Cell, Vol. 103, 181–184, October 13, 2000, Copyright ©2000 by Cell Press

Translocation and Reversible Localization of Signaling Proteins: A Dynamic Future for Signal Transduction

Minireview

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An increasing amount of experimental evidence has accumulated over the last years suggesting that most signal transduction processes utilize colocalization of sequentially acting signaling proteins for the selective activation of downstream functions (for reviews, see Pawson and Scott, 1997; Tsunoda et al., 1997). In many cases, the activation of signaling proteins by upstream activators or their activation of downstream effectors has been shown to involve binding interactions with adaptor complexes, cytoskeletal structures, and subcellular membranes or with the targets and activators themselves. These localization mechanisms can be highly regulated and rapidly reversible, leading to a dynamic view of signal transduction that diverges from the historic "hardwired signaling concept" where receptors and other signaling proteins stay largely in place and spatial signal transmission is made possible by the rapid diffusion of second messengers. In contrast, the current "softwired signaling concept" is built on the idea that signaling proteins translocate and undergo reversible binding interactions as key steps of the signal transmission process. The questions are then raised, whether translocation is an active or passive process, how fast it can be, and which type of mechanisms can regulate the dynamic organization of large numbers of signaling proteins in space and time.

The current evidence for this dynamic model of signal transduction stems from immunofluorescence, cell fractionation and related biochemical approaches which have allowed in many cases to obtain snapshots of the changes in the subcellular localization of signaling proteins. For example, the recruitment of cytosolic SH2 domain containing proteins by phosphorylated tyrosine residues at the plasma membrane or the nuclear translocation of many cytosolic signaling proteins and transcription factors has been extensively explored by such methods.

Until recently the translocation of signaling proteins could only be followed "live" in cells in selective cases where signaling proteins could be fluorescently labeled in vitro and microinjected into cells for experimentation (Harootunian et al., 1993; Gough and Taylor, 1993). The discovery that GFP and its variants can be used as expressed fluorescence tags for signaling proteins and signaling domains led to a new strategy to track the spatio-temporal dynamics of signaling processes by analyzing stimulus-induced changes in their subcellular localization (translocation and colocalization analysis).

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A Model for Translocation Based on Diffusion and Reversible Binding Interactions

The investigation of fluorescently conjugated proteins by different microscopy techniques has led to new insights into the dynamic mechanisms of signaling processes. In nearly all cases studied, a significant fraction of cytosolic, membrane-bound or transmembrane proteins diffuse relatively freely within the cytosol or membrane. Fluorescence recovery after photobleaching (FRAP) studies of plasma membrane receptors, as well as of membrane-bound and cytosolic signaling proteins, have shown that the diffusion coefficients of proteins are quite variable and can be up to 0.05 $\mu m^2/s$ for transmembrane receptors, up to 0.5 $\mu m^2/s$ for membrane-bound proteins and higher than 10 $\mu m^2/s$ for cytosolic proteins (for example, Jans et al., 1990; Niv et al., 1999; Arrio-Dupont et al., 2000). As one of the fastest diffusing examples, the 27 kDa EGFP (enhanced GFP; Clontech, CA) has been measured to have a diffusion coefficient between 30 and 80 $\mu m^2/s$ (i.e., Arrio-Dupont et al., 2000). It is interesting to note that the size of proteins, even conjugated with GFP, is not a major obstacle for diffusion since the diffusion coefficient is inversely proportional to the radius (or the third root of its mass). Instead, specific and unspecific binding interactions in the cytosol are the main reason for lower than expected cytosolic diffusion coefficients. The average distance ($<s>$) that a protein diffuses from its origin along a given axis can be calculated as, $<s> \approx (4 \cdot D \cdot t / \pi)^{1/2}$ (see Endnote 1), with D as the diffusion coefficient and t as the time of diffusion. As an example, a cytosolic signaling protein with a D of 10 $\mu m^2/s$ diffuses on average 4 μm in a 1 s time period. In contrast, membrane-bound and transmembrane proteins diffuse in the same period less than 0.8 and 0.25 μm, respectively. It should be noted that the diffusion distances do not scale linearly but that the average diffusion distance for proteins increases with the square root of the time (twice as far in 4 s compared to 1 s). Figure 1 shows calculated views of a typical random path of a diffusing cytosolic, membrane-bound and transmembrane signaling protein (during a 4 s period). Markedly, cytosolic signaling proteins can rapidly diffuse across the cell, while transmembrane and membrane bound signaling proteins can be regionally localized for time periods of seconds to even minutes.

