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Review

Four-dimensional organization of protein kinase signaling cascades: the roles of diffusion, endocytosis and molecular motors

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Summary

Extracellular signals received by membrane receptors are processed, encoded and transferred to the nucleus *via* phosphorylation and spatial relocation of protein members of multiple component pathways, such as mitogen activated protein kinase (MAPK) cascades. The receptor-induced membrane recruitment of the cytoplasmic protein SOS results in the activation of the Ras/MAPK cascade. It has been suggested that the membrane recruitment of signaling proteins causes an increase in the diffusion-limited rates. We have recently shown that this increase is too small to be responsible for enhanced signal transduction. Instead we demonstrate that the function of membrane localization is to increase the number (or average lifetime) of complexes between signaling partners. A hallmark of signaling pathways is the spatial separation of activation and deactivation mechanisms; e.g. a protein can be phosphorylated at the cell surface by a membrane-bound kinase and dephosphorylated in the cytosol by a cytosolic phosphatase. Given the measured values of protein diffusion coefficients and of phosphatase and kinase activities, the spatial separation is shown to result in precipitous phospho-protein gradients. When information transfer is hampered by slow protein diffusion and rapid dephosphorylation, phospho-protein trafficking within endocytic vesicles may be an efficient way to deliver messages to physiologically relevant locations. The proposed mechanism explains recent observations that various inhibitors of endocytosis can inhibit MAPK activation. Additional mechanisms facilitating the relay of signals from cell-surface receptors to the nucleus can involve the assembly of protein kinases on a scaffolding protein and active transport of signaling complexes by molecular motors. We also discuss long-range signaling within a cell, such as survival signaling in neurons. We hypothesize that ligand-independent waves of receptor activation or/and traveling waves of phosphorylated kinases emerge to spread the signals over long distances.

Key words: signal transduction, protein kinase, diffusion, endocytosis, molecular motor, mitogen activated protein kinase (MAPK), traveling wave.

Introduction

The deciphering of the genome of several organisms including humans has generated a list of the macromolecular parts of living cells. A challenge of current biology is to understand how this list of ‘genetics parts’ gives rise to a four-dimensional (i.e. space- and time-varying) behavior, governed by intracellular regulatory networks. Activation of signal transduction networks by extracellular stimuli is encoded into complex temporal and spatial patterns of activation and relocation of numerous proteins, leading to important cellular decisions ranging from cell survival, growth and proliferation, to growth arrest, differentiation or apoptosis. During the past decade, there has been increasing realisation that cytoskeletal structures and intracellular movement, driven by myosin, kinesin and dyenin motors, play crucial roles in the regulation of metabolism and signal transduction (see, for example, reviews by Hochachka, 1999; Hollenbeck, 2001; Verhey and Rapoport, 2001). Our current understanding of the spatio–temporal organization of signaling processes and its control by cellular topology, diffusion and intracellular movement, however, is far from complete.

Signaling through a plethora of cell-surface receptors, such as G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors, activate mitogen activated protein kinase (MAPK) cascades, which function as central integration modules in information processing (Chang and Karin, 2001; Kholodenko, 2002; Lewis et al., 1998). MAPK cascades relay extracellular stimuli from the plasma membrane to crucial cellular targets distant from the

membrane, e.g. transcription factors. Elucidation of the spatio–temporal organization and regulation of MAPK signaling is becoming increasingly important for understanding signaling specificity and the diverse nature of cellular responses. We have recently shown that simple diffusion of activated kinases may be insufficient for the effective propagation of phosphorylation signals through MAPK cascades (Kholodenko, 2002). In this paper, we analyze the functional implications of membrane targeting of cytosolic proteins, examine the consequences of the spatial separation of certain kinases and phosphatases in MAPK pathways and discuss multiple mechanisms used by a cell to propagate the signals over long distances.

Regulation of signaling by membrane recruitment of cytosolic proteins

There are numerous examples of the membrane localization of cytoplasmic proteins following receptor activation (Mochly-Rosen, 1995). For instance, stimulation of RTKs is linked to the activation of the extracellular signal regulated kinase (ERK) Ras/MAPK cascade through the recruitment of a cytoplasmic protein SOS to the plasma membrane (Egan et al., 1993). SOS (a homolog of the *Drosophila melanogaster* Son of sevenless protein) is the guanine nucleotide exchange factor for the small GTP-binding protein Ras, anchored to the cell membrane (Fig. 1). SOS-Ras interactions convert inactive Ras-GDP to active Ras-GTP. SOS binds to RTKs, such as the epidermal growth factor receptor (EGFR), not directly, but through the adaptor protein Grb2 (growth factor receptor binding protein 2). Grb2, in turn, can bind to the activated receptor either directly or through another adaptor protein, Shc (src homology and collagen domain protein) (Schlessinger, 2000; Schlessinger and Bar-Sagi, 1994). Importantly, RTKs do not phosphorylate SOS, and SOS catalytic activity towards Ras does not change upon its binding to the receptor (Buday and Downward, 1993). Note also, that the Grb2-SOS complexes can exist in the cytoplasm of unstimulated cells due to the high affinity of Src homology (SH) 3 domains of Grb2 to proline-rich regions of SOS (Sastry et al., 1995).

