moesin (ERM) domain to bind to the actin cytoskeleton through association with ERM proteins. Importantly, mutations generated to disrupt the interaction of NHERF with either the  $\beta_2$ -AR carboxyl terminus or with ERM proteins lead to significant agonist-induced lysosomal degradation of the  $\beta_2$ -AR following endocytosis, rather than recycling.<sup>105</sup> As noted above, multiple additional GPCRs have been shown to interact with the NHERF proteins. Although NHERF has been shown to play a role in the recycling of other GPCRs such as the  $\kappa$  opioid receptor (reviewed by Liu-Chen<sup>106</sup>), the generalizability of the role that NHERF plays in the recycling of other GPCRs, particularly in the cardiovascular system, remains to be determined.

### N-Ethylmaleimide-Sensitive Fusion Protein

In a study to identify  $\beta$ -arrestin binding partners,  $\beta$ -arrestin1 was found to interact in both yeast 2-hybrid and in vitro assays with N-ethylmaleimide-sensitive fusion protein (NSF), an ATPase essential for many intracellular transport functions.<sup>107</sup> Furthermore, overexpression of NSF in HEK293 cells led to enhanced agonist-induced  $\beta_2$ -AR internalization and could rescue the effects of a  $\beta$ -arrestin1 mutant (S412D) previously shown to function as a dominant negative for  $\beta_2$ -AR internalization. Interestingly, the  $\beta_2$ -AR is also able, via its extreme carboxyl terminus, to bind directly to NSF.<sup>108</sup> The  $\beta_2$ -AR/NSF interaction is agonist dependent and requisite for efficient agonist-mediated  $\beta_2$ -AR internalization. Importantly, whereas wild-type  $\beta_2$ -ARs recycle to the cell surface following exposure to the antagonist propranolol,  $\beta_2$ -ARs containing mutations in their distal carboxyl termini remain sequestered intracellularly, demonstrating the importance of the  $\beta_2$ -AR/NSF interaction for proper  $\beta_2$ -AR recycling. Thus, although it is clear that proteins that bind to the extreme carboxyl termini of GPCRs, such as NHERF and NSF, serve to regulate the intracellular sorting of the recep-

tors such as the  $\beta_2$ -AR, the extent to which the trafficking of other GPCRs is modulated by these or similar as yet unidentified proteins remains to be elucidated.

### Sorting Nexin 1

Following internalization, GPCRs may be either recycled to the plasma membrane or undergo sorting to the endosomal/lysosomal pathway for degradation. Although fundamentally important as a mechanism for downregulation of certain GPCRs, such as the PAR1, which recognizes the coagulant protease thrombin, comparatively less is known about the molecular components that direct postendosomal sorting and recycling of the receptors.

Although sorting nexin 1 (SNX1) was originally identified as a membrane-associated protein that interacts with the receptor tyrosine kinase, epidermal growth factor receptor (EGFR) and functions in EGFR sorting to lysosomes,<sup>109</sup> SNX1 has also been demonstrated to interact with PAR1 in an agonist-dependent manner.<sup>110</sup> Overexpression of a SNX1 carboxyl terminal-deletion mutant did not significantly impair agonist-induced PAR1 internalization and accumulation in early endosomes but was able to impede endosome to lysosome sorting of PAR1 and thus markedly inhibit PAR1 degradation.<sup>110</sup> Additional studies, in which depletion of endogenous SNX1 in HeLa cells via small interfering RNA technology markedly impaired agonist-induced PAR1 degradation, buttress the suggestion that SNX1 plays a role in the intracellular trafficking of PAR1 from endosomes to lysosomes.<sup>111</sup> Whether SNX1 or other SNX proteins can mediate the endosomal to lysosomal sorting of GPCRs other than PAR1 remains to be determined but is certainly possible given the general conservation of trafficking themes shared across members of this large protein family.

### Rab GTPases

A second class of proteins shown to regulate the movement of GPCRs both during and subsequent to endocytosis are the Rab proteins, a family of Ras-like small GTP binding proteins. Importantly, different Rab GTPase family members selectively associate with specific intracellular structures including both recycling and sorting endosomes, where they mediate multiple steps of vesicular membrane trafficking including vesicle budding, docking, and fusion (reviewed by Seachrist and Ferguson<sup>112</sup>).

