Signaling Networks: The Origins of Cellular Multitasking

Review

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One characteristic common to all organisms is the dynamic ability to coordinate constantly one's activities with environmental changes. The function of communicating with the environment is achieved through a number of pathways that receive and process signals, not only from the external environment but also from different regions within the cell. Individual pathways transmit signals along linear tracts resulting in regulation of discrete cell functions. This type of information transfer is an important part of the cellular repertoire of regulatory mechanisms. However, as increasingly larger numbers of cell signaling components and pathways are being identified and studied, it has become apparent that these linear pathways are not free-standing entities but parts of larger networks. Several articles in this review series describe in exquisite detail how individual classes of signaling pathways are organized and function. As we understand the details of such functional organization and move to the next level of analyzing integrated cellular functions, it will become increasingly important to identify and study the properties and capabilities of signaling networks as a whole.

One of the more surprising revelations that is coming from the initial studies of networks and component interactions in different cell types is that there may be a general signaling network that receives signals from cell type–specific inputs (i.e., receptors) and engage cell type–specific machinery. The molecular identity of the signaling components and their interacting partners may be cell type–specific, but the overall function of these components and the logic of the circuitry is preserved from cell type to cell type. We will compare two cell types, T cells (Dustin and Chan, 2000 [this issue of *Cell*]) and the postsynaptic region of glutamatergic synapses, to develop this argument. Signaling networks are likely to have a variety of emergent properties and capabilities. We will describe some of our current insights into how signaling networks are organized and how this dynamic spatial organization can lead to higher order cellular capabilities. As an example of such capabilities, we further develop the concept that the ability of a cell to regulate spatially resolved multiple functions in a coordinated manner arises from the organization of signaling pathways into networks.

Signaling Networks: Junctions and Nodes
Networks result from interconnections between signaling pathways. Such interconnections occur because the

same signaling component is capable of receiving signals from multiple inputs. Such networking may occur within similar classes of signaling pathways, such as between the Ras and Rho pathways (see Bar-Sagi and Hall, 2000 [this issue of *Cell*]), and between different pathways, such as the Gsα/cAMP and MAP kinase pathways (Wu et al., 1993; Chen and Iyengar, 1994). There are two general classes of interconnections: junctions, which are signal integrators and nodes, which split the signal and route them to multiple outputs. An immediate complexity that arises from these definitions is that they are not mutually exclusive properties for signaling components. A recent example of a molecule with dual identity is TOR, which appears capable of receiving signals from a number of sources as well as regulating a number of processes (Schmelzle and Hall, 2000 [this issue of *Cell*]). In the biological context, junctions and nodes should be considered as operational definitions for input and outputs at any given locus within the network (see Figures 1 and 2).

An early example of signal integrators was adenylyl cyclase, which was shown to produce cAMP in response to signals from Gs-coupled receptors as well as Ca^{2+}. As adenylyl cyclases were cloned and characterized, it became obvious that the different isoforms were capable of receiving signals from a wide variety of inputs (Pieroni et al., 1993) and thus cAMP levels in the cell could serve as an indicator of the balance of signals between many pathways. Additionally, from junctions signals may be routed to regulate numerous physiological events, as is the case with protein kinase A. The adenylyl cyclases-cAMP-PKA module is a junction at the adenylyl cyclase end and a node at the protein kinase A end. This complex situation detailing the signal-receiving capabilities of adenylyl cyclases, as well as the cellular machinery and processes regulated by the cAMP-activated protein kinase A, is depicted in Figure 1.

Signal integration at junctions can be both positive and negative. Raf, serving as a junction between the MAP kinase and cAMP pathways, best exemplifies this. Here, opposite types of connections are observed when different isoforms of Raf are present. c-Raf is inhibited by protein kinase A (Wu et al., 1993) while B-Raf is stimulated by the cAMP pathway (Vossler et al., 1997; Kawasaki et al., 1998). Thus, to understand signal integration at this junction, the molecular identity and relative proportions of the junctional components need to be known. This type of knowledge about concentrations of various cellular components in the natural context will be quite important for the development of accurate models of signaling networks.