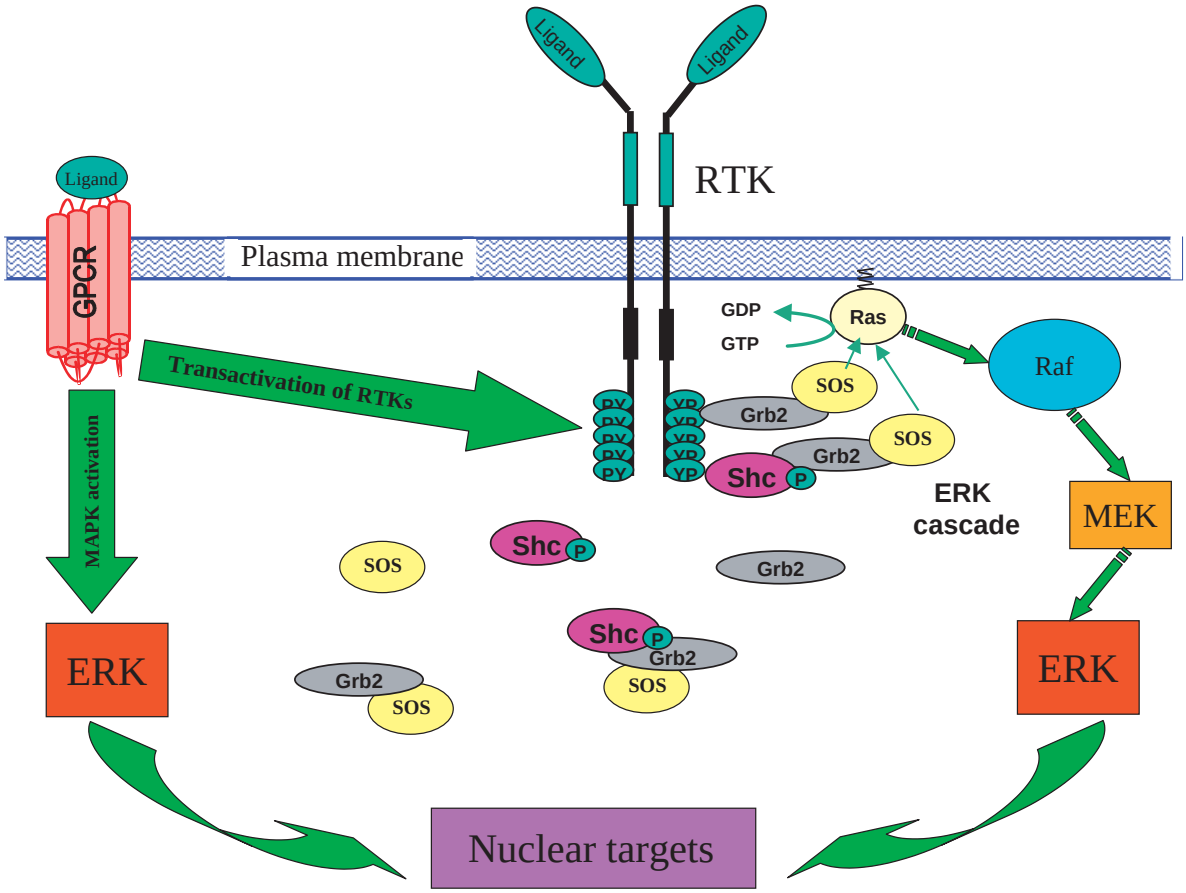

Fig. 1. Signaling through the ERK (MAPK) cascade following activation of receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). RTKs activate the ERK cascade by recruiting guanine exchange factor SOS to the plasma membrane in close proximity to the membrane-anchored Ras. The upper right corner of the figure details the signaling activation route near the membrane surface. Below, it is shown that some of the signal transduction complexes, e.g. the Shc-Grb2-SOS and Grb2-SOS complexes, reside in the cytosol, raising the question of whether Ras activation occurs through direct interactions of SOS from the cytosol. Activated Ras-GTP promotes the activation of Raf-1, which phosphorylates MEK, which in turn activates ERK. Left, GPCRs stimulate MAPK cascades either through transactivation of RTKs, such as EGFR, or directly by using β-arrestin as a scaffold for the ERK and JNK cascades. For abbreviations, see list. See text for further details.

This interaction pattern raises a number of questions about the role of the membrane recruitment in signal transfer. For instance, why should SOS relocate to the membrane if its catalytic activity is not activated by RTKs? What prevents direct interaction of cytosolic SOS or Grb2-SOS complexes with the membrane-bound Ras from catalyzing GDP/GTP exchange on Ras? To clarify the effect of membrane localization, we consider two extreme cases. When two signaling molecules form a productive complex, transducing the signal after each diffusive encounter, the signal-transduction process is called 'diffusion-limited'. If only a small fraction of the collisions leads to binding that lasts long enough to transfer the information, the signal transduction is called 'reaction-limited'. In the latter case, the two protein molecules associate and dissociate a number of times before signal transduction takes place.