Given these considerations, how can one interpret an observed subcellular change in the localization of a GFP conjugated signaling protein? A rough analysis of currently reported translocation time courses suggests that nearly all of them are consistent with a random diffusion process and are likely triggered by the encounter of a diffusing signaling protein with a newly enabled localized binding site. Over time, the diffusing signaling protein will screen a large region of a cell or membrane for the presence of potential binding partners, giving rise to the observed local enrichment or translocation. Since cellular binding interactions with affinities in the tens of nanomolar to micromolar range are typically rapidly reversible, individual proteins at these target sites can

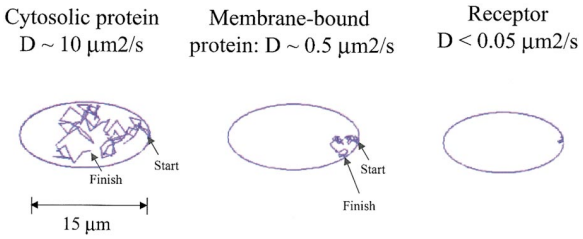

Figure 1. Diffusion-Mediated Random Walk of Signaling Proteins

Schematic representation of a 4 s long random walk of (left) a cytosolic protein, (middle) a membrane-bound protein, and (right) a receptor. Simulated with MATLAB.

still dynamically exchange and find different subcellular sites until the system is in a steady state.

From the perspective of individual proteins, a plasma membrane translocated signaling protein would then not be persistently localized but instead undergo sequences of random walks in the cystosol, interspersed with plasma membrane binding and dissociation events. One of the most striking examples of dynamic translocation has been observed for conventional protein kinase C isoforms, which can translocate to plasma membrane within less than 1 s of an increase in calcium concentration and still rapidly exchange between the cytosol and plasma membrane at elevated calcium concentrations (Oancea and Meyer, 1998; Figure 2).

It should be noted that while an active transport mechanism for signaling proteins is probably not relevant in many cell types, an interesting exception is in mature neurons where diffusion processes are not sufficient to get signaling proteins from the cell body into extended neurites, suggesting that transport assistance for signaling proteins has also to exist in specialized cell systems.

Molecular Mechanisms for Protein Translocation

Translocation events in signal transduction are thought to be driven by one of two processes: (1) by the generation of new protein–protein binding interactions or (2) by second messenger–mediated binding interactions between signaling proteins and lipid partners in the plasma membrane or other subcellular structures. Some of the best studied examples in the first category are the generation of GTP-bound small GTPases which can lead to the diffusion-mediated recruitment of kinases and other enzymes to the plasma membrane or other subcellular structures. Furthermore, the role of tyrosine phosphorylation is often to trigger the recruitment of SH2 domain–containing proteins to the plasma membrane, while the phosphorylation of G protein–coupled receptors at serine/threonine residues can lead to the recruitment of arrestin and its isoforms. Some of the best studied examples in the second category include the Ca^{2+}-, phosphoinositide-, or diacylglycerol-mediated plasma membrane translocation of proteins with C2 (a Ca^{2+} and negative phospholipid binding domain that was first described as the second conserved region in protein kinase C), PH (pleckstrin homology domain that bind phosphoinositide lipids), or C1 domains (first identified as the first conserved domain in protein kinase C and which binds diacylglycerol). This list of mechanisms for the translocation of signaling proteins to different membranes and microdomains is rapidly increasing.

Thus, by utilizing second messenger binding, phosphorylation, GDP to GTP exchange, conformational changes, and other signaling mechanisms, cells can rapidly and reversibly induce new binding sites for signaling proteins and thereby change the localization state of a large class of continuously diffusing signaling proteins. Since these translocation events often have a significant delay due to the required diffusion and binding interaction, cells can utilize the delayed assembly of signaling intermediates to suppress "activation noise," to generate thresholds for activation and to increase the specificity of signaling responses.