Within the cardiovascular system, Rab5 plays a central role in the agonist-dependent CCV-mediated internalization and early endosomal localization of some GPCRs, including the  $\beta_2$ -AR.<sup>113</sup> Expression of a dominant negative Rab5 (S34N) blocked  $\beta_2$ -AR internalization, whereas a constitutively active Rab5 (Q79L) did not significantly alter  $\beta_2$ -AR internalization. Interestingly, whereas the  $\beta_2$ -AR is not able to directly associate with Rab5, the AT<sub>1</sub>R does bind Rab5 directly via the carboxyl terminus of the receptor and localizes to Rab5-containing endosomal structures following endocytosis.<sup>114</sup> Furthermore, the interaction of Rab5 with the AT<sub>1</sub>R on endosomes appears to be necessary for preventing sorting of the internalized receptor to lysosomes.<sup>115</sup> Unlike internalization of the  $\beta_2$ -AR, however, endocytosis of the AT<sub>1</sub>R does not appear to be dependent on Rab5,<sup>114</sup> demon-

strating the intracellular diversity in GPCR trafficking roles played by even single members of the Rab protein family.

Whereas Rab5 plays a role in GPCR trafficking events associated with GPCR internalization and/or early endosomal formation, multiple additional Rab family members have been implicated in other GPCR-trafficking events, including Rab4 and Rab11 in receptor recycling, and Rab7 in endosomal to lysosomal sorting.<sup>115</sup> In a transgenic mouse model in which a dominant-negative Rab4 (S27N) was overexpressed in the heart, mice had impaired responsiveness to both endogenous and exogenous catecholamines.<sup>116</sup> Furthermore, when these mice were crossbred with mice also overexpressing a cardiac-specific human  $\beta_2$ -AR, the resultant mice had an abnormal intracellular vesicular  $\beta_2$ -AR accumulation, as well as significantly reduced cardiac contractility. Such results suggest that proper Rab4-mediated regulation of  $\beta_2$ -AR receptor recycling is necessary for maintenance of normal catecholamine responsiveness and receptor resensitization. Whether Rab4 also plays a similar role *in vivo* in the recycling of other GPCRs within the cardiovascular system is currently unknown. For a more complete review of the diverse roles that members of the Rab protein family play throughout nearly all aspects of GPCR trafficking, refer to several recent excellent reviews.<sup>100,112</sup>

### Other Proteins With Roles in GPCR Endocytosis and Postendocytotic Trafficking

Many additional proteins have been identified and characterized that play fundamental roles in the endocytosis and postendocytotic trafficking of GPCRs. These include, but are not limited to, proteins involved in CCV formation and membrane scission such as AP-2, clathrin, dynamin, Hrs, and GASP,<sup>117–119</sup> as well as multiple scaffolding proteins that have been demonstrated to interact with the  $\beta_1$ -AR including postsynaptic density 95 (PSD95),<sup>120</sup> membrane-associated guanylate kinase inverted-2 (MAGI-2),<sup>121</sup> and the endophilins.<sup>122</sup> Because of the limited scope of this review, we have chosen to highlight only selected proteins as above.

### Internalization/Trafficking and Signaling

Although internalization mechanisms leading to receptor sequestration away from the source of stimulus have been primarily considered as signal desensitization pathways, a large body of research now exists in support of continued signaling that is coupled with GPCR endocytosis.<sup>123</sup> Moreover, although it is generally believed that endocytic “addresses” determine the extent of signaling, continued signaling mechanisms during the trafficking of proteins may actually determine the exact endocytic route by regulating processes such as vesicle fusion and cargo transfer. As such, it is increasingly evident that the 2 processes, trafficking and signaling, are not only connected but likely inextricably intertwined (Figure, D).