Membrane localization does not significantly enhance diffusion-limited signal transfer

It has earlier been suggested that localization of signaling proteins close to the cell membrane causes an increase in the first-encounter rate (Adam and Delbruck, 1968); the solutes do not 'get lost' by wondering off into the bulk phase (Axelrod and Wang, 1994; Berg and Purcell, 1977). However, such an increase appears to be too small to account for significantly enhanced signal transduction (Kholodenko et al., 2000b). The diffusion-limited rates in the membrane and in the cytosol can be compared in terms of the ratio (h) of the corresponding association rate constants in two dimensions and in three dimensions. For diffusion-limited association of two proteins located in the plasma membrane of a spherical cell or delocalized over the cytosol volume, the ratio h was estimated to be $(0.02–0.05)\times(D_{membr}/D_{cyt})\times(r_{cell}/r_{prot})$ (Kholodenko et al., 2000b). For instance, if the cell radius (r_{cell}) is 10 μm and the sum (r_{prot}) of typical protein radii is approximately 10^{-2} μm, then $h\approx(20–50)\times D_{membr}/D_{cyt}$. Because the diffusion coefficients of proteins in the membrane (D_{membr}) are about two orders of magnitude lower than in the cytosol (D_{cyt}) (Cherry, 1979; Kusumi et al., 1993), we conclude that the function of the membrane recruitment is unlikely to be an enhancement of the encounter rates. In fact, it has already been suggested that the fastest route to diffuse is through the cytosol, not through the membrane, because of two orders of magnitude difference in the diffusion coefficients (Bray, 1998).

Gain in the number of signaling complexes is more critical than an increase in the association rate

In the reaction-limited extreme, the first-encounter rate is much faster than the signal transduction rate, which can be estimated as the fraction of molecules in the associated state (as if this step were at equilibrium) multiplied by the reaction rate constant. An increase in the effective local concentration of a cytosolic protein due to membrane relocation brings about an increase in the apparent affinity to a binding partner in the membrane (Ferrell, 1998). Haugh and Lauffenburger (1997) estimated that an increase in the reaction-limited protein association rate could be as high as $10^2–10^3$. In the general case, however, the rate enhancement depends on many kinetic and molecular details including the probability of success per diffusion encounter, the concentration depletion zones near the targets, the reversibility of the binding and other factors (Axelrod and Wang, 1994). The binding reversibility suggests that the dissociation rate constant, in addition to the association rate, may change upon the membrane relocation, as in fact was observed for affinity enhancement due to 'macromolecular crowding' (Rohwer et al., 1998). Therefore, although the speed of signal transduction increases by the translocation of signaling proteins, it should not be assumed that any such enhancement is due to an increase in the rate of formation of protein complexes. Instead, the underlying mechanism can be an increase in the number (or average lifetime) of signaling complexes, which act as catalysts activating downstream processes. We submit that membrane localization serves to enhance the extent of complex formation of signal-transduction proteins, and hence increase the intensity of the signal that is being transduced.

Receptor-mediated membrane recruitment significantly increases the number of complexes formed by a cytoplasmic protein and a membrane-anchored protein

A gain in the number of signaling complexes involving a cytosolic protein X and a membrane-bound protein Y can be brought about by a two-step, 'piggyback' mechanism. The first step is binding of X to an activated membrane receptor R ('piggy'), which confines X to a membrane shell, where Y is located. The second step is a 'piggyback riding' of X until it meets membrane-anchored Y. X then forms a complex with Y, while continuing to ride piggyback on R. The quantitative analysis shows that this piggyback riding leads to a strong reduction of the apparent dissociation constant, $K_{d,app}$, which can be expressed through the equilibrium dissociation constant K_d of cytosolic X and membrane-bound Y, as follows (Kholodenko et al., 2000b):

$$K_{d,app}\ (\text{piggyback riding}) = K_d(V_m/V_c)(1+\kappa)\ ,$$

where

$$\kappa = K_{d,R}/C_R\ . \qquad (1)$$

V_c is the cytosol volume and V_m is the volume of a water layer adjacent to the membrane where protein Y is confined. The value of the dimensionless factor κ is determined by the ratio of the dissociation constant $K_{d,R}$ for the binding of cytosolic X to the activated receptor R and the concentration of the latter (C_R). Experimental data show that the $K_{d,R}$ values appear to be in the range of 1–100 nmol l^{-1}, whereas the total RTK concentrations (based on the whole cytoplasmic volume) are in the range of 100–1000 nmol l^{-1} and approximately 20–50% of the total amount is activated by the stimuli (see Kholodenko et al., 1999; Moehren et al., 2002, and the references therein). Therefore, for membrane recruitment by RTKs, the value of κ does not exceed 1. We conclude that when the activated receptor is present in excess of cytosolic X, the apparent dissociation constant decreases by a factor as high as V_m/V_c,

i.e. by 2 or 3 orders of magnitude in comparison with the actual K_d of binding X and Y.

An alternative mechanism of an enhancement in signal transfer rate may be an increase in the efficiency of the reaction between cytosolic X and membrane-bound Y due to a conformational change of X upon binding to a membrane anchor, such as receptor R. However, for SOS-Ras interactions, structural and kinetic data on the catalytic mechanism and activity rule out this possibility (Buday and Downward, 1993; Corbalan-Garcia et al., 1998; Lenzen et al., 1998).

Recruitment to a scaffold

Our results apply not only to the case of membrane translocation, but to translocation into any subcellular compartment. Scaffolds of various sorts should be mentioned here. They act as templates, bringing together signaling proteins, organizing and coordinating the function of entire signaling cascades (Bray, 1998). Importantly, our results suggest that the number of signaling complexes will increase only if these complexes do not dissociate from a scaffold. Even if the interacting proteins were brought to close vicinity on a scaffold, the dissociation of the protein complex from the scaffold will result in further dissociation of the complex, which will be in thermodynamic equilibrium with its components.

