The role of protein–protein interaction domains in signal transduction has been a topic of intensive study for the last ten years. Many of these studies have focused on the role of protein–protein interactions in signaling downstream of receptor and non-receptor tyrosine kinases. However, recent evidence has pointed to the extensive role of protein-protein interaction domains in the localization and scaffolding of signaling molecules. This Perspective series reviews the role of protein–protein interactions in localization and downstream signaling. The first Perspective in the series, by Schillace and Scott, provides an overview of targeting in signal transduction by protein kinases and phosphatases. The second in this issue, by Fanning and Anderson, explores PDZ domains, important for receptor targeting and clustering in a variety of cell systems. This series continues in the April 1999, no. 7, issue; two Perspectives discuss the role of protein–protein interactions in signaling downstream of tyrosine kinases. Clements and Koretzky highlight new insights into signaling by lymphocytes, while Virkamäki, Ueki, and Kahn examine the role of protein–protein interactions in insulin signaling and insulin resistance.

Perspective

SERIES
on protein–protein interaction domains in signal transduction

Alan R. Saltiel and Benjamin L. Margolis, Editors

This series continues on pages 767–772 and in the April 1999, no. 7, issue

Organization of kinases, phosphatases, and receptor signaling complexes

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The issue of specificity in cellular signaling has attracted the interest of many researchers for four decades. At its heart lies a rather simple question: How do the protein kinases and phosphatases that govern changes in the phosphorylation state of cellular proteins modify the correct substrate? This is a complex problem when one considers that over 2,000 protein kinases and 1,000 protein phosphatases are estimated to be present in the human genome (1). Although many effector-mediated signaling pathways employ a similar repertoire of protein kinases and phosphatases to implement their intracellular effects, somehow fidelity is retained to ensure that the appropriate intracellular responses occur. One hypothesis that has gained acceptance over the past decade is the idea that targeting of kinases and phosphatases close to their substrates is crucial to ensure tight regulation of the phosphorylation events. The basis for this postulate has developed from evidence that a molecular framework of adapter, anchoring, and scaffold proteins exists to maintain kinases and phosphatases in defined subcellular compartments (2). Evidence is now also accumulating in support of the clustering and organization of receptors and channels with intracellular signaling cascades. This Perspective will highlight the importance of protein domains in the compartmentalization of signaling enzymes close to their activators and targets, with a focus on kinase-mediated signal transduction.

Scaffolding of MAP kinase cascades. A classic example of kinase anchoring is the organization of components involved in the mitogen-activated protein (MAP) kinase cascades (Fig. 1*a*). For example, in the budding yeast *Saccharomyces cerevisiae*, the pheromone mating response is initiated through G protein–linked activation of the kinase Ste20. This leads to stimulation of a MAP kinase cascade in which Ste11 phosphorylates and activates Ste7, which in turn phosphorylates and activates the MAP kinase homologs Fus 3 or Kss1 (3). This signaling pathway can be tightly controlled because each of the component enzymes is physically associated with a scaffold protein called Ste5. Through this type of organization, many of the early components in the pheromone mating pathway are localized together. This probably facilitates the rapid transduction of signals through the complex and also may ensure that there is a segregation of this MAP kinase module from related signaling cascades in yeast. This view is supported by the identification of a second yeast scaffold protein called Pbs2 that coordinates components of the osmoregulatory pathway. The transmembrane osmosensor Sho1 is coupled to a MAP kinase cascade containing Ste11, Pbs2, and the MAP kinase Hog1. Pbs2 serves a dual function in this pathway as a component of the MAP kinase cascade and as the scaffold protein that maintains the signaling complex (4). The importance of Pbs2 and Ste5 in segregating signaling specificity is emphasized by the observation that although the pheromone mating and osmosensory pathways share a common kinase component, Ste11, they show no crosstalk.

