Current Biology, Vol. 12, R32–R40, January 8, 2001, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(01)00646-7

Signaling Complexes : Junctions on the Intracellular Information Super Highway

Review

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An enigmatic yet fundamental principle of signal transduction is that parallel signaling pathways assembled from a common repertoire of enzymes are able to propagate diverse physiological responses. A key feature of such a mechanism is that separate signaling pathways are organized into localized transduction units, each tailored to respond optimally to a particular signal. Protein–protein interactions maintained by anchoring, adapter and scaffolding proteins provide the molecular glue that holds these signal transduction units together. A major objective of the signaling community is to ascertain how signals flow through compartmentalized transduction units that contain transmembrane receptors, protein kinases, phosphatases and their substrates.

Introduction

Recent advances in our understanding of cell signaling have highlighted the complexity of biological information processing. While many components of these signaling cascades are well known, it is still unclear how multiple signaling pathways interact with each other and how this communication is regulated. Increasingly, attention is shifting towards understanding the subcellular organization of signaling enzymes. In this review, we will discuss the emergence of 'signal-directing' molecules that target the activity of broad specificity protein kinases and phosphoprotein phosphatases to predetermined cellular microenvironments. We will also discuss the use of kinase scaffolds in the segregation of parallel signal transduction pathways that emanate from G-protein coupled receptors. Finally, we will consider the implications of these findings for future research directions that may further elucidate the cellular role of these signaling complexes.

Signaling Networks and Compartmentalization

A cellular response to hormone action or growth factor stimulation is often the engagement of signaling complexes that include modulatory proteins, signaling enzymes and their substrates. The integrity of these multienzyme complexes is often sustained by adapter or scaffolding and anchoring proteins [1]. A variety of binding partners can interact with these organizing molecules, making it possible to construct intricate signaling complexes from a simple set of building blocks. Several recent reviews provide insightful perspectives on the roles of signaling complexes maintained by these modular adapter proteins in the control of cellular processes [2–6]. This article will highlight the added levels of complexity that are conferred by the compartmentalization of these signaling networks. Once tethered to distinct sites, kinases and phosphatases gain a measure of selectivity in their action due to their restricted access to a subset of substrates. Subcellular localization provides a means of spatially segregating broad specificity kinases and phosphatases to avoid indiscriminate phosphorylation and dephosphorylation events [7].

Organization of MAP Kinase Pathways

Signaling complexes facilitate the processing of signals that are directed through Mitogen Activated Protein (MAP) kinase cascades. Each transduction unit is composed of three sequentially activated protein kinases: an upstream MAP kinase kinase kinase (MKKK), an intermediate MAP kinase kinase (MKK) and a terminal MAP kinase. Scaffolding proteins are believed to organize each consortium of enzymes to facilitate and focus the rapid transfer of signals from one kinase to the next. In the budding yeast *Saccharomyces cerevisiae*, distinct MAP kinase cascades are assembled from a limited repertoire of enzymes to control diverse processes such as mating, osmoregulatory sensing and pseudohyphal growth [8]. Genetic and biochemical approaches show that a scaffolding protein called Sterile 5 (Ste5) organizes the yeast pheromone response pathway consisting of Sterile 11, Sterile 7 and the MAP kinases Kss1 or Fus3 [9–11] (Figure 1). Activation of the terminal kinase causes specific changes in gene expression and cell cycle arrest that are a prelude to cell–cell fusion [11]. Recent data suggest that there are additional levels of regulation within the Ste5 signaling complex, as kinase units that contain Fus3 selectively phosphorylate the substrate protein Far1 [12]. Furthermore there may be crosstalk between parallel signaling scaffolds as Ste5-associated Fus3 seems to attenuate Kss1 kinase activity in another scaffold [13] (Figure 1).