In the case of GPCRs,  $\beta$ -arrestin has been clearly demonstrated to play an essential role in the desensitization of G protein-mediated second messenger signaling. More recently, however, the  $\beta$ -arrestin proteins have also been shown to facilitate both receptor internalization via CCVs, as well as to couple the receptors to and activate nonreceptor tyrosine

kinases (eg, members of the c-Src family), thereby mediating a second wave of signaling.<sup>124,125</sup> In addition, on binding to activated GPCRs such as the AT<sub>1a</sub>R,  $\beta$ -arrestin has also been shown to be capable of sequestering and scaffolding signaling kinases, as well as targeting active signaling complexes into cytoplasmic endosomal microcompartments.<sup>125,126</sup> Importantly, chemical or molecular inhibition of receptor endocytosis (or, alternatively, expression of  $\beta$ -arrestin mutants that impair receptor internalization), can lead to a decline in such downstream signaling, demonstrating a coupling between endocytosis and signaling.<sup>124,127</sup>

In addition to c-Src activation,  $\beta$ -arrestin has been shown in multiple studies to function as a signaling intermediate in GPCR signal transduction to mitogen-activated protein kinase (MAPK) pathways, under conditions where G protein coupling is virtually absent.<sup>125,128,129</sup> Although this  $\beta$ -arrestin-dependent signaling has been most well characterized for ERK activation, it may also apply to other signaling kinases and appears to play roles in a variety of cellular responses including chemotaxis and antiapoptosis. Although it has not yet been conclusively determined that receptor endocytosis is required for this  $\beta$ -arrestin pathway, recent data suggest that the trafficking patterns of  $\beta$ -arrestin/receptor complexes may play a significant role.<sup>130,131</sup> Interestingly,  $\beta$ -arrestin ubiquitination at specific acceptor sites (eg, Ang II–induced ubiquitination of lysines 11 and 12 in  $\beta$ -arrestin2) is crucial for stable  $\beta$ -arrestin/AT<sub>1</sub>R interaction as well as the formation and endosomal localization of AT<sub>1</sub>R-MAPK signalosomes. Whether this observation may be broadly applicable to other GPCRs relevant to cardiovascular biology is currently unknown and is an area of active investigation.

### Conclusions

Within the cardiovascular system, regulation of GPCR trafficking is of fundamental importance both for physiological homeostasis and the molecular response to physiological perturbation. There is efficient and coordinated movement of GPCRs from synthesis to the cellular surface, where they can interact with components of the extracellular milieu to transduce signals to intracellular effectors and subsequent GPCR retrieval from the cell surface. These processes highlight the intricate cellular balance that has developed to exquisitely regulate hormonal responsiveness at the tissue level. Indeed, much has been learned about GPCR trafficking within the cardiovascular system over the past 2 decades, as evidenced by the ever-expanding ensemble of identified proteins crucial to this process. The recent recognition that GPCR signaling may occur within the cardiovascular system via G protein-independent/ $\beta$ -arrestin-dependent pathways raises the fascinating possibility that pharmacologically relevant cardiovascular ligands may be developed that allow for selective GPCR signaling via the activation of proteins initially believed only to have a role in limiting GPCR signaling and promoting GPCR removal from the cell surface.

### Acknowledgments

We thank Donna Addison and Elizabeth Hall for excellent secretarial assistance.

### Sources of Funding

This work was supported by NIH grants HL 16037 and HL 70631 (to R.J.L.) and HL080525 and American Heart Association grant 0530014N (to S.K.S.). M.T.D. is supported by NIH training grant T32-DK-00701. R.J.L. is an investigator with the Howard Hughes Medical Institute.

### Disclosures

None.