Recently, parallel levels of organization have been proposed for mammalian MAP kinase cascades (Fig. 1*b*). The identification of JIP-1, a scaffold protein that binds the kinases MKK7, MLK1, and JNK *in vitro*, suggests that mammalian kinase cascades are scaffolded in a manner

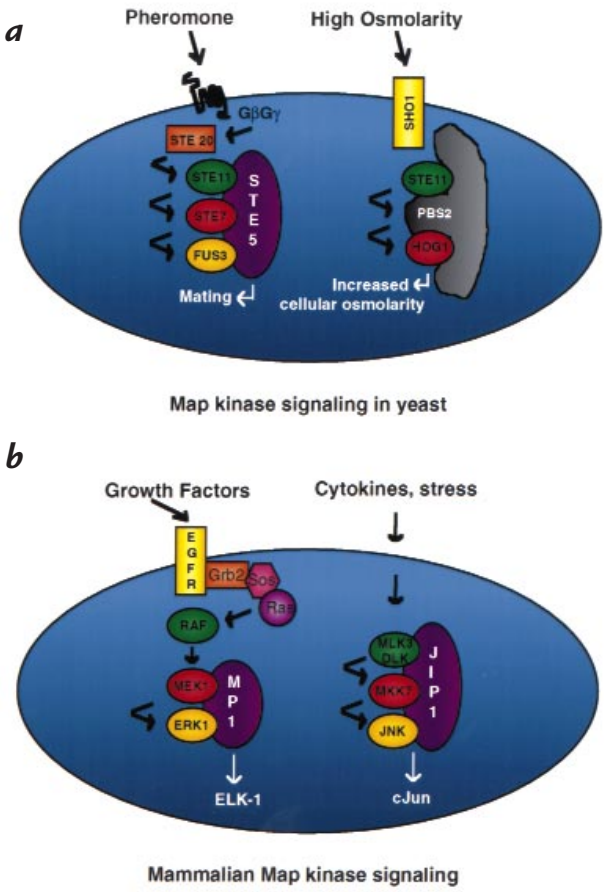

Figure 1
MAP kinase scaffolding. A schematic representation of proteins involved in scaffolding yeast and mammalian MAP kinases. (***a***) The yeast pheromone mating response is initiated through G protein–linked activation of the kinase Ste20. This leads to stimulation of a MAP kinase cascade composed of Ste11, Ste7, and Fus3, all of which are bound to the scaffold Ste5. Osmotic regulation results from activation of the receptor SHO1, which initiates a phosphorylation cascade of Ste11, Pbs2, and HOG1. The signaling molecules are targeted to SHO1 through interactions with Pbs2. This cascade prompts the cell to increase cellular osmolarity in response to external conditions. (***b***) Localization of adapter proteins, such as GRB2, with signaling molecules mediates growth factor stimulation of the RAF/MEK/ERK kinase cascade, which is scaffolded through interactions with the adapter protein MP1. Additionally, JIP-1 sequesters MLK3/DLK, MKK7, and JNK for response to cytokine or stress signaling. *MAP*, mitogen-activated protein.

similar to those in yeast. Each kinase binds to a distinct region of JIP-1, and overexpression of the scaffold protein specifically enhances the transduction of signals through the pathway (5). Mammalian MAP kinase pathways may also be ordered by the adapter protein MP1. MP1 enhances activation of the classical MAP kinase cascade by bringing together MEK1 and ERK1. Overexpression of MP1 favors the formation of MEK1/ERK1 complexes with a concomitant increase in ELK1 transcription, a commonly used index of MAP kinase activation (6). MP1 seems to represent a novel class of MAP kinase adapter protein, as it only binds two members of the kinase cascade, whereas JIP-1, Pbs2, and Ste5 organize and segregate complete MAP kinase pathways. As yet, it is unclear what the functional role of MP1 may be, but it has been proposed to facilitate interactions specifically involving MEK1. It will be of interest to establish whether MP1 has other binding partners.