These sophisticated mechanisms of molecular organization in yeast MAP kinase cascades highlight the usefulness of scaffolding proteins for organizing distinct combinations of enzymes from a pool of common

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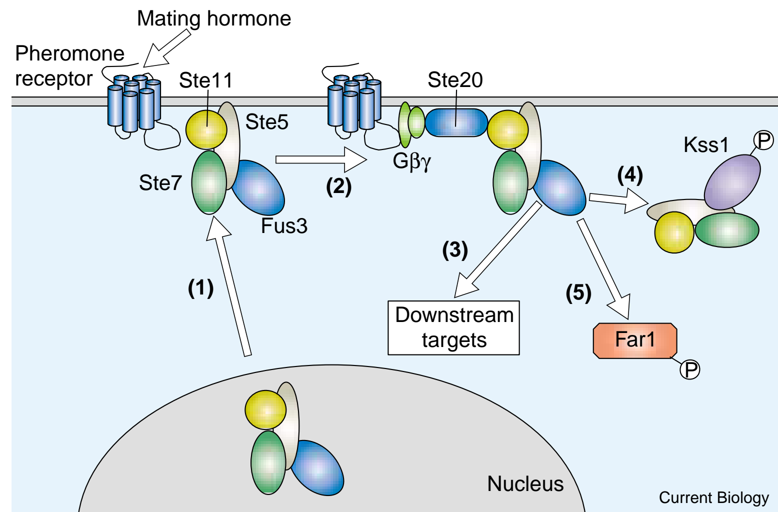

Figure 1. Pheromone Activation of Yeast MAP Kinases.

This process involves multiple stages. (1) Binding to cell surface GPCRs results in translocation of the Ste5 kinase scaffold from the nucleus to the plasma membrane. (2) The proximal kinase in the Ste5 kinase scaffold, Ste11, is activated upon recruitment of Gβγ subunits and another protein kinase called Ste20. (3) Signals relayed through the Ste5 kinase scaffold to the terminal enzyme Fus3 lead to the phosphorylation of various downstream substrates. (4) One example is the phosphorylation of scaffolds containing Kss3 to attenuate other MAP kinase cascades that regulate filamentous growth. (5) Phosphorylation of another substrate, Far1, may also contribute to the inhibition of filamentous growth. The name of each component kinase and scaffolding protein is shown.

components. For example, Ste11 also functions as the MKKK in the high osmolarity response pathway where the MKK Pbs2 acts both as the scaffolding protein and the intermediate kinase that signals to the downstream MAP kinase Hog1 [8] (Figure 2). Evidence that the same enzyme, Ste11, is a component of the pheromone response and high osmolarity response pathways shows the importance of kinase scaffolds as a mechanism to confer specificity on the signals relayed through individual MAP kinase cascades.

In mammalian cells, the JIP family of scaffolding proteins coordinates activation of the Jun kinase cascade, whereas a scaffolding protein called MP-1 may increase the efficacy of signaling through the ERK1/2 pathway [14–17]. Although both proteins undoubtedly provide platforms for the assembly of kinase cascades, it is clear that other interactions between the component kinases further contribute to the integrity of each complex. Association of MAP kinase cascade components with scaffolding proteins may regulate the responsiveness of the cascade to activating stimuli. Nihalani *et al.* [18] have shown that JIP maintains DLK, an upstream kinase in the JNK pathway, as an inactive, monomeric enzyme. Activation of this enzyme requires recruitment of the terminal kinase, JNK, into the scaffold. This event releases DLK from the scaffold to permit its dimerization, autophosphorylation and activation of the soluble enzyme within the vicinity of the scaffold [18]. Presumably, activated DLK is then able to activate MKK7, the next enzyme in the cascade. This model proposes that recruitment of the terminal kinase JNK is required to induce activation of upstream enzymes that are components of a preassembled and inactive JIP scaffold. Interestingly, other MAP kinase scaffolding proteins, such as KSR, may act in a similar manner as activated MAPK is recruited into the complex upon cascade activation [19,20].