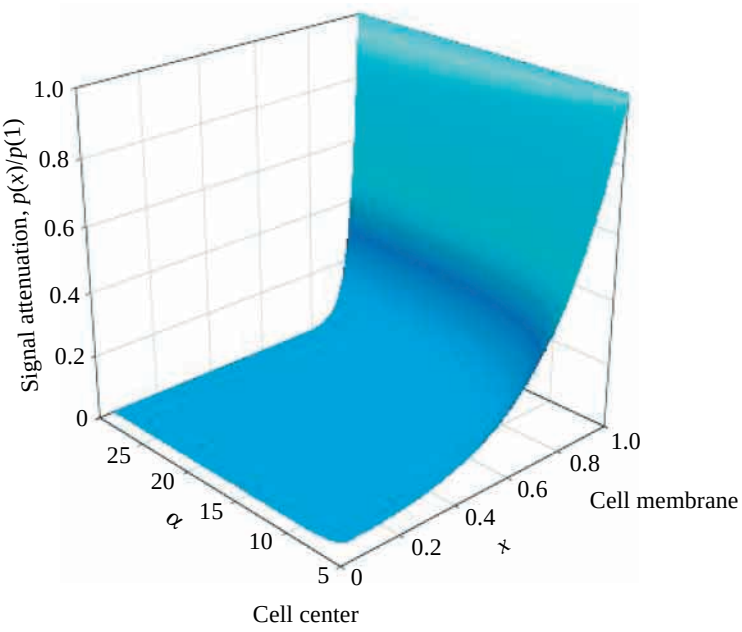

Fig. 2. Decline in the phospho-protein concentration with distance from the cell membrane at different values of the parameter α. The parameter α is determined by the ratio of the characteristic times of diffusion and the phosphatase reaction ($\alpha^2=K_p\times L^2/D$, where L is the cell radius, $K_p=V_{max,p}/K_{m,p}$ is the observed first-order rate constant of the phosphatase and D is the protein diffusion coefficient). The decline, $p(x)/p(1)$, is calculated as the ratio of the concentrations of the phosphorylated form at distance xL from the cell center and at the plasma membrane, where x=1. Reproduced with permission from Kholodenko (2002).

Specifics of SOS-Ras and GAP-Ras interactions

The calculations demonstrate that activation of Ras by SOS from the cytosol is two or three orders of magnitude less effective than the catalysis, mediated by the RTK-bound SOS, for instance, mediated by the EGFR-Grb2-SOS and EGFR-Shc-Grb2-SOS complexes (Haugh and Lauffenburger, 1997). After the dissociation of phosphorylated Src homology and collagen domain protein (Shc) from EGFR, the Shc-Grb-SOS complexes may bring about an additional route for Ras activation. However, quasi-equilibrium association of these complexes with Ras may be insignificant compared to receptor-mediated association unless the Shc-Grb-SOS complexes are also targeted to the plasma membrane through specific domains, such as phosphotyrosine-binding and pleckstrin homology domains (Drugan et al., 2000; Ugi et al., 2002). Quantification of relative contributions of SOS complexes with the receptor and with phosphorylated Shc to Ras activation is awaiting experimental verification.

Signaling of activated Ras is turned off by the activation of GTPase-activating proteins (GAPs) (Bollag and McCormick, 1992). Similar to SOS, p120 GAP is a cytoplasmic protein. As in the case of SOS signals, the membrane recruitment of GAP is necessary for an appreciable increase in the rate of GTP hydrolysis on Ras.

A variation on the topic of Ras activation by RTKs has been described for fibroblast growth factor receptors (FGFRs) (Kouhara et al., 1997). Stimulation of FGFRs induces cell proliferation, differentiation and migration by activation of the Ras/MAPK signaling pathway. However, unlike other RTKs, activated FGFR cannot bind Grb2 directly. Recently, a novel membrane-anchored protein, phosphorylated by activated FGFR, has been discovered and is known as FRS2 (FGFR substrate 2) (Kouhara et al., 1997). Tyrosine-phosphorylated FRS2 is able to bind Grb2 and, therefore, the Grb2-SOS complex. In a familiar twist, the juxtaposition of the FRS2-Grb2-Sos complex on the membrane may facilitate the Ras activation by SOS, providing a feasible mechanism for linking FGFRs to the Grb2/SOS/Ras/MAPK pathway.

Signal transduction through MAPK cascades can require endocytic trafficking and/or active molecular transport

Our results demonstrate that the membrane recruitment of specific cytosolic proteins can enhance receptor-induced activation of a membrane-anchored target, such as Ras protein or a membrane-bound kinase, by a 1000-fold; but how do signals emanating from membrane-bound proteins spread into the cell interior and reach the nucleus? We will show here that simple diffusion of activated proteins may be insufficient for the signal transfer through MAPK pathways.