Subcellular Localization of Signaling Processes

Polar cells such as epithelial cells and neurons, chemotactic cells such as macrophages and fibroblasts, as well as many other cell types, have a need to restrict signaling processes to a small subregion of the cell. This raises the question of how cells can prevent diffusion and translocation processes which would spread the signaling response across the entire cell. For example, diffusible signaling proteins that are activated by phosphoryla-

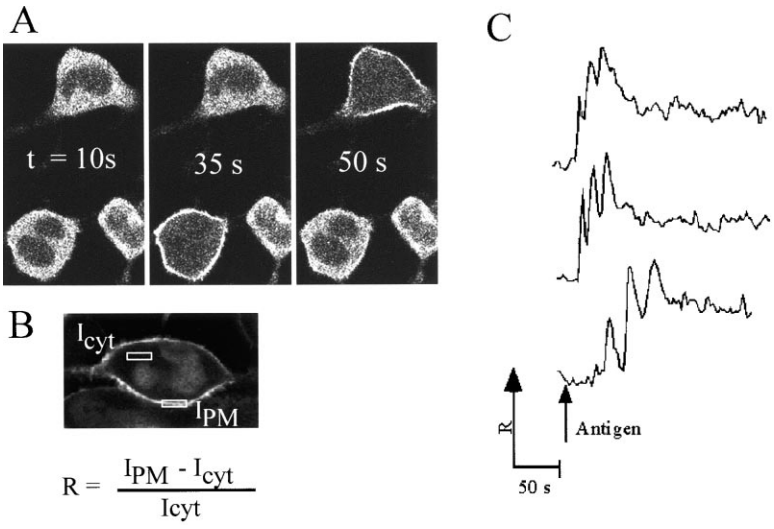

Figure 2. Plasma Membrane Translocation of a Conventional Protein Kinase C Isoform (Oancea and Meyer, 1998)

(A) Receptor activation induced translocation of PKCγ-GFP to the plasma membrane at 10, 35, and 50 s after the addition of antigen (20 μg/ml DNP-BSA). (B) Definition of the relative plasma membrane translocation parameter R. (C) Examples of the time course of plasma membrane translocation of PKCγ-GFP. Maximum translocation of PKCγ-GFP to plasma membrane was R = 1.5 ± 0.6 (N = 30).

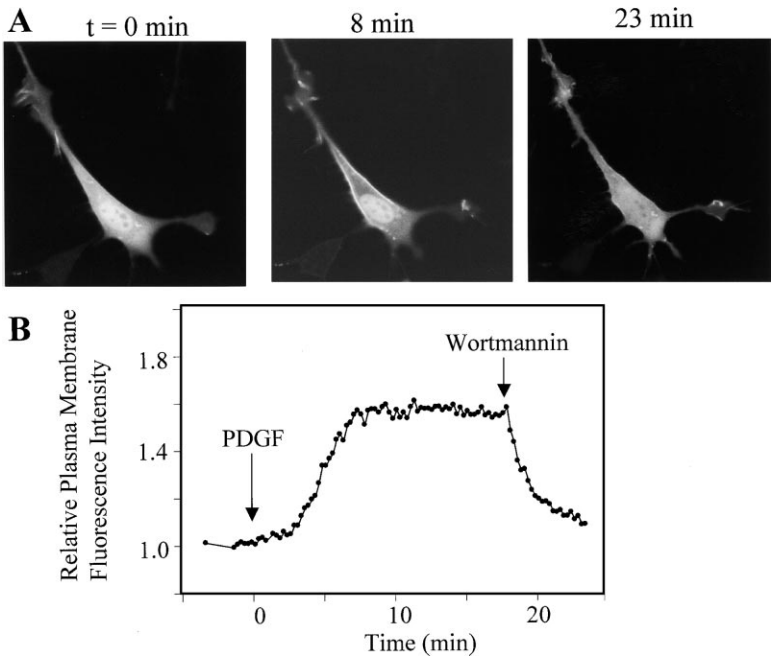

Figure 3. Translocation of GFP-AKT-PH to and from the Plasma Membrane in NIH-3T3 Cells

Before stimulation (right panel), 5 min after the addition of PDGF (5 nM, final concentration, middle panel), and 15 min after the addition of wortmannin (1 μM, final concentration, left panel), measured by confocal microscopy (after Watton and Downward, 1999).

tion could act far from their activation site if they can diffuse and if the relevant phosphatase activity is low.