PKA anchoring proteins. The spatial organization of the cAMP-dependent protein kinase (PKA) is another well-known example of kinase anchoring. Upon binding of cAMP to the tetrameric PKA holoenzyme, the active catalytic (C) subunits are released from the regulatory (R) subunit dimer and are free to phosphorylate substrates in their vicinity. One of the regulatory mechanisms in place to restrict the movement of C subunits and prevent nonspecific phosphorylation events is the subcellular localization of the PKA holoenzyme through association with A-kinase anchoring proteins (AKAPs) (7). The AKAPs are a functionally related family of 30 or so proteins that are classified on the basis of their ability to bind the R subunits of PKA. Although there is little sequence similarity between individual anchoring proteins, each AKAP contains a region of approximately 24 residues responsible for binding the R-subunit dimer. Structural studies suggest that AKAP sequences adopt an amphipathic helical conformation that inserts into a hydrophobic pocket formed by the R-subunit dimer (8). Numerous side-chains participate in the tight packing of the AKAP and R-binding surfaces and may explain why AKAP peptides constitutively bind PKA with nanomolar affinities. Accordingly, AKAP peptides are ideal antagonists of PKA anchoring inside cells and have been used to demonstrate that compartmentalized pools of PKA modulate cAMP-responsive events. For example, cardiac and skeletal muscle L-type calcium channel activity is enhanced by PKA (9). These effects are inhibited by an anchoring inhibitor peptide that is patterned after the conserved AKAP sequence and uncouples PKA localization. Similar experiments have implicated a role for anchored pools of PKA in the modulation of smooth muscle calcium-activated potassium channels, kidney ROMK channels, and neuronal AMPA/kainate glutamate receptors (9, 10).

Targeting of the PKA holoenzyme to discrete intracellular environments is the defining feature of each AKAP. This is achieved through specialized targeting signals that maintain protein–lipid and protein–protein interactions with ligands at defined intracellular sites. The intracellular locations of some well-characterized AKAPs are presented in Fig. 2. For example, membrane attachment of a small-

molecular-weight anchoring protein, called AKAP15/18, is mediated through myristoylation and dual palmitoylation signals (11). Members of the AKAP-KL family are attached to the actin cytoskeleton at the apical membrane in epithelial cells, whereas sAKAP84/DAKAP1 contains a mitochondrial targeting sequence (12, 13). Recently, a protein called yotiao, which binds to the cytoplasmic tail of NMDA receptors containing the NR1/1A subunit, has also been identified as an AKAP. Interestingly, yotiao binds the type I protein phosphatase (PP1), thereby maintaining a signaling complex of two enzymes with opposing actions that are physically associated with their substrate. Functional experiments indicate that anchored PP1 is active and limits channel activity. PKA activation overcomes the phosphatase to confer rapid cAMP- enhancement of NMDA receptor currents. Thus, a recurring theme in PKA-mediated signal transduction is the colocalization of kinases and phosphatases in signaling complexes. This is further exemplified by AKAP79, which coordinates the location of PKA, PKC, and protein phosphatase 2B (calcineurin) at the postsynaptic densities of neurons, and by AKAP220, a vesicle-associated anchoring protein that binds PKA and PP1 (14). The clustering of kinases and phosphatases furnishes an additional level of control to the coordination of signaling events by positioning the enzymes close to selected substrates, where they can respond to signals generated by activators. There is no doubt that additional AKAP signaling complexes remain to be identified.

