2 Signal Transduction and Mammalian Cell Growth: Problems and Paradigms

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INTRODUCTION

To divide or to differentiate, to attach or to move, to survive or to die—these are among the key decisions cells must make during the development and adult life of a metazoan organism. Such decisions must be accurate and well coordinated and are dictated both by factors external to the cell and by internal cues. The process by which cells carry out these decisions is termed signal transduction. This chapter reviews emerging principles that govern signaling pathways germane to cell growth and division, with particular emphasis on the role of protein–protein interactions. In so doing, the crucial role of such protein interactions in mitogenic signal transduction, and the importance of emerging technology for their detection, will become apparent.

Mitogenic signaling pathways are complicated. With the completion of the human genome sequence, the number of recognizable signaling proteins will certainly increase, and the models of these pathways are apt to become more complicated still. Although many signaling pathways seem formidably complex when viewed as a whole, at closer inspection these pathways often can be described in terms of a series of simple interactions of one protein with another. Indeed, so fundamental are these interactions that it is not an exaggeration to say that they form the basis of all signal transduction machinery.

Why are protein–protein interactions so important in mitogenic signaling? The binding of one signaling protein to another can have a number of consequences. For one, such binding can serve to recruit a signaling protein to a location where it is activated and/or where it is needed to

Protein–Protein Interactions: A Molecular Cloning *Manual*, © 2002 by Cold Spring Harbor Laboratory Press, Chapter 2.

carry out its function. A relevant example that illustrates this phenomenon is the behavior of the protein kinase Raf, which, upon cell stimulation, is recruited from the cytoplasm to the plasma membrane by binding to the GTPase Ras (Avruch et al. 1994). A second consequence of protein interactions is that binding of one protein to another can induce conformational changes that affect activity or accessibility of additional binding domains, permitting additional protein interactions. Such is the case for signaling proteins such as p21-activated kinase, which, upon binding the GTPases Cdc42 or Rac, undergoes a profound conformational change that dislodges an autoinhibitory domain and thereby activates the kinase (Lei et al. 2000). Of course, stimulant-induced changes in protein location and conformation are not mutually exclusive. In many instances, recruitment to a signaling complex results in both relocation and enzymatic activation, as is the case, for example, with the protein tyrosine phosphatase SHP2, which is recruited from the cytoplasm to activated receptor tyrosine kinases (RPTKs) at the plasma membrane and is at the same time activated by the engagement of its SH2 domains to phosphotyrosine residues in the RPTK (Barford and Neel 1998; Hof et al. 1998).

THE SCAFFOLDING OF SIGNAL TRANSDUCTION

Binary protein–protein interactions are the cornerstone of signal transduction; however, an emerging theme in this research area is that higher-order assemblages are also critical for efficient transmission of signals. Scaffolds, adapters, insulators, and inhibitors are superimposed on the basic framework of the mitogenic signaling machinery. These additional layers of complexity have changed the way we look at signal transduction, and point to new ways to consider the organization of such pathways. For example, it has been known for some time that multicomponent complexes are assembled at activated RPTKs at the plasma membrane (Schlessinger 2000), whereas other complexes assemble at gene promoters on chromatin in the nucleus (Lee and Young 2000). It has now become clear that such multicomponent complexes also play a significant role in signaling in the cytoplasm and are critical for the regulation of mitogenesis. As one biologically important example, our understanding of the central Ras-Raf-Mek-mitogen-activated protein kinase (MAPK) pathway has evolved with the discovery that many of the elements of this complex are not only physically associated with one another, but also segregated from other cytoplasmic signaling proteins by several distinct scaffolding proteins (Garrington and Johnson 1999; Kolch 2000). These scaffolding proteins usually, but not always, lack catalytic function; however, they play key roles in signal transduction by virtue of their ability to complex with two or more elements of the Ras/MAPK pathway.

