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Specificity Is Complex and Time Consuming: Mutual Exclusivity in Tyrosine Kinase-Mediated Signaling

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ABSTRACT

Most fundamental cellular processes are transduced through tyrosine kinase (TK)-mediated pathways. For transduction without corruption, the protein−protein interactions involved have to be mutually exclusive. Many of these proteins bind via homologous domains whose binding characteristics suggest that their innate specificity is not sufficiently high to account for the integrity of signal transduction. Stimulation of TK-mediated signals is often accompanied by recruitment of a precise, multimolecular protein complex that is itself capable of imposing specificity. Furthermore, this complex provides protection against phosphatase activity, controlling the longevity of the active signaling complex, and thus influencing outcomes in subsequent downstream events.

Introduction

An eukaryotic cell receives communication from the external world through its membrane-bound receptors. These receptors are of several general forms each responding to distinctly different types of stimuli, but all capable of sensing a binding event outside, and responding by initiating a signaling pathway inside the cell. In this article we limit our attention to signal transduction events that are derived from the initial extracellular stimulation of a membrane bound receptor, and the subsequent protein−protein interactions that are mediated by tyrosine kinase (TK) activity. The transduction of this type of signal generally involves the sequential binding and subsequent enhanced activation of a defined sequence of proteins, or groups of proteins. This has the ultimate effect of a net increase in activity of these proteins over a previously maintained basal level. This pulse of signaling activity results in a change to the cell morphology, metabolic function, or survival, usually via chemical modification of proteins, release of secondary messengers or direct transcriptional effects, that is dictated by the specific path that has been activated.

TK-mediated signaling accounts for a large number of important transduction pathways often resulting in commitment to fundamental alteration of cellular condition.[1,2,3] Aberrancies in these pathways are responsible for many disease states. Tyrosine residues on selected proteins in the signal transduction pathway are phosphorylated providing the site of recognition for downstream, effector binding partners. This kinase activity involves the turnover of ATP, and as such is an energetically expensive process. To achieve the appropriate cellular response from activation of a given receptor, these pathways have to ultimately be highly specific. Most TK activity is centered on the early activation events after receptor stimulation and is usually localized proximal to the cell membrane.

In attempting to describe signaling specificity, the original dogma suggested that TK-mediated signal transduction proceeds via a linear relay of bimolecular interactions. The emergence of this view was largely dependent on the experimental techniques used to fish for interacting molecules. These immunoprecipitation or two hybrid-type experiments, by their very nature, were capable of identifying only one (the highest affinity) binding partner at a time. The acceptance of this dogma requires that the individual protein−protein interactions involved would have to be highly specific to avoid any 'crossed-lines' between different signaling processes in the cell. More recently it has become clearer that the stimulation of a TK receptor generally results in the simultaneous recruitment of a number of protein molecules that assemble as a complex at the membrane. In this article we attempt to illuminate some of the features of this type of protein ensemble and address the issue of why such a mechanism has evolved to maintain mutual exclusivity in these pathways.

Specificity of Protein−Protein Interactions in Signaling Pathways. The prevalence of different TK-mediated signaling in cells clearly requires that a pathway committed to a given signal is able to avoid interaction with any other pathway. Many of the proteins found to play a role in the passage of a TK-mediated signal possess structurally homologous domains. For example, in most cases the first binding event after receptor tyrosine phosphorylation involves the noncovalent interaction of a Src homology (SH) 2 domain. However, any given cell can have in excess of a hundred different proteins containing SH2 domains. For signaling to be based on the linear processing dogma alone, it would be necessary for the equilibrium dissociation constant (K_D) between a given SH2 domain and specific cognate ligand to be several orders of magnitude lower than any nonspecific interaction that might occur

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Table 1. Thermodynamic Parameters for the Interaction of the Src SH2 Domain with Tyrosylphosphopeptides at 25 °C[a]

peptide sequence	K_D (μM)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ref
Ac-EPQpYEEIPIYL-NH_2	0.09	−40.1	−38.7	1.4	10[b]
Ac-EPQpYEEVPIYL-NH_2	0.16	−38.8	−28.6	10.2	10[b]
Ac-EPQpYEEEPIYL-NH_2	0.21	−38.1	−32.7	5.4	10[b]
Ac-EPQpYEEWPIYL-NH_2	0.31	−37.1	−32.2	4.9	12[b]
Ac-EPQpYEEDPIYL-NH_2	0.38	−36.6	−27.5	9.1	12[b]
Ac-EPQpYQPGEN-NH_2	14.1	−27.7	−25.7	2.0	10[b]
Ac-LGGQpYEEIPIP-NH_2	0.53	−35.8	−35.3	0.5	7[c]
pY	333.3	−19.7	−0.4	19.3	14[d]