### References

- Rajagopal K, Lefkowitz RJ, Rockman HA. When 7 transmembrane receptors are not G protein-coupled receptors. *J Clin Invest*. 2005;115:2971–2974.
- Vassiliatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA. The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci U S A*. 2003;100:4903–4908.
- Wise A, Gearing K, Rees S. Target validation of G-protein coupled receptors. *Drug Discov Today*. 2002;7:235–246.
- Benovic JL, Mayor F Jr, Staniszewski C, Lefkowitz RJ, Caron MG. Purification and characterization of the beta-adrenergic receptor kinase. *J Biol Chem*. 1987;262:9026–9032.
- Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem*. 1998;67:653–692.
- Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J Biol Chem*. 1992;267:17882–17890.
- Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science*. 1990;248:1547–1550.
- Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS. The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci U S A*. 1999;96:3712–3717.
- Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature*. 1996;383:447–450.
- Ellegaard L, Helenius A. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol*. 2003;4:181–191.
- Chapple JP, Cheetham ME. The chaperone environment at the cytoplasmic face of the endoplasmic reticulum can modulate rhodopsin processing and inclusion formation. *J Biol Chem*. 2003;278:19087–19094.
- Tan CM, Brady AE, Nickols HH, Wang Q, Limbird LE. Membrane trafficking of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol*. 2004;44:559–609.
- Bernier V, Lagace M, Bichet DG, Bouvier M. Pharmacological chaperones: potential treatment for conformational diseases. *Trends Endocrinol Metab*. 2004;15:222–228.
- Sitia R, Braakman I. Quality control in the endoplasmic reticulum protein factory. *Nature*. 2003;426:891–894.
- Pan Y, Metzzenberg A, Das S, Jing B, Gitschier J. Mutations in the V2 vasopressin receptor gene are associated with X-linked nephrogenic diabetes insipidus. *Nat Genet*. 1992;2:103–106.
- Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, Bouvier M. Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Invest*. 2000;105:887–895.
- Liebman PA, Entine G. Lateral diffusion of visual pigment in photoreceptor disk membranes. *Science*. 1974;185:457–459.
- Downer NW, Cone RA. Transient dichroism in photoreceptor membranes indicates that stable oligomers of rhodopsin do not form during excitation. *Biophys J*. 1985;47:277–284.
- Dadi HK, Morris RJ. Muscarinic cholinergic receptor of rat brain. Factors influencing migration in electrophoresis and gel filtration in sodium dodecyl sulphate. *Eur J Biochem*. 1984;144:617–628.
- Lefkowitz RJ, Haber E, O'Hara D. Identification of the cardiac beta-adrenergic receptor protein: solubilization and purification by affinity chromatography. *Proc Natl Acad Sci U S A*. 1972;69:2828–2832.