Networks also contain nodes where signals may be split and routed through several different pathways to regulate distinct cellular functions. Like junctions, nodes may also be upstream or downstream in the network. One of the best upstream examples of a node is the receptor tyrosine kinases (Schlessinger, 2000 [this issue of *Cell*]), which can route growth factor signals through many different pathways. Although such routing can result in regulation of multiple independent cellular func-

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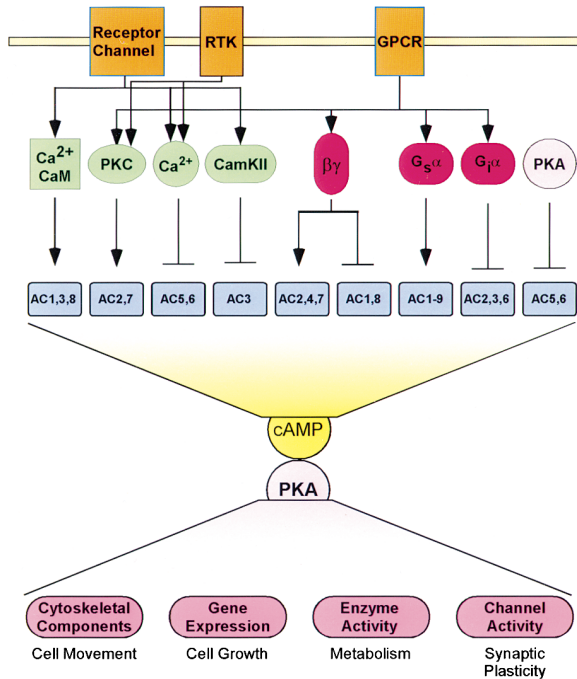
Figure 1. Adenylyl Cyclases as Examples of a Junction

The signal receiving capabilities of the various adenylyl cyclase isoforms and the capability of the cAMP-dependent protein kinase (PKA) to regulate various physiological functions are shown. Receptor channel, ligand gated channel (e.g., NMDA receptor); RTK, receptor tyrosine kinase; GPCR, G protein–coupled receptor. Stimulatory signals are shown as arrows and inhibitory signals as plungers. The various cellular components or processes regulated by PKA are shown in the red ovals and the resultant physiological functions are given below.

tions (e.g., growth factors such as PDGF can regulate vascular smooth muscle cell migration and proliferation), signal routing through multiple pathways can produce combinatorial signal specificity at the level of gene expression (Fambrough et al., 1999; Schlessinger, 2000). Such combinatorial specificity may be used as a mechanism to establish hierarchy amongst the regulated cellular processes.

A downstream example of a node is Cdc42, a member of the Rho family of GTPases. It receives signals from many receptor pathways (Bar-Sagi and Hall, 2000) and in turn can regulate a number of different cell functions through regulation of different effectors. This is depicted in Figure 2. The capability of a network component such as Cdc42 (or another Rho-related GTPase) to regulate a number of different cellular processes endows the network with the ability to regulate multiple cellular tasks.

Dynamics of Signaling Complexes

A general mechanism used for the assembly of signaling networks is the formation of complexes of signaling proteins. The organization of these complexes is dynamic and the complexes are often assembled in response to signal input. Perhaps, the best described of these complexes is the postsynaptic density, an electron-dense region just underneath the postsynaptic

membrane. The region consists of a mesh of cytoskeletal filaments, onto which the signaling complexes are assembled (Kennedy, 1997). We are now beginning to understand how this assembly occurs and the role of scaffolds in such complexes. One of the more noteworthy aspects of these signaling complexes is the number of proteins that are organized together. A recent proteomic analysis of the NMDA type glutamate receptor in the mouse brain revealed that more than 50 signaling proteins are associated with the NMDA receptor complex (Husi et al., 2000). This type of analysis does not provide any information on pair-wise interactions or even about which groups of proteins form functional complexes; nevertheless, the overview provided by this study will be quite useful in framing the contours of such complexes for more detailed functional analysis.