The use of scaffolding proteins in signal transduction is an evolutionarily conserved strategy. Examples of scaffolds for MAPK signaling modules have been found in all commonly studied eukaryotic organisms. At present, the function of these proteins is best understood in yeast (Fig. 1). In *Saccharomyces cerevisiae*, scaffold proteins function to segregate various common elements of MAPK modules. For example, the MAPK Ste11p participates in three distinct MAPK signaling modules: (1) the Ste5p scaffold coordinates components of the pheromone-response MAPK signaling module; (2) the Pbs2p scaffold coordinates components of an osmoregulatory MAPK signaling module; and (3) Ste11p also participates in a MAPK signaling module that regulates filamentation. In this last case, a scaffold protein has not been identified. In the absence of Pbs2p function, osmotic stress induces inappropriate activation of both the filamentous growth pathway and the mating pathway (O'Rourke and Herskowitz 1998; Davenport et al. 1999). Thus, in yeast it seems clear that one function of these scaffold proteins is to specify and insulate the signaling functions of MAPK modules.

In mammalian cells, scaffolds for MAPK modules include kinase suppressor of Ras (KSR) (Downward 1995), growth factor receptor-binding protein 10 (Grb10) (Nantel et al. 1998), and

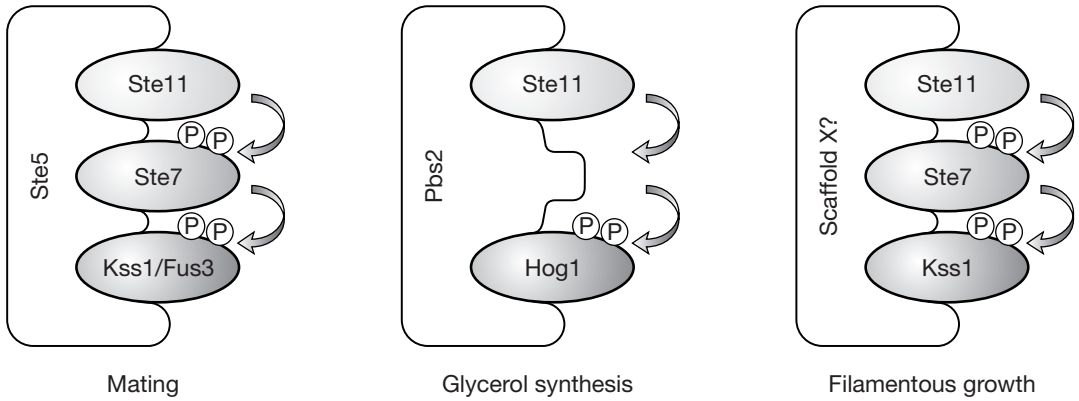

FIGURE 1. Scaffolds in budding yeast. *S. cerevisiae* uses similar signaling proteins toward different ends. The kinase Ste11p is involved in at least three distinct signaling cascades: mating, glycerol synthesis in response to hyperosmotic shock, and filamentous growth. It is thought that scaffolding proteins play a key role in signaling specificity, insulating Ste11p and downstream components from inadvertant activation by inappropriate stimuli. For the mating pathway, Ste5p binds three kinases, Ste11p, Ste7p, and Fus3p (and possibly Kss1p). In the case of hyperosmotic shock, the Ste11p target Pbs2p itself provides a scaffold function, binding both its upstream activator Ste11p and its downstream target Hog1p. It is not known whether the signaling machinery activated during filamentous growth requires an adapter protein.

Mek partner 1 (MP1) (Fig. 2) (Schaeffer et al. 1998). KSR binds to all three members of the canonical MAPK cascade: Raf, Mek, and Erk. Grb10 binds RPTKs, Raf, and Mek, but probably not at the same time, because a single SH2 domain in the carboxyl terminus of Grb10 mediates all these interactions. MP1 tethers the MAPK Erk1 to its activator MEK, and similar scaffolds (e.g,. JIP-1) are known for the stress-activated protein kinase Jnk and its upstream activators MKK7 and MLK (Yassuda et al. 1999). As in budding yeast, such a design may ensure efficient signal transmission and may also serve to prevent excessive interference, or cross-talk, from other signaling pathways in mammalian cells.