Signaling through MAP kinase cascades is terminated in part by a growing class of dual specificity phosphatases whose members dephosphorylate phosphothreonine and phosphotyrosine residues at the active site of various component kinases [21]. Initial studies show that protein–protein interaction with their substrates results in catalytic activation of certain dual specificity phosphatases [21]. Although it is not yet clear whether the phosphatases associate with MAP kinase scaffolding proteins such as JIP, MP-1 or KSR, the recruitment of phosphatases into these scaffolds could provide an efficient mechanism to terminate signaling through each cascade. Presumably, dephosphorylation of the MAP kinases is also regulated by conventional serine/threonine and tyrosine phosphatases [21,22], and it is possible that certain MAP kinase scaffolds will bind these enzymes as well.

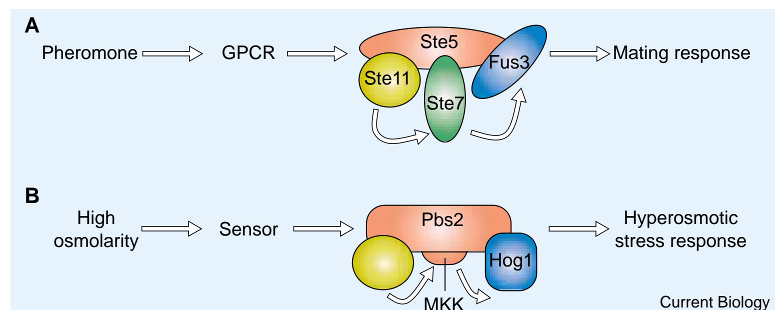

Figure 2. MAP Kinase Pathways in Yeast.

Distinct MAP kinase pathways in yeast have different protein scaffolds. This allows use of the same component in multiple pathways. (A) The pheromone response pathway is composed of the MKKK Ste11, the MKK Ste7 and the MAP kinase Fus3. These enzymes form a signaling complex with the scaffold protein Ste5 at its core. (B) Pbs2 organizes the hyperosmotic stress response pathway through its association with Ste11 and Hog1. The scaffolding proteins, Ste5 and Pbs2, and the component kinases are shown.

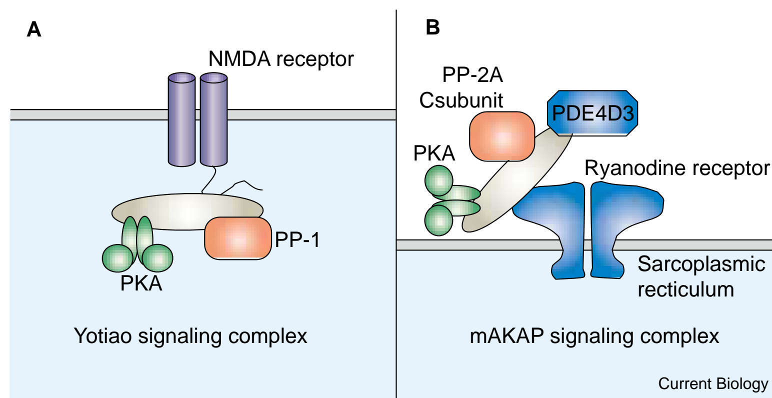

Figure 3. AKAP Signaling Complexes.

A recently appreciated role for AKAPs is the simultaneous targeting of signal transduction and signal termination enzymes. (A) In neurons, Yotiao maintains a signaling complex of active phosphatase PP-1 and PKA that is physically linked to a substrate for both enzymes, the NR1 subunit of the NMDA receptor ion channel. (B) In cardiac myocytes, hypertrophic stimuli induce expression of the muscle selective anchoring protein mAKAP. This leads to the assembly of a signaling complex including PKA, the phosphodiesterase PDE4D3 and the phosphatase PP-2A that is targeted to a substrate, the ryanodine receptor ion channel.