In some signaling pathways, high-affinity cytoskeletal attached adaptor proteins are likely employed to nearly irreversibly localize the signaling proteins within a pathway and thereby prevent their diffusion and translocation. Such a permanent localization mechanism might be in place in signaling systems such as the visual signal transduction in invertebrates that require local signal transmission (Zuker, 1996). In this particular case, the prelocalization of the signaling proteins also eliminates the relatively slow diffusion steps and serves as an effective way to accelerate signal transmission. In the example of chemotactic cells, at least some of the involved signaling proteins, such as the Rho family G proteins and PH domain–containing proteins, appear to be diffusible but act locally within a small region at the leading edge of the cell. Their ability to diffuse is suggested from the measured relatively fast diffusion coefficients of small G proteins (Niv et al., 1999) and from the observed diffusible spread of GFP conjugated PH domains (for example Parent et al., 1998, and Servant et al., 2000).

In the case of chemotaxis and in many other local signaling systems, it becomes critical to know how one can estimate the distances over which activated signaling proteins can transmit information. If local activation of a cytosolic, membrane-bound or transmembrane signaling protein occurs, the average distance that the signaling protein travels before it is inactivated can be defined as a "range of action (r)" dependent on the diffusion coefficient (D) and the lifetime of the activated state (τ), where τ is defined as the time it takes for 63% of the proteins to be inactivated: $r = (2 \cdot D \cdot \tau)^{1/2}$ for 2-dimensional diffusion processes and $r = (3 \cdot D \cdot \tau)^{1/2}$ for 3-dimensional diffusion processes (see Endnote 2).

For example, a locally phosphorylated membrane-bound signaling protein with a diffusion coefficient of 0.5 $\mu m^2/s$ and a lifetime of 10 s would have a range of ~3 μm. This suggests that in order for a signaling pathway to act locally, the key second messengers and diffusible signaling proteins in the pathway have to have diffusion coefficients and lifetimes that restrict them into the localized signaling region. While local signaling events have been well explored for locally produced second messengers such as calcium as well as for some activated plasma membrane receptors, much less is currently known about the range of action of activated membrane bound and cytosolic signaling proteins. Such studies will be important for understanding how protein-based signaling steps are transmitted from the plasma membrane to internal organelles or across polarized cells.

Fluorescent Translocation Biosensors as Tools in Signal Transduction

Many recent studies have shown that the translocation and subcellular localization of signaling proteins or signaling protein domains can be used as versatile tools to study when and where a particular signaling step is triggered (for example Barak et al., 1997; Stauffer and Meyer, 1997), to dissect activation mechanisms of enzymes (Oancea and Meyer, 1998), or to investigate chemotactic and other localized signaling responses (Parent et al., 1998; Meili et al., 1999; Servant et al., 2000). In the study by Oancea and Meyer, 1998, such an analysis of the kinetics of translocation was made by comparing the translocation of the full-length protein versus individual C1 and C2 domains, which led to a model for a sequential activation mechanism of PKC that may enable the kinase to function as a frequency detector for calcium signals and as a delayed coincidence detector for diacylglycerol and Ca^{2+} signals. In the chemotaxis studies (i.e., Servant et al., 2000), the evidence for the dynamic local 3′ phosphoinositide (3′PtdIns) signals at the leading edge have led to a convincing model that local 3′PtdIns signals are of critical importance in distinguishing the front from the back of a chemotactic cell.