MAPK activation by RTKs and GPCRs

MAPK cascades are widely involved in eukaryotic signal transduction, and these pathways are evolutionarily conserved in cells from yeast to mammals (reviewed in Chang and Karin, 2001; Lewis et al., 1998). Mammalian cells express at least four different MAPK families, including the ERK, the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades. A three-tiered cascade comprises MAPK, MAPK kinase (MKK) and MKK kinase (MKKK). A kinase at the first cascade level, MKKK, is activated at the cell membrane. A kinase at the bottom level, MAPK, is activated in the cytosol and phosphorylates target proteins both in the cytosol and nucleus. Mitogenic signaling by RTKs and GPCRs is associated with Ras-dependent stimulation of the ERK cascade. The classical paradigm for GPCR signaling involves the agonist-dependent interaction of GPCRs with heterotrimeric G proteins that stimulates the exchange of bound GDP for GTP, resulting in dissociation of G proteins into α and βγ subunits. Subsequent interaction of α and βγ subunits with effector enzymes or ion channels regulates the generation of soluble second messengers or ionic conductance changes. However, this paradigm should be extended to include the proliferative and differentiative effects of GPCRs. It is presently known that in a multitude of cell types, GPCRs stimulate the MAPK cascades, such as the ERK, JNK and p38 MAPK cascades (Garrington and Johnson, 1999; Gutkind, 1998; van Biesen et al., 1996). Interestingly, the pathways of GPCR and RTK-mediated MAPK activation often converge. Recently, evidence has emerged that the overlap of GPCR and RTK signaling pathways is accounted for in part by GPCR-mediated 'transactivation' of RTKs, such as EGFR (Fig. 1), platelet-derived growth factor receptor and insulin-like growth factor-1 receptor. For instance, EGFR activation by GPCR was shown to be mediated by signaling pathways involving non-receptor tyrosine kinases, Src and Pyk2, and through activation of the metalloprotease leading to the release of heparin binding EGF (Pierce et al., 2001; Prenzel et al., 1999). It is, however, important to realize that although RTK transactivation is now a recognized mechanism for GPCR-induced activation of the ERK cascade, other signaling pathways can also contribute to this link (Andreev et al., 2001; Miller and Lefkowitz, 2001).

Heterogeneous spatial distribution of activated components in MAPK cascades

Multiple cellular proteins are phosphorylated and dephosphorylated at distinct cellular locations. In the Ras-ERK pathway, inactive Raf-1 (MKKK) resides in the cytosol. Upon stimulation of cell-surface receptors, Raf-1 is translocated from the cytosol to the plasma membrane by a high-affinity binding to GTP-loaded Ras. At the membrane, Raf-1 undergoes a series of activation steps involving interaction with 14-3-3 proteins and phosphorylation on specific tyrosine and serine residues (Mason et al., 1999). Although the mechanism of activation is not yet completely understood, the association of Raf-1 with membranes appears to be essential for its activation. The Raf-1 kinase phosphorylates the cytosolic kinase MEK (MKK) at the plasma membrane, whereas soluble serine/threonine phosphatases dephosphorylate the activated MEK in the bulk phase (Kyriakis et al., 1992; Strack et al., 1997). In the cytosol, active MEK kinase phosphorylates ERK (MAPK) and specific ERK phosphatases are localized to the cytosol and nucleus. Spatial separation of protein kinases and phosphatases suggests that there may be cellular gradients of phosphorylated (active) forms of MEK and ERK, with high concentrations in the periplasmic region near the phosphorylation location and low concentrations at a distance from the plasma membrane. Since activated ERK phosphorylates multiple cytoplasmic and nuclear targets, large spatial gradients of this kinase may have very important implications for cell signaling.

Indeed, the quantitative analysis demonstrates that the spatial separation of kinases and phosphatases potentially results in precipitous gradients of phospho-proteins, given measured values of protein diffusion coefficients and phosphatase and kinase activities (Brown and Kholodenko, 1999; Kholodenko et al., 2000a). We illustrate this analysis by calculating the spatial gradients for a single level cascade, in which the kinase is located on the plasma membrane and the phosphatase is distributed homogeneously in the cytoplasm (These calculations and Fig. 2, which illustrates them, are reproduced from Kholodenko, 2002, with permission from Elsevier Science.) For a spherical cell, the following equation describes radial diffusion of the phospho-protein (p) from the cell membrane, where p is produced at the rate, v_{kin}, to the cell interior, where p is dephosphorylated at the rate, v_p,

$$\frac{\partial p}{\partial t} = \left(\frac{D}{L^2}\right)\frac{1}{x^2}\frac{\partial}{\partial x}\left(x^2\frac{\partial p}{\partial x}\right) - v_p\,,$$

$$\left.\frac{\partial p}{\partial x}\right|_{x=1} = \frac{L^2}{3D}\,v_{kin}\,, \qquad \left.\frac{\partial p}{\partial x}\right|_{x=0} = 0\,. \tag{2}$$

Here p is the phospho-protein concentration, D is the protein diffusion coefficient in the cytosol (assumed to be equal for the phosphorylated and unphosphorylated forms), x is a dimensionless coordinate, equal to the distance from the cell center divided by the cell radius, L [the distance d from the cell membrane is expressed in terms of x as $d=(1-x)L$], v_{kin} and v_p are the rates of the kinase and phosphatase, respectively. If we assume that the phosphatase is not saturated by a target phospho-protein (a reasonable assumption for most cytosolic phosphatases), the phosphatase rate v_p can be described as $v_p=K_p\times p$, where K_p is the observed first-order rate constant (equal to the ratio of the maximal activity to the Michaelis constant, $K_p=V_{max,p}/K_{m,p}$). Then, the steady-state solution to Equation 1 can be found readily (Kholodenko et al., 2000a), and the ratio of the phospho-protein concentrations at the distance x from the cell center and at the plasma membrane, $p(x)/p(1)$, is given by:

$$\frac{p(x)}{p(1)} = \frac{e^{\alpha x} - e^{-\alpha x}}{x(e^{\alpha} - e^{-\alpha})}\,, \qquad \alpha^2 = k_p\frac{L^2}{D}\,. \tag{3}$$