A-Kinase Anchoring Proteins

A-kinase anchoring proteins (AKAPs) are examples of proteins that compartmentalize signaling complexes at precise subcellular sites [23]. Members of this family of functionally related proteins bind to regulatory (R) subunit dimers of cAMP-dependent protein kinase (PKA) holoenzymes through a common amphipathic helix motif of 14–18 residues [23–25]. Unique targeting domains on each anchoring protein direct PKA–AKAP complexes to specific subcellular compartments, thereby providing a mechanism that positions PKA to respond to localized changes in cAMP concentration. Another important function of AKAPs is to assemble signaling complexes containing multiple kinases, phosphatases and regulatory proteins. For example, AKAP79 targets PKA, PKC and protein phosphatase 2B (PP-2B/calcineurin) to neuronal plasma membranes and synaptic sites [26]. Functional studies have revealed the importance of the spatial organization of PKA provided by this AKAP signaling complex. Rosenmund *et al.* [27] initially showed that a peptide encompassing the R subunit binding site in the human thyroid anchoring protein, Ht31, antagonizes PKA anchoring inside cells. Perfusion of this peptide into cultured hippocampal neurons causes a rundown of synaptic AMPA type glutamate receptor activity with a time course that is similar to the inhibition of the kinase. Further analyses have shown that the AKAP79 signaling complex is recruited into a larger transduction unit with its substrate, GluR1, through interaction with a bridging protein, the membrane associated guanylate kinase SAP97 [28].

Functional studies show that AKAP79-bound PKA enhances GluR1 phosphorylation on serine 845 in the cytoplasmic tail of the receptor. Serine 845 is an important site for regulation of channel function during the induction of long term synaptic depression, and its phosphorylation state is regulated by PKA *in vivo* [29–31]. These data suggest that AKAP79 plays an important role in the tight coupling of PKA to a substrate, GluR1, and show the importance of compartmentalization of a scaffold and its associated proteins within a restricted subcellular domain. Subsequent experiments have defined a functional role for other AKAP signaling complexes and PKA anchoring in a variety of cAMP dependent events [32,33].

Bidirectional Regulation of Signaling by AKAP Complexes

A valuable feature of some signaling complexes is the presence of signal transduction and signal termination enzymes in the same network. This creates focal points of enzyme activity where the bidirectional regulation of signaling events can be controlled and the phosphorylation status of target substrates is precisely regulated. A common scenario is the clustering of protein kinase and phosphatase activities. For example, Yotiao, a splice variant of the *AKAP450* gene, binds PKA and protein phosphatase 1 (PP-1). Yotiao also interacts with the NR1a subunit of synaptic NMDA glutamate receptor ion channels, which are regulated by PKA and PP-1 [34–38] (Figure 3). The modulation of NMDA receptors containing NR1a requires interactions with the scaffolding protein as peptide mediated displacement of either PKA or PP-1 causes changes in the modulation of channel activity [35]. AKAP450/CG-NAP, a large centrosomal AKAP of unknown function, has been reported to bind three kinases, PKA, PKC and PKN, and two phosphatases, PP-1 and PP-2A [39,40]. Likewise, the simultaneous association of PP-1 and PKA with anchoring proteins such as AKAP149/D-AKAP-1 and AKAP 220 undoubtedly will contribute to the bidirectional regulation of phosphorylation events at mitochondria, endoplasmic reticulum and vesicular organelles [41,42]. Thus, the possibilities for coordinated phosphorylation and dephosphorylation events mediated by the enzymes associated with AKAPs are numerous.