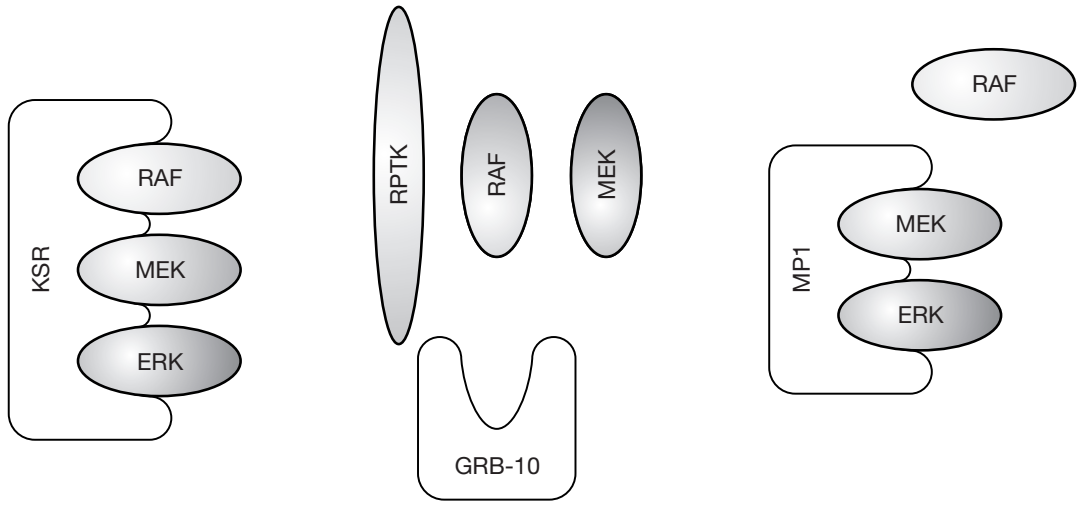

FIGURE 2. Examples of scaffolds in mammalian cells. As in yeast, mammalian signaling pathways also employ scaffolding proteins. KSR is functionally similar to budding yeast Ste5p, in that this protein can bind three members of a MAPK cascade. Grb10 represents a different type of adapter. This protein binds MAPK cascade members as well as RPTKs, but does so via a single binding domain and thus is unlikely to bind all three partners simultaneously. MP1 represents a third type of scaffold, linking together two elements of a MAPK cascade.

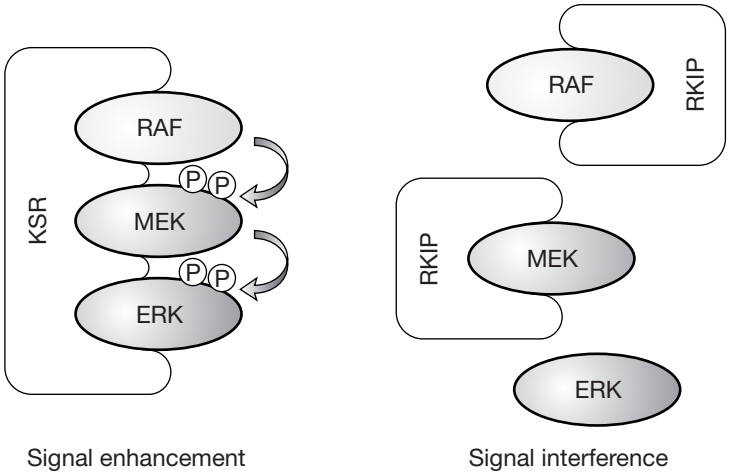

FIGURE 3. Antiscaffolds prevent efficient transmission of signals. Scaffold proteins such as KSR are thought to enhance signal transduction by physically coupling enzymes and their substrates. In contrast, antiscaffolds, such as RKIP, may interfere with signaling by sequestering these elements from one another.

Scaffold-like proteins are involved not only in signal transmission, but also in signal interference (Fig. 3). RKIP, a protein initially identified as a binding partner for Raf, can also bind Mek and Erk, suggesting that it might function in a manner analogous to KSR (Yeung et al. 1999, 2000). However, unlike KSR, RKIP inhibits, not enhances, signaling from Raf to Mek. The structural basis for this phenomenon probably lies in the fact that the binding elements on RKIP for Raf and Mek overlap substantially. Because it is unlikely that RKIP can simultaneously bind Raf and Mek, RKIP may act as a sequestering agent for both these proteins, preventing their effective interaction with one another. It would not be surprising if other antiscaffold proteins should be found that disrupt signaling modules in a similar manner.