21. George SR, O'Dowd BF, Lee SP. G-protein-coupled receptor oligomerization and its potential for drug discovery. *Nat Rev Drug Discov.* 2002;1:808–820.
22. Lee SP, O'Dowd BF, George SR. Homo- and hetero-oligomerization of G protein-coupled receptors. *Life Sci.* 2003;74:173–180.
23. Bulenger S, Marullo S, Bouvier M. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci.* 2005;26:131–137.
24. Angers S, Salahpour A, Bouvier M. Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu Rev Pharmacol Toxicol.* 2002;42:409–435.
25. Park PS, Filipek S, Wells JW, Palczewski K. Oligomerization of G protein-coupled receptors: past, present, and future. *Biochemistry.* 2004;43:15643–15656.
26. Park PS, Wells JW. Oligomeric potential of the M2 muscarinic cholinergic receptor. *J Neurochem.* 2004;90:537–548.
27. Salahpour A, Angers S, Mercier JF, Lagace M, Marullo S, Bouvier M. Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting. *J Biol Chem.* 2004;279:33390–33397.
28. Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, Taulan M, Jockers R, Barberis C, Bouvier M. Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. *Mol Endocrinol.* 2003;17:677–691.
29. Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C, Bouvier M. A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem.* 1996;271:16384–16392.
30. Zhu X, Wess J. Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry.* 1998;37:15773–15784.
31. Lee SP, O'Dowd BF, Ng GY, Varghese G, Akil H, Mansour A, Nguyen T, George SR. Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. *Mol Pharmacol.* 2000;58:120–128.
32. Hague C, Uberti MA, Chen Z, Hall RA, Minneman KP. Cell surface expression of alpha1D-adrenergic receptors is controlled by heterodimerization with alpha1B-adrenergic receptors. *J Biol Chem.* 2004;279:15541–15549.
33. Barki-Harrington L, Luttrell LM, Rockman HA. Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction in vivo. *Circulation.* 2003;108:1611–1618.
34. Hague C, Uberti MA, Chen Z, Bush CF, Jones SV, Ressler KJ, Hall RA, Minneman KP. Olfactory receptor surface expression is driven by association with the beta2-adrenergic receptor. *Proc Natl Acad Sci U S A.* 2004;101:13672–13676.
35. Uberti MA, Hague C, Oller H, Minneman KP, Hall RA. Heterodimerization with beta2-adrenergic receptors promotes surface expression and functional activity of alpha1D-adrenergic receptors. *J Pharmacol Exp Ther.* 2005;313:16–23.
36. Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA. Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc Natl Acad Sci U S A.* 2001;98:343–348.
37. Xiang Y, Devic E, Kobilka B. The PDZ binding motif of the beta 1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J Biol Chem.* 2002;277:33783–33790.
38. Wyse BD, Prior IA, Qian H, Morrow IC, Nixon S, Muncke C, Kurzchalia TV, Thomas WG, Parton RG, Hancock JF. Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. *J Biol Chem.* 2003;278:23738–23746.
39. Barnett-Norris J, Lynch D, Reggio PH. Lipids, lipid rafts and caveolae: their importance for GPCR signaling and their centrality to the endocannabinoid system. *Life Sci.* 2005;77:1625–1639.
40. Insel PA, Head BP, Ostrom RS, Patel HH, Swaney JS, Tang CM, Roth DM. Caveolae and lipid rafts: G protein-coupled receptor signaling microdomains in cardiac myocytes. *Ann NY Acad Sci.* 2005;1047:166–172.
41. Insel PA, Head BP, Patel HH, Roth DM, Bunday RA, Swaney JS. Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem Soc Trans.* 2005;33:1131–1134.
42. Chini B, Parenti M. G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *J Mol Endocrinol.* 2004;32:325–338.
43. Papoucheva E, Dumuis A, Sebben M, Richter DW, Ponimaskin EG. The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *J Biol Chem.* 2004;279:3280–3291.
44. Rapacciuolo A, Suvarna S, Barki-Harrington L, Luttrell LM, Cong M, Lefkowitz RJ, Rockman HA. Protein kinase A and G protein-coupled receptor kinase phosphorylation mediates beta-1 adrenergic receptor endocytosis through different pathways. *J Biol Chem.* 2003;278:35403–35411.
45. Xiang Y, Rybin VO, Steinberg SF, Kobilka B. Caveolar localization dictates physiologic signaling of beta 2-adrenoceptors in neonatal cardiac myocytes. *J Biol Chem.* 2002;277:34280–34286.
46. Chuang DM, Costa E. Evidence for internalization of the recognition site of beta-adrenergic receptors during receptor subsensitivity induced by (-)-isoproterenol. *Proc Natl Acad Sci U S A.* 1979;76:3024–3028.
47. Hein L, Meinel L, Pratt RE, Dzau VJ, Kobilka BK. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol.* 1997;11:1266–1277.
48. Hausdorff WP, Campbell PT, Ostrowski J, Yu SS, Caron MG, Lefkowitz RJ. A small region of the beta-adrenergic receptor is selectively involved in its rapid regulation. *Proc Natl Acad Sci U S A.* 1991;88:2979–2983.
49. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci.* 2002;115:455–465.
50. Bohm SK, Grady EF, Bunnett NW. Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J.* 1997;322(pt 1):1–18.
51. von Zastrow M, Kobilka BK. Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J Biol Chem.* 1994;269:18448–18452.
52. Claing A, Laporte SA, Caron MG, Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol.* 2002;66:61–79.
53. Shenoy SK, Lefkowitz RJ. Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J.* 2003;375:503–515.
54. Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev.* 2001;53:1–24.
55. Penela P, Murga C, Ribas C, Tutor AS, Peregrin S, Mayor F Jr. Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. *Cardiovasc Res.* 2006;69:46–56.
56. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ. Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. *Science.* 1992;257:1264–1267.
57. Stoffel RH, Randall RR, Premont RT, Lefkowitz RJ, Inglese J. Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J Biol Chem.* 1994;269:27791–27794.
58. Kunapuli P, Gurevich VV, Benovic JL. Phospholipid-stimulated autophosphorylation activates the G protein-coupled receptor kinase GRK5. *J Biol Chem.* 1994;269:10209–10212.
59. Premont RT, Koch WJ, Inglese J, Lefkowitz RJ. Identification, purification, and characterization of GRK5, a member of the family of G protein-coupled receptor kinases. *J Biol Chem.* 1994;269:6832–6841.
60. Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem.* 1999;274:32248–32257.
61. Fredericks ZL, Pitcher JA, Lefkowitz RJ. Identification of the G protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic receptor. *J Biol Chem.* 1996;271:13796–13803.
62. Trester-Zedlitz M, Burlingame A, Kobilka B, von Zastrow M. Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. *Biochemistry.* 2005;44:6133–6143.
63. Pelkmans L, Fava E, Grabner H, Hannus M, Habermann B, Krausz E, Zerial M. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature.* 2005;436:78–86.
64. Ferguson SS, Downey WE 3rd, Colapietro AM, Barak LS, Menard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science.* 1996;271:363–366.

65. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ. Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes. *J Biol Chem.* 1999;274:11253–11259.
66. Mundell SJ, Matharu AL, Kelly E, Benovic JL. Arrestin isoforms dictate differential kinetics of A2B adenosine receptor trafficking. *Biochemistry.* 2000;39:12828–12836.
67. Lin FT, Miller WE, Luttrell LM, Lefkowitz RJ. Feedback regulation of beta-arrestin1 function by extracellular signal-regulated kinases. *J Biol Chem.* 1999;274:15971–15974.
68. Lin FT, Chen W, Shenoy S, Cong M, Exum ST, Lefkowitz RJ. Phosphorylation of beta-arrestin2 regulates its function in internalization of beta(2)-adrenergic receptors. *Biochemistry.* 2002;41:10692–10699.
69. Kohout TA, Lin FS, Perry SJ, Conner DA, Lefkowitz RJ. beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci U S A.* 2001;98:1601–1606.
70. Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem.* 2000;275:17201–17210.
71. Pierce KL, Lefkowitz RJ. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci.* 2001;2:727–733.
72. Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ. Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci U S A.* 2003;100:1740–1744.
73. Wojcikiewicz RJ. Regulated ubiquitination of proteins in GPCR-initiated signaling pathways. *Trends Pharmacol Sci.* 2004;25:35–41.
74. Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol.* 2003;19:141–172.
75. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998;67:425–479.
76. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science.* 2001;294:1307–1313.
77. Martin NP, Lefkowitz RJ, Shenoy SK. Regulation of V2 vasopressin receptor degradation by agonist-promoted ubiquitination. *J Biol Chem.* 2003;278:45954–45959.
78. Jacob C, Cottrell GS, Gehringer D, Schmidlin F, Grady EF, Bunnett NW. c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. *J Biol Chem.* 2005;280:16076–16087.
79. Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, Benovic JL. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell.* 2003;5:709–722.
80. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem.* 2001;276:45509–45512.
81. Liang W, Fishman PH. Resistance of the human beta1-adrenergic receptor to agonist-induced ubiquitination: a mechanism for impaired receptor degradation. *J Biol Chem.* 2004;279:46882–46889.
82. Walrafen P, Verdier F, Kadri Z, Chretien S, Lacombe C, Mayeux P. Both proteasomes and lysosomes degrade the activated erythropoietin receptor. *Blood.* 2005;105:600–608.
83. Milojevic T, Reiterer V, Stefan E, Korkhov VM, Dorostkar MM, Ducza E, Ogris E, Boehm S, Freissmuth M, Nanoff C. The ubiquitin-specific protease Usp4 regulates the cell surface level of the A2A receptor. *Mol Pharmacol.* 2006;69:1083–1094.
84. Shenoy SK, Lefkowitz RJ. Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *J Biol Chem.* 2003;278:14498–14506.
85. Gabilondo AM, Hegler J, Krasel C, Boivin-Jahns V, Hein L, Lohse MJ. A dileucine motif in the C terminus of the beta2-adrenergic receptor is involved in receptor internalization. *Proc Natl Acad Sci U S A.* 1997;94:12285–12290.
86. Preisser L, Ancellin N, Michaelis L, Creminon C, Morel A, Corman B. Role of the carboxyl-terminal region, di-leucine motif and cysteine residues in signalling and internalization of vasopressin V1a receptor. *FEBS Lett.* 1999;460:303–308.
87. Schulein R, Hermosilla R, Oksche A, Dehe M, Wiesner B, Krause G, Rosenthal W. A dileucine sequence and an upstream glutamate residue in the intracellular carboxyl terminus of the vasopressin V2 receptor are essential for cell surface transport in COS.M6 cells. *Mol Pharmacol.* 1998;54:525–535.