Although the glutamatergic synapse is a specialized structure, many of the organizational principles learned from the study of this region are likely to be relevant in other mammalian cellular systems. The review on T cells in this series (Dustin and Chan, 2000) presents a picture of the signaling components interacting with the T cell receptor that are very similar to those identified by Husi et al. (2000) as interacting with the NMDA type glutamate receptor. If the different classes of signaling components such as small G proteins, soluble tyrosine kinases, phosphatases, adaptor proteins, and cytoskeletal elements are considered, the composition of the complex in the two cell types and hence the network is essentially identical. However, each cell has cell type–specific isoforms of the different components. Thus, it appears possible that there may exist a general intracellular signaling network in diverse cell types. Of course the inputs and outputs are different. The extracellular signal input in the case of the neuron is the diffusible neurotransmitter glutamate and in the case of the T cell are cell–cell interactions with the antigen-presenting cell. The receptors are different as well, but nevertheless they engage a similar intracellular signaling network. The physiological outputs are cell type–specific: in the case of the neuron there is a change in the excitatory postsynaptic potential and in the case of T cells there is the secretion of IL-2. However, the cellular machinery such as the movement apparatus as well as the transcriptional and translational apparatus engaged by the signaling network is the same. It is also noteworthy that many of the components in the T cell and neuronal networks are also found in networks that transmit proliferative signals, giving credence to the idea that there is a general signaling network in diverse cell types. This hypothesis needs to be tested rigorously in many other cell-types.

The molecular mechanisms by which the signaling complexes are organized are beginning to emerge. As with many other general signaling concepts (Iyengar, 1996), the cAMP pathway provided some of the earliest evidence for the role of anchoring proteins (AKAPs) in the assembly of signaling complexes and in providing a spatial dimension to signaling. A large number of AKAPs have been identified (Edwards and Scott, 2000) as has another very important class of scaffold proteins, the PDZ domain proteins (Ziff, 1997; Garner et al., 2000). AKAPs are multivalent and bind a number of protein kinases and phosphatases to form signaling complexes that should have the intrinsic capability to both consoli-
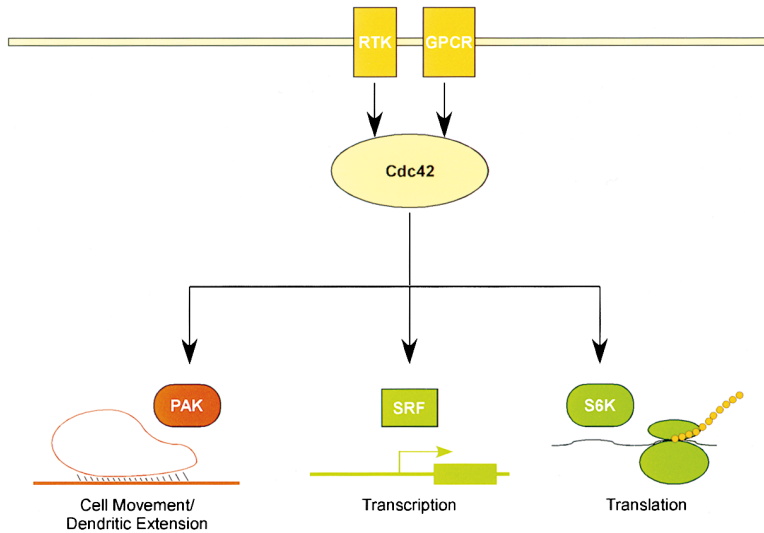
Figure 2. Cdc42, a Member of the Rho Family of GTPases as an Example of a Node

Cdc42 may be stimulated both by receptor-tyrosine kinases (RTK) as well as G protein–coupled receptors (GPCR), and in turn regulate different cellular functions by regulating the distinct downstream kinases Pak, S6K (S6-kinase), or the serum response factor (SRF).