In mammalian cells, other classes of cytoplasmic scaffolding proteins have been found that link proteins operating in common signaling pathways. Such proteins are sometimes termed anchoring scaffolds. Examples of such anchoring scaffolds that are relevant for mitogenic pathways include the A-kinase anchoring proteins (AKAPs), the cytoplasmic domains of many RPTKs, insulin receptor substrate 1 (IRS1), the T-cell protein linker for activation of T cells (LAT), and Src homology 2 domain-containing protein of 76 kD (SLP76) (Burack and Shaw 2000). These types of scaffolds differ from those involved in the Raf-Mek-MAPK pathway in that the complexed proteins do not act enzymatically upon one other (e.g., Raf phosphorylates Mek, which phosphorylates MAPK), but rather are all involved in a particular signaling function. The anchoring function of this class of scaffold protein may be important in the assembly of signaling elements, imposing a spatial and temporal restriction on their distribution.

PROTEIN–PROTEIN INTERACTIONS AND THE REGULATION OF SIGNALING CROSS-TALK

Although the existence of multicomponent signaling modules in the cytoplasm of mammalian cells is no longer in dispute, their purpose is not completely clear. Is it, as is apparently the case in yeast, to provide insulation against cross-talk from competing signaling pathways? Is it to increase the efficiency of signal transmission by bringing the relevant components in proximity with one another? The prevalence and relevance of cross-talk in mammalian signal transduction have been hotly debated items in the signaling literature. At issue is whether signaling pathways in mammalian cells are best described in terms of linear flow diagrams, or as interconnected webs (Tucker

et al. 2001). In simple eukaryotes such as budding yeast, *Drosophila*, and *Caenorhabditis elegans*, the majority of signaling pathways appear to operate as discrete, linear entities, perhaps due to the presence of insulating adapter proteins. This facet of signal transduction can be clearly seen in the mating pathway in *S. cerevisiae*. Activation of the pheromone receptor sets off a chain of events that can be likened to a bucket brigade, with the signal passing from the pheromone receptor to a set of protein kinases arranged in series (and with some components held together by the Ste5p scaffold protein) to a transcription factor (Posas et al. 1998). Experimentally, the essential linearity of the system can be appreciated by the fact that the transcriptional response to mating pheromone is completely dependent on a single transcription factor, Ste12p (Roberts et al. 2000). If significant cross-talk occurred, pheromone signals would be expected to diverge and induce expression of additional, non-Ste12p-dependent genes.

Whether mitogenic signaling pathways operate as discrete linear units or as interconnected webs in higher eukaryotes, it is known that stimulation of a given receptor can activate multiple pathways, and stimulation of similar receptors can yield different outcomes. A classic example of the latter phenomenon is seen in PC12 cells, where switching on the epidermal growth factor (EGF) receptor leads to proliferation, whereas stimulation of the nerve growth factor (NGF) receptor causes growth arrest and differentiation (Marshall 1995). Many of the same signaling proteins are activated by these two receptors, so any description of the differing outcomes of EGF versus NGF signaling will have to invoke either spatial or temporal issues or subtle differences in the identity of the effectors that are recruited to the receptors. We know that EGF leads to transient MAPK activation, whereas NGF leads to prolonged MAPK activation (Qui and Green 1992; Traverse et al. 1992), which is likely to account for their opposing cellular effects; however, the molecular basis for these temporal differences remains uncertain. One strong possibility is that differential use of scaffolds is a key factor. Landreth and colleagues have shown that transient activation of the MAPKs by EGF is a consequence of the formation of a short-lived complex assembling on the EGF receptor itself, comprising the adapter Crk, the exchange factor C3G, the GTPase Rap1, and the protein kinase B-Raf (Kao et al. 2001). In contrast, NGF stimulation of PC12 cells results in the phosphorylation of a scaffold protein, FRS2, on which is assembled a stable complex of Crk, C3G, Rap1, and B-Raf, resulting in the prolonged activation of the MAPKs. Thus, although the same set of signaling proteins is recruited by NGF and EGF, alterate scaffolding arrangements are induced by these different stimuli and may result in the observed unique outcomes.