Two recent reports [43,44] show that phosphodiesterases (PDEs), the enzymes that catalyze cAMP metabolism, are present in signaling complexes with the cAMP dependent protein kinase. These findings add a novel twist to PKA regulation as they show that an anchored pool of phosphodiesterase may tightly control local cAMP levels. Dodge *et al.* [43] found that the muscle selective mAKAP directly binds PKA and a splice variant of the cAMP-specific, type 4

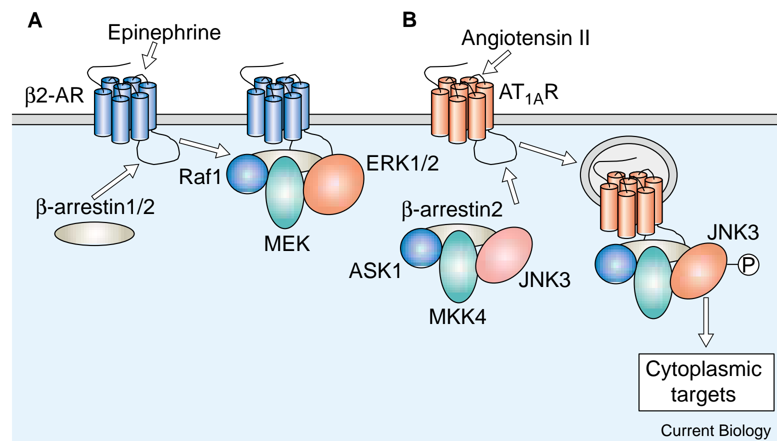

Figure 4. β-Arrestins Function as Mammalian MAP Kinase Scaffolds.

Accumulating evidence suggests that a secondary function of β-arrestins may be to facilitate the assembly of MAP kinase scaffolds in response to G-protein coupled receptor-signaling events. (A) Upon activation of the β2 adrenergic receptor, β-arrestin 1 and 2 act as scaffolds for the component kinases of the ERK1/2 MAP kinase cascade. (B) β-arrestin 2 constitutively binds a JNK kinase module consisting of ASK1, MKK7 and JNK3 that is responsive to AT_{1A} angiotensin receptors. The receptor–kinase scaffold is internalized on clathrin coated vesicles along with active JNK3. The component kinases are shown.

phosphodiesterase, PDE4D3 (Figure 3). Simultaneously, Tasken *et al.* [44] reported the interaction of PDE4D3 with AKAP450, a large centrosomal AKAP in testicular Sertoli cells. Biochemical and immunofluorescent analyses show that both enzymes are constitutively associated with the centrosomes during interphase of the cell cycle [44]. The implications of both studies are that the role of PDE4D3 within these complexes is to depress cAMP levels within the vicinity of anchored PKA. At rest, PDE4D3 inhibits basal PKA activity associated with mAKAP, possibly acting to dampen noise and increase gain in the system. Furthermore, PKA phosphorylation is known to up-regulate PDE4D3 activity 2–3 fold, establishing a negative feedback loop that rapidly terminates the cAMP signal [45,46]. It would appear that the close proximity of PDE4D3 and PKA in the mAKAP signaling complex facilitates this process as peptide mediated displacement of the kinase from the signaling complex prevents the phosphorylation and up-regulation of the phosphodiesterase [43].

Although PDE4D3 is a substrate for the kinase, it is clear that there are other PKA substrates associated with the mAKAP scaffold (Figure 3). For example, the regulation of ryanodine receptor (RyR) phosphorylation is important for maintaining contractility in response to β-adrenergic signaling and increases in intracellular Ca^{2+} concentration in the heart. Hyperphosphorylation of sarcoplasmic reticulum RyR leads to increased Ca^{2+} sensitivity of the channel and decreased sensitivity to β-adrenergic stimulation [47–49]. These changes are manifest in human heart tissue undergoing heart failure where changes in RyR phosphorylation are also detected [47]. The abnormal regulation of RyR function may be due to several factors that regulate cAMP/PKA signaling in heart, including loss of phosphatase activity from the RyR complex [47] and defects in regulation of cAMP levels by PDE activity associated with the complex [43] (Figure 3). Thus, the composition and assembly of this signaling network may be altered in disease states.