date and dissipate biochemical signals (see below). AKAPs themselves are targeted to distinct regions of the cell. For instance, recent studies have shown that WAVE, a member of the WASP family of scaffolding proteins, is an AKAP, binding both protein kinase A and the tyrosine kinase c-Abl as well as specifically binding to actin. These interactions allow WAVE to recruit PKA and c-Abl to the sites of actin reorganization induced by growth factors (Westphal et al., 2000). Such complexes may be dynamically regulated by other signals. The association of *Drosophila* AKAP200 with the cytoskeleton is regulated by protein kinase C (Rossi et al., 1999). Therefore, the cell has the means to assemble signaling complexes at specified locations in an activity-dependent manner.

The other major class of scaffolding proteins is the PDZ domain–containing proteins that are involved in the assembly of signaling complexes at the glutamatergic synapse (Ziff, 1997; Garner et al., 2000). The number of proteins found to contain PDZ domains has steadily increased and so have their interacting partners. A recent study has shown that AKAPs can interact with PDZ domain–containing proteins PSD-95 and SAP-97 allowing for protein kinase A to be targeted to AMPA type glutamate receptor (Colledge et al., 2000). This type of interaction between classes of scaffolding molecules can lead to the formation of higher order complexes. The organizational format here appears quite intricate but nevertheless can be understood in terms of the signaling networks present in the postsynaptic region.

A general theme that emerges from the study of the various classes of scaffolding proteins is that these proteins possess bidirectional specificity. At one end they specifically recognize one or a group of signaling components and at the other end a location within the cell, thus providing the molecular basis for spatial organization of signaling pathways. Bidirectional specificity itself is a general mechanism for routing signals. Heterotrimeric G protein α subunits couple to selective classes of receptors and specific effectors and thus provide specificity in linear signal transfer (Gilman, 1987). Mechanisms of signal transfer such as binding of phosphoty-

rosine residues to PTB domains are also used as scaffold assembly mechanisms. An example of this is the JIP scaffolding proteins for the JNK kinase pathways that contain PTB and SH3 domains (Davis, 2000 [this issue of *Cell*]). Such assembly using signal transfer mechanisms is not restricted to scaffold proteins alone. Effectors themselves, functioning as scaffolds, can use this mechanism to assemble signaling complexes in order to regulate the timing of signal flow. In the case of the N-type calcium channel, the tyrosine-phosphorylated channel has been shown to recruit the heterotrimeric GTPase-activating protein RGS-12 through its PTB domain to regulate the rate of desensitization of the receptor response.

Although there are a large number of components and interactions, the studies with the scaffolding proteins allow us to reach the following conclusions about the assembly of signal networks. Scaffolds are the building blocks onto which signaling nodes and junctions are assembled. Such assembly provides a natural mechanism to achieve selective separation of signaling components and thus achieve specificity of signal routing. The scaffolds also provide a mechanism by which signals can be spatially resolved within the cell and thus provide the spatial dimension to signaling networks. Since interactions between components in the signaling complexes can be regulated by signal inputs, a unique feature of biological signaling networks is that junctions and nodes can be assembled and disassembled in an activity-dependent manner. This property sets biological signaling networks apart from physical networks where network architecture is preset and cannot be reorganized by signal input. The molecular complementarity between interacting partners and the spatial constraints provided by the anchors, scaffolds, and other organizing centers provide the physio-chemical basis for activity-dependent self-organization as a unique emergent property of biological signaling networks.

Signal Consolidation

A major function of signaling networks is to place a value on the signal such that it is either converted into further