88. Barak LS, Tiberi M, Freedman NJ, Kwatra MM, Lefkowitz RJ, Caron MG. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *J Biol Chem.* 1994;269:2790–2795.
89. Gabilondo AM, Krasel C, Lohse MJ. Mutations of Tyr326 in the beta 2-adrenoceptor disrupt multiple receptor functions. *Eur J Pharmacol.* 1996;307:243–250.
90. Paing MM, Temple BR, Trejo J. A tyrosine-based sorting signal regulates intracellular trafficking of protease-activated receptor-1: multiple regulatory mechanisms for agonist-induced G protein-coupled receptor internalization. *J Biol Chem.* 2004;279:21938–21947.
91. Diviani D, Lattion AL, Abuin L, Staub O, Cotecchia S. The adaptor complex 2 directly interacts with the alpha 1b-adrenergic receptor and plays a role in receptor endocytosis. *J Biol Chem.* 2003;278:19331–19340.
92. Claing A, Chen W, Miller WE, Vitale N, Moss J, Premont RT, Lefkowitz RJ. beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. *J Biol Chem.* 2001;276:42509–42513.
93. Premont RT, Claing A, Vitale N, Freeman JL, Pitcher JA, Patton WA, Moss J, Vaughan M, Lefkowitz RJ. beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. *Proc Natl Acad Sci U S A.* 1998;95:14082–14087.
94. Vitale N, Patton WA, Moss J, Vaughan M, Lefkowitz RJ, Premont RT. GIT proteins, A novel family of phosphatidylinositol 3,4,5-trisphosphate-stimulated GTPase-activating proteins for ARF6. *J Biol Chem.* 2000;275:13901–13906.
95. Claing A, Perry SJ, Achiriloaie M, Walker JK, Albanesi JP, Lefkowitz RJ, Premont RT. Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. *Proc Natl Acad Sci U S A.* 2000;97:1119–1124.
96. Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. *J Biol Chem.* 2001;276:18953–18959.
97. Naga Prasad SV, Laporte SA, Chamberlain D, Caron MG, Barak L, Rockman HA. Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. *J Cell Biol.* 2002;158:563–575.
98. Backer JM. Substrate specificity: PI(3)Kgamma has it both ways. *Nat Cell Biol.* 2005;7:773–774.
99. Naga Prasad SV, Jayatilake A, Madamanchi A, Rockman HA. Protein kinase activity of phosphoinositide 3-kinase regulates beta-adrenergic receptor endocytosis. *Nat Cell Biol.* 2005;7:785–796.
100. Gaborik Z, Hunyady L. Intracellular trafficking of hormone receptors. *Trends Endocrinol Metab.* 2004;15:286–293.
101. Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ. Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem.* 1991;60:653–688.
102. Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S, Lefkowitz RJ. The beta2-adrenergic receptor interacts with the Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor to control Na<sup>+</sup>/H<sup>+</sup> exchange. *Nature.* 1998;392:626–630.
103. Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ, Lefkowitz RJ. A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci U S A.* 1998;95:8496–8501.
104. Mahon MJ, Donowitz M, Yun CC, Segre GV. Na<sup>(+)</sup>/H<sup>(+)</sup> exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature.* 2002;417:858–861.
105. Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. *Nature.* 1999;401:286–290.
106. Liu-Chen LY. Agonist-induced regulation and trafficking of kappa opioid receptors. *Life Sci.* 2004;75:511–536.
107. McDonald PH, Cote NL, Lin FT, Premont RT, Pitcher JA, Lefkowitz RJ. Identification of NSF as a beta-arrestin1-binding protein. Implications for beta2-adrenergic receptor regulation. *J Biol Chem.* 1999;274:10677–10680.