New Roles for Arrestins as Scaffolding Proteins

The β2 adrenergic receptor (β2-AR) is the prototypic example of a G protein-coupled receptor (GPCR) that transiently elevates intracellular cAMP through the stimulation of adenylyl cyclase activity. However, persistent β2-AR activation also leads to receptor desensitization and internalization through a process that involves phosphorylation of the cytoplasmic tail of the receptor by G protein-coupled receptor kinases (GRKs) and the recruitment of arrestins [50]. Although one function of arrestins is to promote desensitization, it is now evident that arrestin binding also favors the assembly of β2-AR signaling complexes and the nucleation of clathrin cages to mediate endocytosis [50]. β2-ARs, like many other GPCRs, form higher order receptor complexes which signal to mammalian MAP kinase cascades [50]. Lefkowitz and colleagues ([51–53] and references therein) have shown that β2-AR nucleates an endocytic signaling complex including β-arrestin, Src, and EGF receptors that signals to the ERK1/2 cascade (Figure 4). Internalized protease-activated receptors are present in a macromolecular complex that includes β-arrestin 1, Raf-1 and activated ERKs [54]. Although the molecular mechanisms that link each receptor to MAP kinase activation are not precisely defined, the arrestins are key players in this process.

A further example of arrestin mediated scaffolding was recently provided by McDonald *et al.* [55] who showed that β-arrestin 2 maintains a c-Jun N-terminal kinase (JNK) kinase module of ASK1, MKK4 and JNK3. Cell based studies suggested that sequestration of JNK3 and ASK1 by their simultaneous association with β-arrestin 2 enhances activation of JNK3 [55] (Figure 4). Importantly, β-arrestin 2 enhances JNK3 activity upon application of angiotensin II, showing that cascade activation is responsive to a GPCR ligand (Figure 4). This property is reminiscent of the Sterile 5 complex in yeast where occupancy of G protein-coupled pheromone receptors leads to the translocation of the entire Ste5-associated MAP kinase cascade to the membrane [56,57]. Other

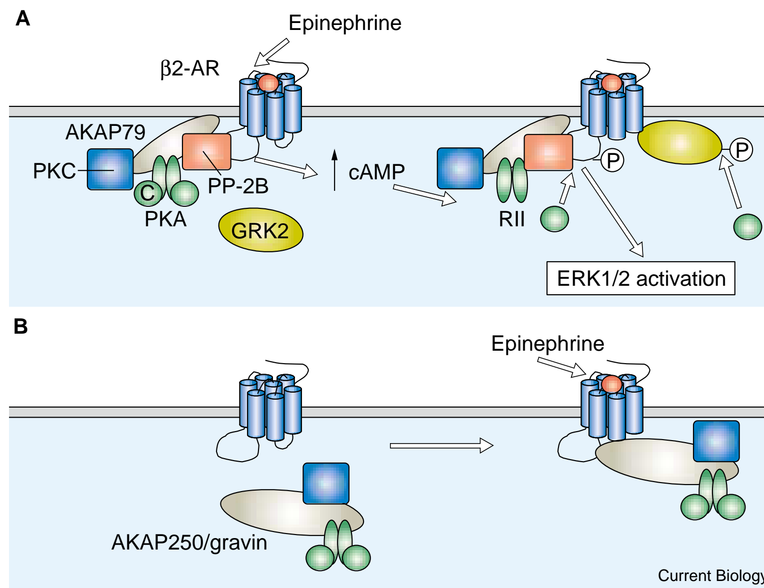

Figure 5. AKAP Mediated Regulation of β2-AR Signaling.