biochemical event and subsequently a biological response or safely dissipated within the network. The signal can come from a single input such as Ca^{2+} in the postsynaptic region, where it can activate many signaling pathways that comprise a network, or from multiple inputs, each of which individually activates one or more signaling pathways within the network. The issue is how can the signal within the network be evaluated such that the appropriate physiological response is mounted. Generally, when signals are of sufficient amplitude for a specified duration, they evoke a physiological response and such signals can be considered consolidated signals. In the laboratory the simplest way to obtain a consolidated signal is to provide a high-amplitude (pharmacological dose) extracellular signal for an extended period. Although this approach has been very useful in tracking the linear signaling pathways, it is not reflective of physiological situations. Here, extracellular signals are generally subsaturating and often pulsatile in nature. How are these signals consolidated? Consolidation depends on network architecture and the regulatory mechanisms such architecture provides. Most linear signaling pathways themselves have a variety of mechanisms to dissipate signals at various levels of signal flow. At the receptor level the process of desensitization can rapidly limit signal flow. This type of regulation is seen in heterotrimeric G protein pathways, where receptor kinases (GRKs) rapidly uncouple the receptors from the G proteins (Pitcher et al., 1998). Receptors can also be downregulated (i.e., removed from the site of action), although this mechanism is slower and most often used to limit the effect of subsequent stimuli.

The second locus of regulation to achieve signal consolidation is at the level of signal transducers. In both small and large G protein pathways, the duration of the activated state of the G protein determines the amplitude and duration of signal flow. Persistent activation of G proteins by inhibition of the intrinsic GTPase activities has profound physiological and pathophysiological consequences. Inhibition of the GTPase activity of Gsα by cholera toxin leads to inhibition of water reabsorption in the intestine and consequently dysentery, a major symptom in cholera. Similarly, mutations that inhibit the GTPase activity of Ras are associated with a significant portion of human tumors. There are a large number of proteins that regulate the GTPase activities of both small and large G proteins. These GTPase activating proteins are called GAPs for small G proteins and RGS proteins for the heterotrimeric G proteins. A common feature between both small and heterotrimeric G protein pathways is that both the amplitude and duration of signal propagation beyond the G protein is regulated by the relationship between the receptor signal and the regulation of G protein activity. Since the G protein regulators themselves may be regulated through the network, signal consolidation at the level of G proteins is a network property. Additionally, interactions between the GAPs may result in junctions between the signaling pathways (Bar-Sagi and Hall, 2000).

The third major locus of signal consolidation is at the level of protein kinases. Persistently activated protein kinases are capable of triggering physiological functions. Examples abound, including persistently activated protein kinase A in cholera, persistently (muta-

tionally) activated tyrosine kinases and MAP kinases in proliferation and neoplastic transformation (Marshall, 1995), and persistently activated calcium-calmodulin kinase II in long-term potentiation of synaptic responses (Soderling, 2000). Often these key protein kinases are activated by phosphorylation in response to upstream signals. The duration of activation of the key protein kinase is determined by the balance of signal input and the phosphatases that limit the amplitude and duration of the activated protein kinase. Alternatively, sequential inhibitory phosphorylation by protein kinases from other pathways can also regulate signal consolidation. Such gating interactions involving protein kinases and phosphatases form one class of junctions in the assembly of signaling networks (Iyengar, 1996). Since anchors such as AKAPs bring together protein kinases and phosphatases, it is not only the interactions but also the organization of the network that determines whether a given signal will be consolidated at the level of protein kinases to obtain a physiological response.

The mechanisms of signal consolidation described above result in two emergent properties of the network. The first is the setting of threshold for the physiological response. Thresholds can be set at multiple levels and are dependent on the concentration of the signaling components, interactions between the components, and the colocalization of the interacting components. Thus, selective movement of the consolidated signal (i.e., either an activated protein kinase such as MAP kinase or an activator such as cAMP) to the appropriate location could function as a mechanism to set local thresholds for the conversion of biochemical reactions into physiological responses (for a more detailed discussion see Teruel and Meyer, 2000 [this issue of *Cell*]). The second system property that emerges from signal consolidation is the ability to propagate responses across different time scales. The distribution of consolidated signals to different cellular locations where they can stay active for various lengths of time could be one mechanism by which signals can be used to regulate physiological responses that depend on the integrated functioning of several cellular machines and that operate over different time scales. These predicted system properties need to be demonstrated by explicit experimentation.