Therefore, the phospho-protein concentration decreases toward

the cell interior approximately exponentially, as illustrated in Fig. 2. The phospho-protein concentration profile, $p(x)/p(1)$, depends on the dimensionless parameter α (recognizable as the square root of the Damkohler number), which compares the phosphatase and the diffusive time scales. Therefore, all we need to know are the observed first-order rate constant of the phosphatase reaction K_p, the diffusion coefficient D and the cell radius. A typical value for a hepatocyte radius is 10 μm, D is estimated to be of the order of 10^{-8} cm^2 s^{-1} (Arrio-Dupont et al., 2000; Dayel et al., 1999; Gershon et al., 1985; Jacobson and Wojcieszyn, 1984), and values for K_p were found to range from roughly 0.1 to 10 s^{-1} (see Haugh and Lauffenburger, 1998; Kholodenko et al., 2000a; Zhao and Zhang, 2001, and references therein). With K_p values of 0.25 or 1 s^{-1}, the distance from the plasma membrane, at which the phosphorylation signal is attenuated by a factor of 10, is equal to 6.8 or 2.6 μm, respectively. Importantly, the exponential character of the rapid decrease in the phosphorylation signal with the distance from the cell membrane, $d=L(1–x)$, does not depend on the specific activity and kinetics of the membrane kinase, provided that the phosphatase is far from saturation.

This analysis suggests that unless there is an additional mechanism (besides slow protein diffusion) for signal propagation through the MAPK cascade, the levels of activated MEK and ERK will drop precipitously in the cell interior. At distances larger than several μm from the plasma membrane, the phosphorylation signal should decrease to subthreshold levels, provided that the cytosolic phosphatase activity is sufficiently high. Since eukaryotic cell radii vary from 5 to 50 μm, we conclude that propagating a message from the plasma membrane to the nucleus can require an additional vehicle besides diffusion in the cytoplasm.

A novel role of endocytosis in activation of MAPK signaling

Upon ligand binding and activation, many GPCRs and RTKs internalize *via* clathrin-coated pits. For instance, in hepatocytes over 50% of phosphorylated EGFR is transferred to early endosomes during the first 10 min after EGF stimulation (Di Guglielmo et al., 1994). Internalization takes receptor–ligand complexes and other signaling proteins from the plasma membrane and brings them inside the cell. Molecules that were not recycled back to the cell membrane are degraded in lysosomes. Although internalized GPCRs continuously recycle back to the cell surface after dephosphorylation in endosomes, a significant proportion of receptors are located internally (Koenig and Edwardson, 1997). Therefore, traditionally, clathrin-mediated endocytosis has been implicated in downregulation of signaling by plasma membrane receptors. A novel role of endocytosis in ‘turning on’ activation of the ERK cascade by cell surface receptors was first reported for the EGF receptor (Vieira et al., 1996). A conditional defect in endocytosis can be imposed by the regulated expression of a mutant form of dynamin (Dyn1-K44A), a GTPase that is required for clathrin-coated vesicle formation. In HeLa cells, this expression led to a marked decrease in EGF-induced ERK activation, whereas Shc phosphorylation was enhanced in endocytosis-defective cells. Subsequent studies have demonstrated that both GPCR- and EGFR-mediated activation of ERK is sensitive to various distinct inhibitors of clathrin-mediated endocytosis, including monodansylcadaverine, depletion of intracellular K^+ or cholesterol, cytochalasin D and a mutant dynamin (Ceresa et al., 1998; Daaka et al., 1998; Kranenburg et al., 1999; Maudsley et al., 2000; Rizzo et al., 2001; Vieira et al., 1996). Therefore, a possible mechanism of control over signal transduction may engage receptor endocytosis (Clague and Urbe, 2001; Di Fiore and De Camilli, 2001; Haugh et al., 1999). However, whereas experimental evidence points to an essential role of receptor endocytosis in the activation of MAPK cascades, the reason for the involvement of the endocytic machinery remains poorly understood (Ceresa and Schmid, 2000; Kranenburg et al., 1999; Pierce et al., 2000). Interestingly, in some cellular systems endocytosis was not required to activate ERK (for a review, see Leof, 2000).

The relationship between receptor internalization and ERK activation allows us to suggest that trafficking of signaling intermediates within endocytic vesicles may be an efficient way of propagating the signal (Kholodenko, 2002). Indeed, it was reported that the engagement of the endocytic machinery is essential for ERK activation by MEK, but not for activation of Ras (Kranenburg et al., 1999). Interestingly, the data on the subcellular distribution of activated MEK demonstrated that biphosphorylated MEK is detectable only at the plasma membrane and in intracellular vesicles, whereas the total MEK pool is cytosolic (Kranenburg et al., 1999). Endocytic trafficking of active MEK can help to avoid the formation of steep spatial gradients of phosphorylated MEK and ERK, since this mechanism overcomes the spatial separation of kinases and phosphatases within the MAPK cascade. Therefore, the endocytosis of phosphorylated MEK (or a protein complex containing activated MEK) rather than of activated receptors appears to be critical for ERK activation.