TECHNOLOGY AND SIGNAL TRANSDUCTION—A VIEW OF THE NEAR FUTURE

Given the importance of scaffolds and antiscaffolds in regulating cross-talk in mitogenic signal transduction, and the fact that the diverse structures of these proteins make their identification by sequence inspection or complementation difficult, emerging technologies for finding proteins of this type could be useful. For example, the multiple-bait interaction trap systems (see Serebriiskii et al. 1999), in which pairs of baits are examined for binding to a common partner, could be used to identify proteins that bind both Raf and Mek, or any other pair of signaling proteins in which scaffolds are suspected to play a role. Because overexpression of scaffolds often inhibits signal transduction (Dickens et al. 1997), probably by altering the stoichiometry of the endogenous signaling components, it is also conceivable that proteins with scaffold or antiscaffold function could be identified by expression screens for inhibitors of mitogenesis. Finally, the rapid evolution of mass spectrometry techniques may make it likely that the components of cytoplasmic mitogenic signaling complexes will be increasingly amenable to direct identification in immunoprecipitates (Pandey and Mann 2000).

If mitogenic signaling in higher eukaryotes in fact operates in such a complex manner, then to understand signaling pathways it will be necessary to develop a means to analyze the operation and interaction of large networks of proteins rather than to consider the behavior of a few indi-

vidual components in isolation. However, at present we lack good tools to perform such multicomponent analyses. Until now, our understanding of signaling pathways has been built mainly on the backbone of biochemistry, the careful and often painstaking analysis of single proteins in vitro. Is a new analytic paradigm for signal transduction required, and, if so, what will it be?

Clearly, data from array analyses of mitogenic signals are telling us something, but the messages are not easy to decipher. Serum stimulation of fibroblasts in culture induces expression of a bewildering array of genes (Iyer et al. 2000). Activated Ras induces or impedes expression of scores, if not hundreds, of genes (Zuber et al. 2000). How does one begin to make sense of these data? Some have suggested that proteins whose expression is coordinately regulated might work together, affecting similar processes. In other words, temporal concordance of expression might imply functional similarities. Perhaps the best case for this theory can be made in the budding yeast mating pathway, where many of the genes involved are coordinately up-regulated. This may also be true in mammalian systems, but there is not much evidence to support it in a general way.

Beyond temporal concordance, it is, in principle, possible to impose a second level of organization of the data, that of spatial location. If we knew where all these proteins were located in the cell, we could begin to sort out what proteins interact with what others. The colocalization of candidate binding proteins can be established by fluorescence resonance transfer methods and related techniques (Bastiaens and Squire 1999; Pollok and Heim 1999). General schemes for localizing proteins have also been published, usually involving screening libraries of green fluorescent protein (GFP) fusions (Ding et al. 2000; Misawa et al. 2000). On a genome-wide scale, it could be useful to combine such localization data with those obtained by independent means.

A similar approach, whose utility has recently been validated, is to determine the composition of signaling networks by combining biochemical data with two-hybrid analyses to analyze protein–protein interactions. In budding yeast, a meta-analysis of existing two-hybrid and biochemical data has confirmed the presence of several clusters of interacting proteins and, in some cases, has suggested functions for proteins whose roles were previously obscure (Schwikowski et al. 2000). Finally, evolutionary relationships may also provide a key to understanding signaling complexes, because conserved proteins that operate together in model eukaryotes such as yeast, worms, or flies are often good indicators of a similar design in humans.

We are entering a new era, and our old ideas about mitogenic signaling are undergoing a rapid revision. It has become abundantly clear not only that protein–protein interactions form the basis for signal transduction, but also that these interactions occur on a scale and with a degree of complexity not previously suspected. It will be a great challenge to integrate the onslaught of data from the genome sequencing projects to determine the organization of signaling proteins in mitogenesis. However, meeting this challenge might allow us to learn how to manipulate protein–protein interactions in the treatment of human diseases such as cancer, where the proper regulation of signal transduction has by definition gone awry.

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