Protein domains involved in targeting. Often the targeting of kinases and phosphatases is mediated by specialized domains or sequences that interact with other proteins. Perhaps the best understood example is the Src homology 2 domain (SH2), which is found in many protein tyrosine kinases and signaling components of growth factor–mediated transduction cascades. The SH2 is a module of approximately 100 amino acids that binds to specific phosphotyrosine sequences on its partner proteins, often providing a physical link to the activated growth factor receptor (15). Other domains that facilitate protein–protein interactions of signaling molecules include PTB domains, which also bind phosphotyrosine sequences, and src homology 3 (SH3) and WW domains, which bind proline-rich sequences. Often, signaling proteins contain more than one of these protein modules; for example, the adapter protein Grb2 contains two SH3 domains and an SH2 domain. Simultaneous association of Grb2 or its relatives with multiple binding partners permits the formation of oligomeric signaling complexes containing both protein tyrosine kinases and protein tyrosine phosphatases (Fig. 1*b*). The involvement of these complexes in insulin signaling and diabetes is the focus of another Perspective in this series and therefore will not be discussed further here.

Coupling receptor activation to signaling cascades is essential for the transmission of intracellular signals. Ample research has now demonstrated that another protein module, the PDZ domain, coordinates the location of transmembrane proteins with cytoskeletal elements and signaling enzymes (16). The PDZ domain takes its name from the three proteins in which it was originally identified: PSD-95, at the postsynaptic density, Discs Large, of *Drosophila* imaginal discs; and ZO-1, of epithelial tight junctions. PDZ proteins often contain multiple PDZ domains that permit simultaneous association with several proteins via recognition of the COOH-terminal motif E(S/T)DV. The role of PDZ domains in epithelial cells will be the focus of another essay in this series. It is appropriate, however, for this Perspective to discuss briefly the contribution of PDZ interactions to the localization of certain second messenger–regulated kinases and phosphatases and the function of PDZ domains in neuronal receptor clustering and targeting.

In neurons, the AMPA-type glutamate receptor ion channel is in part regulated by the type 1 protein phosphatase (PP1). This phenomenon has recently been associated with a PDZ protein called spinophilin, which binds PP1 and is proposed to colocalize with the AMPA receptor in the postsynaptic membranes of neostriatal neurons (17). EBP50/NHERF is a PDZ protein involved in regulation of sodium–hydrogen transport in the apical membrane of epithelial cells. Two studies demonstrate that EBP50/NHERF binds to the cystic fibrosis transmembrane conductance regulator (CFTR) and ezrin, a cytoskeletal com-

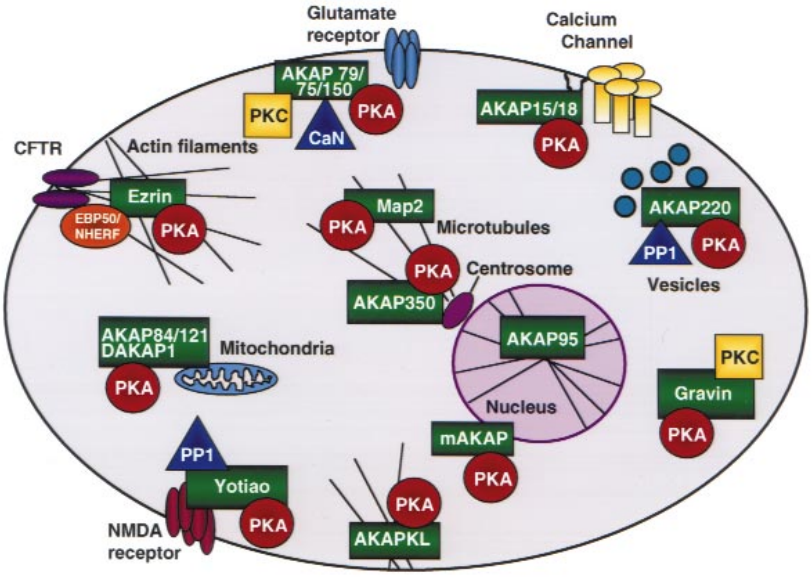

Figure 2
A schematic representation of the subcellular localization of AKAPs. The localization of PKA to different cellular compartments is mediated through interactions with AKAPs. A selection of AKAPs, the signaling molecules that they bind, and their subcellular location are depicted here. The targeting of mAKAP to the perinuclear membrane is a recent observation from our lab. See text and ref. 1 for details on targeting of the remaining AKAPs pictured here. *AKAPs*, A-kinase anchoring proteins.