In many cases, the localization of multidomain signal-

ing proteins is dependent on several signaling events and binding interactions. In these cases, GFP conjugated minimal domains have been found to be useful tools to monitor a particular signaling step more selectively. For example, GFP conjugated PH domains of Akt (Figure 3 and Watton and Downward, 1999) and GFP conjugated C1 domains from PKC (Oancea et al., 1998) were found to be more selective and higher affinity probes than the full-length proteins for the visualization of localized changes in plasma membrane 3′ PtdIns and diacylglycerol concentration, respectively. The list of such biosensors is rapidly increasing with the identification of domains that can be used to monitor phosphatidic acid production (a domain from RAF; Rizzo et al., 2000), InsP3 signals (Hirose et al., 1999), Ca^{2+} signals (C2 domains from PKC; Oancea and Meyer, 1998), more selective PtdIns(3,4,5)P3 signals (Venkateswarlu et al., 1998) and other signaling events.

While translocation studies in living cells generated a lot of new insights into the spatial and temporal organization of signaling processes, they have also shown some of the challenges that still lay ahead in utilizing fluorescent reporter constructs. Cellular autofluorescence and photobleaching of GFP make it often necessary to use relatively high concentrations of reporters to monitor subcellular signaling events. Typically, cellular expression levels have to be larger than 0.1 to 1 μM in order to identify transfected cells and to obtain sufficiently good signal-to-noise for subcellular localization analysis. This requires in many cases that more signaling protein constructs have to be expressed than are present in the native cell, which can lead to interference with downstream signaling (as has been shown in the case of expressed fluorescent SH2 domain reporters, Stauffer and Meyer, 1997). Finally, it is important to mention that light microscopy limits the spatial resolution to about 300 nm, requiring that complementary assays have to be used to directly demonstrate that colocalized signaling proteins directly interact with each other.

Future Microscopy Signaling Studies

Despite these current limitations, a rapidly growing number of studies are demonstrating that the ability to track signaling processes in space and time opens up new possibilities to dissect the dynamic mechanisms in signal transmission and to gain a spatial and temporal understanding of intracellular signal transduction that would not be possible by using biochemical approaches. With the possibility to now study CFP as well as YFP (cyan and yellow fluorescent protein) conjugated signaling proteins and domains in the same cells, the subcellular analysis of the distribution of signaling proteins can be made quantitatively using subtraction analysis or ratioing between the two fluorescent channels. This will advance this microscopy signaling approach into an era where signaling events are not just observed but can actually be measured as a function of time. Furthermore, in some cases it will be possible to measure protein–protein binding interactions by fluorescence resonance energy transfer (FRET), although the biological usefulness of FRET for intracellular binding studies has been limited by signal-to-noise issues due to the typically only small changes in energy transfer between CFP and YFP interaction partners.

There is now much interest in making the currently low throughput fluorescence imaging approaches better suited to attack the more complex question of how entire signaling networks are coordinated in space and time. In such measurements, selected sets of fluorescence biosensors could be used for measuring intermediate or final signaling steps. Such measurements could then be combined with perturbation strategies using antisense oligonucleotides, pharmacological libraries, or sets of expressed dominant-negative and constitutively active constructs in order to gain insights into the wiring diagram and the feedback time constants of cellular signal transduction networks.

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Endnotes

(1) The diffusion profile of a protein that originates in a point source can be described as (Crank, J., The Mathematics of Diffusion, Clarendon, Oxford, 2nd ed., 1975): $F(x,t) = 1/(4 \cdot \pi \cdot D \cdot t) \exp[-x^2/(4 \cdot D \cdot t)]$. If the question is how far a protein will get on average away from its origin, the problem can be reduced to integration of $2 \cdot x \cdot F(x,t)$ from 0 to infinity. This calculation uses the mirror principle of diffusion described in the same reference. The result was used in the equation in the text.

(2) The calculation of the range of action of a continuously but locally produced active protein can be obtained by multiplying the probability that a protein will be inactivated at a time t: $1/\tau \cdot \exp(-t/\tau)$, with the average diffusion distance of the protein in the same time: $(4 \cdot D \cdot t/\pi)^{1/2}$ and by calculating the integral of the product over time from 0 to infinity. This calculation was made for two and three dimensions for the equations that were included in the text.