Scaffolding, cytoplasmic streaming and active transport as mechanisms facilitating signal propagation

Other mechanisms besides endocytosis can help to spread the phosphorylation signals from the plasma membrane further into the cell by preventing the formation of steep spatial gradients of phosphorylated ERK. For instance, cytoplasmic streaming, i.e. solvent fluxes brought about by the movement of cytoplasmic organelles along actin cables, can contribute to intracellular transport of activated MAPK kinases (Agutter et al., 1995). Recent evidence indicates that the MAPK cascade components can bind to scaffolding proteins, e.g. MP1 and JIP-1 in mammalian cells (for a review, see Garrington and Johnson, 1999). Dephosphorylation of the MAP kinases in scaffold complexes may be decreased or even precluded because of sterical obstructions, as was suggested by Levchenko et al. (2000).

Scaffolding may also help to deliver an entire signaling complex containing the MAP kinases to endocytic vesicles. Novel mechanisms have been discovered that link GPCRs to

MAPK activation through use of β-arrestin as a scaffold for the ERK and JNK cascades (Miller and Lefkowitz, 2001; Pierce et al., 2001). Besides its role in GPCR desensitization, β-arrestin has been shown to promote the targeting of the receptor to clathrin-coated pits. As β-arrestins can also recruit and activate Src, it is likely that the entire ERK and JNK cascades can be activated and recruited for clathrin-mediated internalization.

Recent data suggest that molecular motors can be involved in transport of signaling complexes. In fact, in nerve cells, a cargo for the molecular motor kinesin has been identified as scaffolding proteins for the JNK pathway, known as JIPs (Verhey and Rapoport, 2001). Endocytic vesicles and signaling complexes, loaded on molecular motors, can be transported along microtubules to remote cellular locations.

Long-range signaling

Retrograde transport of signaling complexes

One of the most interesting puzzles facing neurobiologists is concerned with unraveling the molecular mechanisms used by neurons to transfer signals over long distances. The survival and function of developing neurons depends on growth factors, the neurotrophins, such as nerve growth factor (NGF) and its receptor, TrkA. NGF, which is made by peripheral tissues, binds to TrkA receptors on distal axons, located up to 1 m away from the neuronal soma. How does the survival signal propagate over a remarkably long distance to the cell body in a physiologically relevant span of time? Diffusion is ruled out as a mechanism of the retrograde signaling, since it would be prohibitively slow. Indeed, we have seen above that diffusion may be insufficient even for spreading the phosphorylation signals within the cell body from the plasma membrane to the nucleus.

Several models were suggested to explain the retrograde transduction of neuronal survival signals (for a review, see Ginty and Segal, 2002). According to a widely accepted model, shortly after NGF binding to TrkA at nerve terminals, the NGF-TrkA complexes are internalized into endosomes by means of clathrin-mediated endocytosis. Signaling endosomes containing activated TrkA with associated ligand are retrogradely transported to the cell bodies. Data on blocking the NGF-TrkA transport by the dynein ATPase inhibitor (EHNA) suggest that vesicular transport can be carried out by molecular motors (Reynolds et al., 1998). In fact, Trk neurotrophin receptors were found to bind directly to a dynein light chain (Yano et al., 2001). The retrograde axonal transport was also shown to be inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, by cytochalasin D, a disruptor of actin filaments, and by the microtubule inhibitor colchicine (Reynolds et al., 1998). Taken together, these studies support a model where the retrograde transport of the NGF-TrkA complexes along axon structural elements (e.g. microtubules) can provide survival signals to neurons.

Ligand-independent waves of receptor activation

Cells have developed multiple mechanisms to overcome the problem of long-distance signaling. It was recently discovered that survival signals might not depend on retrograde transport of NGF (MacInnis and Campenot, 2002). Application of NGF, which was covalently cross-linked to beads to prevent internalization, showed that survival signals were able to reach the cell bodies unaccompanied by the ligand that initiated TrkA activation. One possibility is that activated TrkA can be internalized and retrogradely transported without NGF, implying that the active state of TrkA can be maintained in the absence of the ligand. Another possibility is that downstream TrkA targets, such as PI3K or Akt, can mediate the retrograde signal. An intriguing possibility is that activation of TrkA receptors in nerve terminals brings about an NGF-independent wave of TrkA activation, which rapidly propagates through the neuronal axon (Ginty and Segal, 2002). Indeed, ligand-independent waves of receptor (EGFR) activation, emanated from a point EGF source, were recently reported (Verveer et al., 2000).

How can waves of receptor activation emerge? A possible scenario is that an activated receptor, monomer or dimer, is capable of transphosphorylating and activating an inactive receptor molecule upon diffusive encounter, potentially resulting in lateral spread of receptor activation. In fact, spontaneous activation and signaling by overexpressed EGFR has been reported recently (Thomas et al., 2003). Let us sketch the functional implications of this local amplification assumption for the dynamics of the receptor system on the cell surface. For simplicity, a one-dimensional diffusion is analyzed, and only monomer or dimer forms of the receptor are taken into account (the model can be generalized to include monomer-dimer association/dissociation).