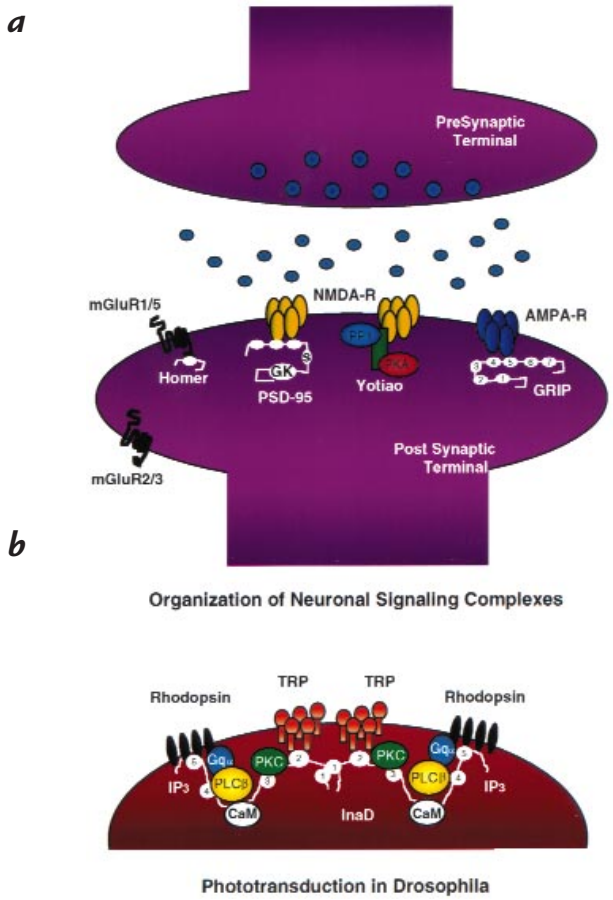

Figure 3

Localization of signaling complexes with channels and clustered receptors. (***a***) A cartoon representation of neuronal receptor clustering and regulation. PDZ domain–containing proteins are responsible for clustering several types of glutamate receptors (see text for details). Evidence is now also accumulating in support of the regulation of receptor activity through targeted kinases and phosphatases, as illustrated by the interaction of the AKAP yotiao with PKA, PP1, and the NR1 subunit of the NMDA receptor. (***b***) The targeting and clustering of receptors and channels with intracellular signaling molecules responsible for phototransduction in *Drosophila* eye. The PDZ domains of InaD target PLC-β, PKC, and calmodulin to the membrane and cluster the TRP channels with these signaling molecules. Homomeric and heteromeric InaD interactions facilitate the formation of this transducisome complex.

ponent that has been proposed to anchor PKA (18,19). Because PKA phosphorylation has been implicated in regulation of CFTR, these data indicate a potential role for anchored PKA via an ezrin/EBP50 complex. These studies suggest that CFTR may be compartmentalized in a complex with its intracellular effector, PKA.

An elegant series of neurobiological experiments have demonstrated that glutamate receptors and other ion channels also are organized through PDZ interactions at the postsynaptic membranes of neurons (20). A main player in this system is PSD-95, a member of the MAGUK family of PDZ proteins. PSD-95 contains three PDZ domains, a SH3 domain, and a guanylate kinase–like sequence. The first and second PDZ domains of PSD-95 bind the NMDA receptor subunit NR2B and the potassium channel Kv 4.1. Ion channel clustering occurs through homomeric PSD-95 interactions. It has been further proposed that stabilization of these clusters may be mediated through an adapter protein called CRIPT, which binds the third PDZ domain of PSD-95 and may tether the complex to the neuronal cytoskeleton. PSD-95 also organizes signaling molecules such as neuronal nitric oxide synthase (nNOS), an enzyme that catalyzes the formation of the second messenger nitric oxide in response to Ca^{2+} entry across the postsynaptic membrane. Simultaneous binding of PSD-95 to NMDA receptors and nNOS may provide a scaffold to bring the enzyme close to its calcium source. Furthermore, PSD-95 may participate in the regulation of Ras-controlled signaling pathways through binding to a Ras–GTPase activating protein called SynGAP (21).