The Orderly Engagement of Multiple Cellular Machinery

Consolidated signals produce changes in cellular functions. Many mammalian cells respond to extracellular signals with changes in a number of cellular functions, and it is the combination of these altered functions that constitutes the physiological response. For example, stimulation of the CA1 glutamatergic neuron can result in an immediate increase in synaptic efficiency (Siegelbaum and Kandel, 1991), dendritic outgrowths (Maletic-Savatic et al., 1999), stimulation of local protein synthesis (Frey et al., 1993), altered patterns of gene expression, biochemical remodeling of the synapse, and persistent changes in synaptic efficiency (Winder et al., 1998). Similarly, in the case of immune responses, the T cell moves toward and contacts the antigen-presenting cell, alters patterns of gene expression and eventually secretes
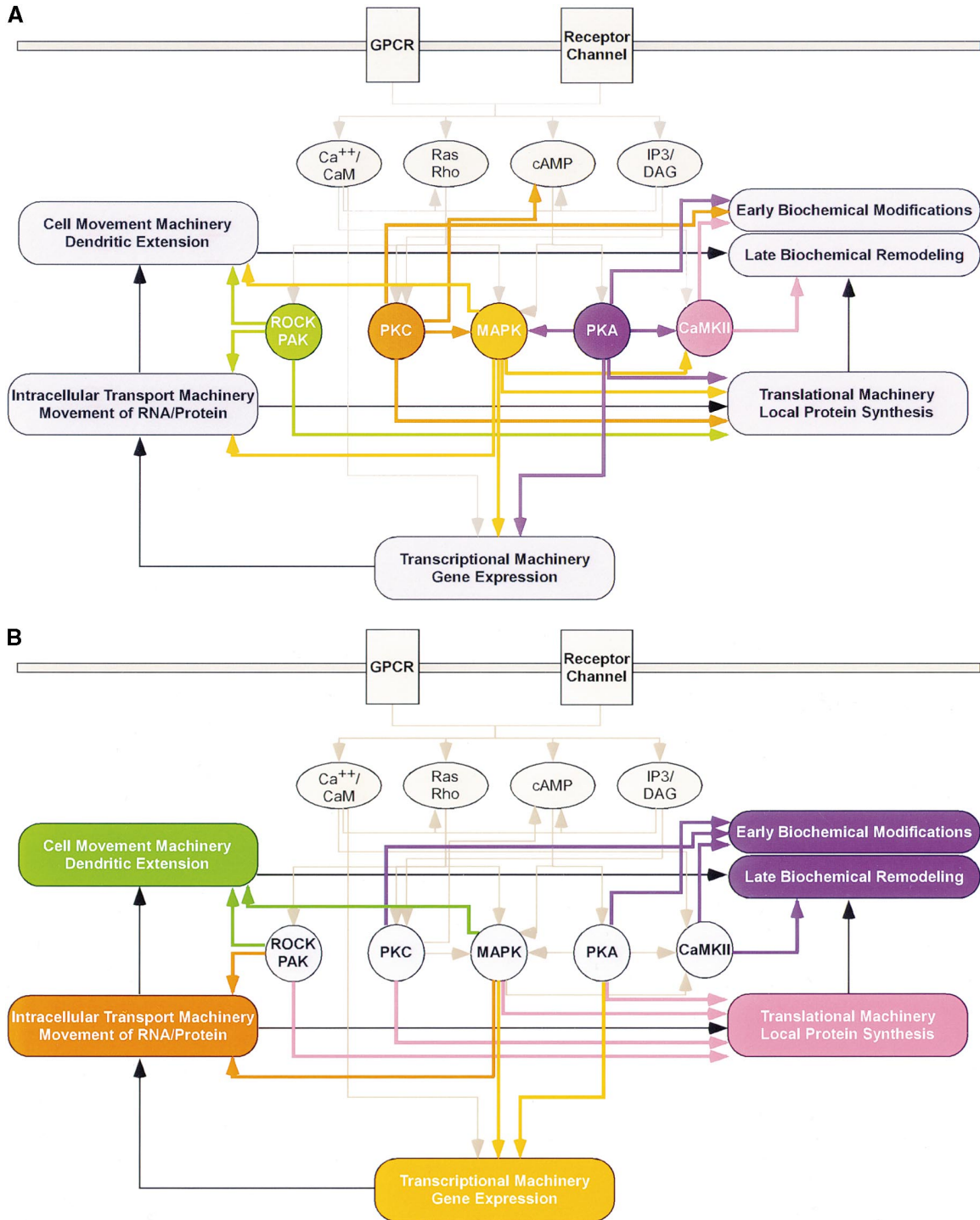
Figure 3. Postulated Postsynaptic Signaling Network in the CA1 Pyramidal Neuron