Two AKAPs nucleate signaling complexes that are assembled on β2-ARs. (A) Agonist independent association of the AKAP79 signaling complex through interactions with sites within the third intracellular loop and carboxy-terminal tail of the β2-AR. Phosphorylation of the third cyto-plasmic loop of the β2-AR (3a) results in activation of the ERK1/2 kinase via a Gαi coupled mechanism [58]. Anchored PKA also phosphorylates GRK2 kinase resulting in increased association of GRK2 with the plasma membrane and enhanced receptor phosphorylation to stimulate β2-AR desen-sitization. (B) Receptor occupancy recruits the AKAP250/gravin–PKA complex through interactions with the carboxy-terminal tail of the β2-AR.

similarities to yeast MAP kinase scaffolds include the ability of individual arrestins to interact with distinct MAP kinase cascades. For example, β-arrestin 1 and 2 recruit members of the ERK cascade upon agonist stimulation [51,52,55], yet β-arrestin 2 constitutively associates with members of the JNK cascade [55] (Figure 4). It will be important to determine if β-arrestin 2 acts as a scaffold for different MAP kinase components in a cell-type specific manner, or whether multiple arrestin–MAP kinase modules are segregated within the same cell. If the latter is the case, what are the mechanisms that allow coupling of one signal to a specific arrestin–MAPK module?

GPCR Regulation by AKAP Signaling Complexes

PKA has been implicated in the regulation of GPCR coupling to multiple G proteins [58,59]. Daaka *et al.* [58] showed that β2-ARs normally coupled to cAMP production through Gαs are also able to activate MAP kinase pathways in a pertussis toxin-sensitive manner via a mechanism that involves a switch in coupling through Gαi/o proteins. They proposed that direct phosphorylation of β2-AR by PKA enhances coupling through Gαi leading to activation of ERK1/2. Two AKAP signaling complexes have been implicated in the regulation of β2-AR function [60,61] (Figure 5). AKAP79 binds to regions within the third cytoplasmic loop and carboxyl terminal tail of the β2-AR in an agonist-independent manner and peptide disruption of PKA anchoring attenuates β2-AR mediated ERK activation in HEK293 cells [61] (Figure 5). AKAP complexes may also coordinate β2-AR phosphorylation, desensitization and recycling as AKAP250/gravin recruits PKA and PKC to the receptor in an agonist-dependent manner [50,60,62] (Figure 5). Anchored PKA enhances GRK2 mediated receptor phosphorylation and β2-AR desensitization due to enhanced membrane localization of active GRK2 [63] (Figure 5). Dephosphorylation of β2-AR and GRK2 are likely to be important signal termination events in this process. Interestingly, this function could be mediated by AKAP79, which also binds to the phosphatase PP-2B.

Implications of Compartmentalization

One surprising outcome of the human genome project is that the total number of genes expressed in an individual cell is less than was anticipated [64,65]. Yet the complexity of biological processes requires these genes to be used in different ways to meet the demand for cellular diversity. Scaffolding proteins contribute to this diversity through the assembly of signaling networks in which enzymes are compartmentalized with a subset of their substrates. There are several implications of this combinatorial model. First, diversity may be obtained by the selective assembly of signaling enzymes that associate with a multivalent anchoring protein. For example AKAP79/150 can simultaneously interact with PKA, PKC and the phosphatase PP-2B, yet theoretically seven distinct signaling complexes could be assembled that contain various combinations of these signaling enzymes. This would create a number of slightly different signaling complexes that could integrate different second messenger signals. Diversity may also be achieved by the preferential use of targeting signals that are contained within the anchoring protein. A good example is D-AKAP-1/AKAP149 which has distinct targeting signals within the anchoring protein that differentially target signaling complexes of PKA and PP-1 to the endoplasmic reticulum, mitochondria or perinuclear membranes [42,66]. Diversity in targeting can also be obtained through alternative splicing of targeting signals [67].