In addition to being regulated by spinophilin and PP1, the AMPA-sensitive glutamate receptors (GluR2 and GluR3) are clustered at postsynaptic sites through association with other PDZ domain–containing proteins (Fig. 3*a*). For example, GRIP is an adapter protein with seven PDZ domains, the fourth and fifth of which interact with the AMPA receptor subunits, leaving the remaining domains free to interact with signaling molecules or cytoskeletal components (22). Likewise, the G protein–linked metabotropic glutamate receptors, mGluR1 and mGluR5, bind Homer, a scaffold protein with a PDZ-like domain. Homer also binds phospholipase C (PLC) and may regulate phosphoinositide signaling at excitatory synapses (23).

A particularly sophisticated example of the involvement of PDZ domains in signaling complexes is the InaD transducisome in *Drosophila* eye phototransduction (Fig. 3*b*). The InaD (inactivation no-afterpotential) protein contains five PDZ domains that interact with various signaling partners, including calmodulin, PLC-β, the eye isoform of protein kinase C, and the transient receptor potential protein (TRP) store–operated calcium channel. Light-stimulated rhodopsin activates PLC-β through Gαq, which induces channel activity via IP3. The channel is then inactivated by PKC phosphorylation. Thus, InaD functions to rapidly activate phototransduction through stimulation of the TRP channel by sequestering signaling enzymes with their activators and substrates. Importantly, the reverse process is also favored, as InaD maintains PKC close to TRP. It appears that the interactions in this complex are not simple, but are multivalent, forming a web of signaling molecules and receptors that functions to regulate phototransduction through the speed of activation and feedback regulation (24, 25).

Conclusions and future directions. Now that many adapter, anchoring, and scaffolding proteins are known, the challenge facing researchers is to define how these interactions are regulated and what functional advantages are provided by multienzyme signaling complexes. Thus far, the formation and breakdown of these complexes appear to be regulated by a variety of mechanisms, but phosphorylation is implicated in several cases. For example, SH2 domains and most PTB domains bind after tyrosine phosphorylation of their binding partners, illustrating how extracellular signals can promote the assembly of multiprotein complexes when and where they are required. Second messengers,

however, can promote the disassembly of such complexes, as illustrated by the uncoupling of the inwardly rectifying potassium channel Kir 2.3 from PSD-95 by PKA phosphorylation of a serine within the PDZ recognition motif (26). SH2, PTB, and PDZ domains recognize short sequences of only four to six amino acids; therefore, signaling events that modify these acceptor sequences may have profound effects upon complex formation. It is likely that the development of small molecules that perturb these interactions will have some therapeutic potential, in addition to advancing knowledge about the regulation of receptor and signal cascade targeting. Finally, we envision that many future advances in our understanding of the functional implications of signaling complexes and receptor targeting will arise from genetic manipulations. This view is supported by recent analysis of mice strains engineered to produce a mutant PSD-95 protein. NMDA receptor clustering is normal, but the mutant PSD-95 is unable to interact with the neuronal cytoskeleton or engage downstream signaling pathways. Electrophysiological and behavioral studies show that certain cognitive processes of the mutant mice, such as increased long-term potentiation (LTP) and aberrant spatial learning, are altered (27). We therefore anticipate that transgenic and targeted gene disruption experiments will have an increasingly important role in developing our understanding of the functional importance of multiprotein scaffolding in the process of signal transduction.

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