A simplified version of some of the key elements of the network and its interface with the various cellular machinery is shown. The purpose of the two figures is to highlight the interconnections between the key protein kinases within the network and the different cellular machinery. In both figures signal flow from the receptor through signal transducers and second messengers is shown in gray. The black arrows denote connections between the various cellular machinery. Panel (A) highlights the connections between the different kinases and the various cellular machinery. It can be readily seen that each protein kinase in the network can regulate multiple cellular machinery. Panel (B) highlights the view from the cellular machinery perspective to indicate that most cellular machines are regulated by multiple protein kinases. It is predicted that such meshing results in a system in which multiple machinery can be coordinately regulated by the signaling network in a robust manner.

cytokines (Dustin and Chan, 2000). In both cell-types each function is executed by distinct cellular machinery that is located in a defined region within the cell. How are these functions regulated in a spatially and temporally coordinated manner? We propose that the signaling network, by virtue of its capability to regulate the different cellular machines, will integrate the function of these machines to produce the physiological response. Here the cell may be considered analogous to a chemical plant with a number of reactors. The overall control system for the plant (i.e., the signaling network) will ensure that the different reactors function in a coordinated manner such that raw materials (corresponding to extracellular signals) introduced into the chemical plant results in the appropriate products (corresponding to changed physiological functions) at the output. We should be careful to limit this analogy since the architecture of the chemical plants (reactors and control system) is fixed while the architecture of the signaling networks and even some of the cellular machines is constantly changing. This analogy does raise the issue of how engineering design principles may be used to develop a function-based understanding of signaling networks and their regulation of cellular machines.

The changes associated with stimulation of the CA1 neuron that results in long-term potentiation of synaptic responses is a reasonable system to illustrate how a signaling network may regulate multiple cellular machines. A simplified version of the signals and processes in the CA1 neuron are schematically depicted in Figure 3. The glutamate signal is recognized both by a receptor channel (the NMDA receptor) as well as the metabotropic glutamate receptors, which are G protein–coupled receptors (GPCR). These receptors activate multiple signaling pathways including the Ca^{2+}/CaM, the cAMP, the IP_3/DAG, and probably the Ras/Rho GTPase pathways. Consequently, several protein kinases such as CaMKII, PKA, MAPK, PKC, and the Rho kinases (ROCK) and the Pak kinases would be activated. The interactions between these protein kinases and phosphatases play a very important role in setting the thresholds for signal consolidation within the network. One of the elements of flexibility in the architecture of a biological signaling network is that there is likely to be a different threshold for the different protein kinases, thus allowing for selective engagement of some cellular machines since different protein kinases can regulate different functions. For example, CaMKII phosphorylates and alters the activity of the AMPA channel (Barria et al., 1997) and this early biochemical modification may lead to increased synaptic efficiency. In contrast, some of the other protein kinases such as protein kinase A and MAP kinase, in addition to maintaining CAMKII active, may be involved in the orchestration of the later events by regulating the transcriptional and translational machinery. The initial activation of the protein kinases may occur in the postsynaptic region at or near the PSD; however, some of the protein kinases may move into the soma and even translocate into the nucleus. Activated MAP kinase is a well-known example of such a moving protein kinase. Such movement may allow for the coordination of dendritic outgrowth, marking of the stimulated dendrite, and the regulation of gene expression. The movement of activated protein kinases to different locations can also allow the protein kinases to stay active for different lengths of time depending on which local regulatory components are present. Thus, spatial resolution of signals within the cell can lead to temporal propagation of signals. Figure 3A demonstrates how the networks of protein kinases engage the different cellular machines while simultaneously making interconnections within the signaling network, while in Figure 3B we highlight the fact that the different cellular machines are regulated by multiple protein kinases. We anticipate that it is this interweaving of connections that will make coordination between the various cellular machines robust. It should also emphasized that the cellular machines themselves are functionally interconnected since the activities and/or products of one cellular machine are often required for the functioning of another cellular machine. Thus, the system as a whole is integrated both at the control level of the signaling network and at the functional response level of effector cellular machines. The dynamic flow of signal through the network and the capability of the signaling network to engage each of the effector cellular machines allow for coordinated regulation of these different cellular machines. The temporal coordination within the system is determined by the duration for which the consolidated signal regulates the individual machine, and the intrinsic rates of functioning of the different cellular machines themselves. This type of coordination becomes quite complex and cannot be readily analyzed or understood at an intuitive level. Systematic quantitative analysis of the models, in conjunction with experiments where multiple changes are measured both simultaneously and sequentially in a controlled fashion, will be required to develop an integrated picture.