Given that the total receptor concentration (or surface density) is c and the concentration of activated receptors at a distance x from a ligand source is $a(x)$, the amount of newly activated receptors per unit time and volume/area will be $k{\times}a(x){\times}[c{-}a(x)]$, where the constant k is proportional to the probability of phosphorylation per diffusive encounter. Active receptors are dephosphorylated by the phosphatases at the rate $v_p(a)$. Under these assumptions, the spatial propagation of activated receptor (from a source at x=0) is described by the following equation:

$$\frac{\partial a}{\partial t} = D\left(\frac{\partial^2 a}{\partial^2 x}\right) + ka(c-a) - v_p(a)\,, \qquad (4)$$

where D is the diffusion coefficient. When the phosphatase activity is low, Equation 4 reduces to the Fisher-like equation, the first partial differential equation known to generate traveling waves (Murray, 1993). A traveling wave propagates without change of shape, i.e. the shape and speed of propagation of the front is constant over time. From Equation 4, it follows that an increase in total receptor amount (c) and a decrease in the phosphatase activity (v_p) may trigger global RTK activation. The situation in which a cell can be 'switched on' by a localized stimulus is in contrast to the current paradigm of 'restricted' signaling by RTKs activated by local growth factor stimulation reported recently for EGF

signaling in COS cells (Sawano et al., 2002). Remarkably, overexpression of EGF receptors in these same cells resulted in global EGFR activation over the entire cell surface following a localized stimulation with EGF (Sawano et al., 2002). We conclude that an initial localized stimulation can be responsible for triggering global RTK activation in cells. Such a mechanism may have implications for the pathogenesis of cancer, where RTKs are overexpressed due to mutations (Thomas et al., 2003). Studies of the dependence of traveling wave solutions on molecular mechanisms of RTK activation and kinetic parameters may help facilitate our understanding of crucial control points in tumor development.

Traveling waves of activated protein kinases

Another possible scenario of how a survival signal can spread over the axon includes traveling waves that may arise in a bistable protein kinase cascade. Bistability is a common theme in cell signaling cascades that contain a positive feedback loop, or a double-negative feedback loop (Bhalla and Iyengar, 1999; Ferrell and Machleder, 1998; Gardner et al., 2000; Thron, 1997, 1999; Tyson and Novak, 2001). A bistable protein kinase cascade can switch between two different stable states, one corresponding to a low and another to a high activity, but cannot exist in an intermediate unstable state. When the signal strength is below a given threshold, a kinase cascade, such as the Mos-MEK-p42 MAPK cascade and the JNK cascade in *Xenopus* oocytes (Bagowski and Ferrell, 2001; Ferrell, 1999), and the ERK cascade in mouse NIH-3T3 cells (Bhalla et al., 2002), remains in a low activity state. An increase in the signal above the threshold switches the cascade to a high activity state. Importantly, the cascade will remain in this high activity state even when the initial signal decreases to subthreshold values.

It was proposed that cells utilize bistable signaling circuits that would enable them to be capable of ‘all-or-none’ switching and to ‘remember’ a transient differentiation stimulus long after the stimulus was removed (Ferrell, 2002). Here we suggest that yet another role for bistable protein kinase cascades may be to support traveling waves of activated phospho-proteins, propagating signals to remote cellular locations. Bistability produces local amplification of the spreading signal, and the combination of local amplification and diffusion may generate traveling waves. In fact, traveling waves in bistable systems have been extensively studied in physics, chemistry and biology (Castiglione et al., 2002; Christoph et al., 1999; Keener and Sneyd, 1998; Shvartsman et al., 2002; Zhabotinsky and Zaikin, 1973). MAPK cascades and the PI3K/PDK1/Akt cascade coupled with the PIP3 synthesis pathway might be candidates for bistable protein kinase cascades that are capable of propagating traveling waves.

Importantly, all of the models of long-range signaling considered are not mutually exclusive. We hypothesize that multiple mechanisms of information transfer have evolved in neurons to transmit signals over long distances. Future experimental work will show if waves of activated kinases emerge in cells.

Concluding remarks

Spatio-temporal organization of mitogenic pathways analyzed here is central for understanding the control over intracellular signal transfer. A picture is emerging, in which simple diffusion has a limited role in intracellular transport of signaling complexes. Endocytosis, scaffolding, molecular motors and traveling waves of phospho-proteins appear to be involved in the propagation of signals to different cellular locations. These mechanisms control cellular decisions that determine cell fate.

Abbreviations

EGF, epidermal growth factor
EGFR, EGF receptor
ERK, extracellular signal regulated kinase
GAP, GTPase-activating protein
GDP/GTP, guanosine di/triphosphate
GPCR, G-protein coupled receptor
Grb2, growth factor receptor binding protein 2
JNK, c-Jun N-terminal kinase
MAPK, mitogen activated protein kinase
MKK, MAPK kinase
MKKK, MKK kinase
NGF, nerve growth factor
PI3K, phosphatidylinositol 3-kinase
RTK, receptor tyrosine kinase
SH, Src homology domain
Shc, Src homology and collagen domain protein
SOS, Son of Sevenless homolog protein
TrkA, the NGF receptor

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