108. Cong M, Perry SJ, Hu LA, Hanson PI, Claing A, Lefkowitz RJ. Binding of the beta2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling. *J Biol Chem.* 2001;276:45145–45152.
109. Kurten RC, Cadena DL, Gill GN. Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science.* 1996;272:1008–1010.
110. Wang Y, Zhou Y, Szabo K, Haft CR, Trejo J. Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1. *Mol Biol Cell.* 2002;13:1965–1976.
111. Gullapalli A, Wolfe BL, Griffin CT, Magnuson T, Trejo J. An essential role for SNX1 in lysosomal sorting of protease-activated receptor-1: evidence for retromer-, Hrs-, and Tsg101-independent functions of sorting nexins. *Mol Biol Cell.* 2006;17:1228–1238.
112. Seachrist JL, Ferguson SS. Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. *Life Sci.* 2003;74:225–235.
113. Seachrist JL, Anborgh PH, Ferguson SS. beta 2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by rab GTPases. *J Biol Chem.* 2000;275:27221–27228.
114. Seachrist JL, Laporte SA, Dale LB, Babwah AV, Caron MG, Anborgh PH, Ferguson SS. Rab5 association with the angiotensin II type 1A receptor promotes Rab5 GTP binding and vesicular fusion. *J Biol Chem.* 2002;277:679–685.
115. Dale LB, Seachrist JL, Babwah AV, Ferguson SS. Regulation of angiotensin II type 1A receptor intracellular retention, degradation, and recycling by Rab5, Rab7, and Rab11 GTPases. *J Biol Chem.* 2004;279:13110–13118.
116. Odley A, Hahn HS, Lynch RA, Marreez Y, Osinska H, Robbins J, Dorn GW 2nd. Regulation of cardiac contractility by Rab4-modulated beta2-adrenergic receptor recycling. *Proc Natl Acad Sci U S A.* 2004;101:7082–7087.
117. Hislop JN, Marley A, Von Zastrow M. Role of mammalian vacuolar protein-sorting proteins in endocytic trafficking of a nonubiquitinated G protein-coupled receptor to lysosomes. *J Biol Chem.* 2004;279:22522–22531.
118. Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray SR, Von Zastrow M. Modulation of postendocytic sorting of G protein-coupled receptors. *Science.* 2002;297:615–620.
119. Zhang J, Ferguson SS, Barak LS, Menard L, Caron MG. Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem.* 1996;271:18302–18305.
120. Hu LA, Tang Y, Miller WE, Cong M, Lau AG, Lefkowitz RJ, Hall RA. beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with N-methyl-D-aspartate receptors. *J Biol Chem.* 2000;275:38659–38666.
121. Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA. beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. *J Biol Chem.* 2001;276:41310–41317.
122. Tang Y, Hu LA, Miller WE, Ringstad N, Hall RA, Pitcher JA, DeCamilli P, Lefkowitz RJ. Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta1-adrenergic receptor. *Proc Natl Acad Sci U S A.* 1999;96:12559–12564.
123. Gonzalez-Gaitan M, Stenmark H. Endocytosis and signaling: a relationship under development. *Cell.* 2003;115:513–521.
124. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science.* 1999;283:655–661.
125. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. *Science.* 2005;308:512–517.
126. Shenoy SK, Lefkowitz RJ. Angiotensin II-stimulated signaling through G proteins and beta-arrestin. *Sci STKE.* 2005;2005:cm14.
127. DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol.* 2000;148:1267–1281.
128. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem.* 2006;281:1261–1273.
129. Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM, Lefkowitz RJ. Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem.* 2006;281:10856–10864.
130. Wei H, Ahn S, Barnes WG, Lefkowitz RJ. Stable interaction between beta-arrestin 2 and angiotensin type 1A receptor is required for beta-arrestin 2-mediated activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem.* 2004;279:48255–48261.
131. Shenoy SK, Lefkowitz RJ. Receptor-specific ubiquitination of beta-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *J Biol Chem.* 2005;280:15315–15324.