Models of signaling networks and their interface with cellular machines are likely to be quite complex. They will be in part deterministic and in part stochastic. Several signaling components are likely to be parts of both types of reactions. Consider the case of MAP kinase which when activated in the dendrite may travel through the dendrite and the soma into the nucleus. It is likely that in some areas like the nucleus, because of anchoring, the effective concentration of MAP kinase is high enough that its phosphorylation of targets can be considered deterministic. In contrast when it is traveling through the dendrite at any given section, its concentration may be low such that its phosphorylation of targets in the dendrite is a stochastic process. Specific synapses are known to be tagged during long-term potentiation (Frey and Morris, 1997) and stochastic reactions involving MAP kinase in the dendrites could lead to tagging of activated connections. To develop robust models of signaling network control of synaptic plasticity, including changes in early and late postsynaptic functions, tagging, local protein synthesis, and gene expression, we need to develop reasonably accurate mathematical representations of processes that are in part deterministic and in part stochastic. Similarly, we also need to deal with systems where some reactions occur in two dimensions (such as for membrane-bound entities) while others are in three dimensions (for soluble entities). An immediate challenge is to develop appropriate algorithms to deal with these complex situations. If we are to build accurate models of complex cellular

processes, then we need proper representation of both space and time in addition to concentration of cellular components and their interactions.

Future Directions

The systematic cataloging of the genes, their products, and the interactions between cellular components provide us with an unimaginable wealth of information about the molecular entities in the cell. One of most exciting research challenges today is how we can integrate this information and move from component analysis to system analysis. The construction and analysis of a signaling network such as the one described in Figure 3 could be a starting point. This may be done by an iterative cycle of modeling and experiments. Systems properties of various components predicted by modeling may be quickly tested by experiments. Valid properties would be incorporated into the model and the model modified and further constrained by data. A preliminary connections map for such a network developed in our laboratory contains some three hundred components and upwards of a thousand interactions. Although large, these are manageable numbers. In such a model every known pair-wise interaction could be tested for use within the context of the network, and new interactions identified as the network properties are analyzed both experimentally and computationally. This would require a fairly high-throughput computational analysis whereby a family of models is developed. As the cost of computation has become progressively cheaper this approach is becoming quite feasible. However, since most experimental biologists including the authors do not routinely design experiments based on computational models, it will be necessary to develop user-friendly modeling systems and databases of functional constants to bring such analysis into most laboratories. The "Virtual Cell Project" is one such attempt (http://www.nrcam.uchc.edu). We expect that such analysis should allow us to move to a more integrated phase of research where cell functions as a whole can be understood in terms of the cellular components and interactions between them. As such understanding develops, we predict that we will find that signaling networks constitute the functional glue that holds the cell together.

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