A second postulate is that additional diversity can be achieved through the dynamic assembly of signaling networks. This can occur on a short time scale via

the organization of existing components into a signaling network as is the case of agonist induced assembly of β2-AR signaling complexes that include β-arrestin, non-receptor tyrosine kinases and MAP kinases [52,55]. In addition, *de novo* synthesis of a scaffolding protein can provide a developmentally regulated component to the assembly of signaling complexes. This has been reported for mAKAP, expression of which is induced 10–15 fold in cardiomyocytes upon exposure to agonists that induce hypertrophy [68]. Increased mAKAP results in the assembly of a signaling complex that incudes PKA, PP-2A and the type 4 phosphodiesterase at the perinuclear membranes of hypertrophic cardiomyocytes [43,69]. Fluorescent tracking of proteins in living cells by techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) [70] is likely to extend our appreciation of the dynamics of signaling scaffolds. A particularly exciting development is the use of green fluorescent protein (GFP) tagged lipid and protein interaction domains to track dynamic associations in real time [71–74]. Likewise, the use of fluorescently coupled phospho-specific antibodies *in situ* allows the resolution of phosphorylation events inside living cells [75]. These probes allow visual detection of signaling events and can, in some cases, be used for quantitative calculations of spatial distribution and kinetics of signaling.

An equally important component of dynamic signaling networks may be the disassembly of these complexes. This could be achieved in two ways: mislocalization of the signaling scaffold in relation to its substrates, or targeted degradation of the anchoring or scaffold protein. Both forms of complex disassembly could involve protein ubiquitination pathways. An E3 ubiquitin ligase is recruited into the IκB–NFκB transcriptional complex to promote poly-ubiquitination and degradation of phosphorylated IκB. This process allows active NF-κB to translocate to the nucleus to regulate transcription of target genes [76–78]. In *Aplysia* sensory neurons, ubiquitination and degradation of PKA regulatory subunits creates a pool of free, active catalytic subunit that is important for the induction of long term synaptic facilitation [79,80]. It would be interesting to determine whether ubiquitination alters AKAP signaling complexes in *Aplysia.* Ubiquitination has been implicated in the endocytosis and degradation of pheromone receptors in yeast [81–83] and EGF receptor complexes in mammalian cells [84,85]. Shenoy *et al.* [86] have recently shown that both β2-AR and β-arrestin are targets for ubiquitination and that ubiquitination of these proteins contributes to agonist induced receptor trafficking and degradation. In addition, Marchese and Benovic [87] have shown that another GPCR, the human immunodeficiency virus coreceptor CXCR4 undergoes agonist induced ubiquitination which promotes receptor sorting to lysosomes. It is likely that future studies will identify other signaling networks that are subject to ubiquitin mediated regulation.

Future Directions

We anticipate that increased use of techniques such as mass spectrometry, genome-wide two-hybrid analysis and cell imaging will facilitate the detection and analysis of signaling complexes. The integration of these approaches has already led to the development of protein–protein interaction maps for the yeast genome [88,89] and no doubt future ventures will focus on similar analyses of mammalian genomes. When these data are combined with information regarding tissue distribution and subcellular localization of individual components it should be possible to develop a more complete picture of the composition of signaling complexes in individual cells. Genetic approaches including gene targeting, antisense ablation of gene expression and RNA interference (RNAi) [90] provide powerful tools to manipulate the expression and composition of particular signaling networks in mammalian systems. In addition, highly sophisticated mass spectrometry techniques can be employed to assess the degree of covalent modification and possibly the stoichiometry of proteins within signaling complexes [91]. One persistent limitation to the mass spectrometry approach is that some of the conditions used in sample preparation, such as immunoprecipitation or the expression of recombinant epitope tagged scaffolding proteins, may disrupt or alter the association of binding partners with the signaling complex. Nevertheless, these approaches could be used to compare the composition and covalent modification state of certain signaling complexes in normal and diseased cells. Undoubtedly, these advances will shed new light on the molecular complexity of cellular signaling processes.

Acknowledgments

The authors wish to thank members of the Scott lab for critical evaluation of this article. F. Donelson Smith and J. D. Scott are supported in part by GM48231.

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