[a] Adapted from ref 12. [b] Experiments in 20 mM MES, pH 6.0, and 50 mM NaCl. [c] Experiments in 50 mM MOPS, pH 6.8, and 100 mM NaCl. [d] Experiments in 20 mM HEPES, pH 7.5, and 100 mM NaCl.

between that SH2 domain and another tyrosyl-phosphorylated site.

Understanding the basis of this specificity was originally investigated through screening of different SH2 domains with a library of tyrosyl phosphopeptides.[4] This work revealed that a given SH2 domain could distinguish its cognate ligand based on the amino acid sequence proximal, and primarily C-terminal, of the phosphotyrosine residue (pY). So, for example, the SH2 domain from the protein Src was reported to preferentially recognize the sequence pYEEI, whereas that from the N-terminal SH2 domain of the p85 subunit of PI3 kinase is specific for pYMXM (where X is any amino acid). The structural basis for this proposed recognition lies in the distinct topology of the majority of binding sites on SH2 domains which have two deep pockets, one of which accepts the phosphotyrosine residue of a binding partner and the other usually accommodates the residue in the pY + 3 position. Thus, the docking of a ligand onto an SH2 domain can be likened to a two-pronged plug.[5,6] The pY binding pocket is highly homologous across the known SH2 domain sequences; however, there is some variation in the pY + 3 pocket. Quantification of these interactions revealed that there was little basis for a high level of specificity. In experiments in which physiological interactions with SH2 domains were mimicked by tyrosyl phosphopeptides, changing the sequence of amino acids proximal to the pY binding site had only limited effects on the K_D.[7−12] For example, extensive binding studies on the Src SH2 domain showed that single amino acid substitutions in the specific sequence (pYEEI see above) gave a difference in the K_D of approximately 1 order of magnitude (see Table 1). Binding of a random sequence (e.g., pYQPG) to the SH2 domain only resulted in a K_D about 2 orders of magnitude weaker than the interaction with the specific ligand.[7] The binding of the same Src SH2 domain to a sequence corresponding that of platelet-derived growth factor (PDGF) receptor with which the protein has no reported physiological interaction was only approximately 50-fold weaker than that of the specific sequence.[13] This suggested that slight variations in concentrations of proteins containing SH2 domains, or phosphorylated substrates, could lead to incorrect signals being initiated. Binding data also revealed that close to 60% of the total free energy of interaction of tyrosyl phosphopeptides with SH2 domains is derived from the pY moiety. Given this large contribution from the pY, the free energy window for a contribution from the other potential interactions of amino acids proximal to the pY is very small.[14,15] These data suggest that there is little to advocate a high level of specificity in the interactions of SH2 domains. On the contrary, the binding of these domains is actually quite promiscuous. Indeed, there is now significant evidence to question the intrinsic ability of a single SH2 domain to selectively recognize tyrosine-phosphorylated targets. This suggests that a single SH2 domain is capable of recognizing a wider range of targets than previously thought. The potential physiological relevance of this is discussed below.

This lack of expected specificity is also apparent for other domains found interacting in the TK-mediated signaling pathways. For example, SH3 domains recognize sequences based around the general motif of PXXP. These proline-rich sequences bind in the PPII helical conformation to binding sites that are hydrophobic and largely topologically amorphous.[13,16] Again, peptide library screening studies derived some suggestion of limited sequence specificity. In binding studies the interactions of a range of SH3 domains with peptides corresponding to sequences from a series of proteins gave no more than 2 orders of magnitude in K_B between specific and nonspecific interactions.[13] Other less common and less well-characterized domains involved in protein−protein interactions such as PTB,[17] WW,[1] or PDZ[18] show a similar limitation in the levels of specificity that they can invoke. Thus, none of the individual domains found in TK-mediated pathways appear to provide a sufficient level of specificity in their interactions to allow confidence in their ability to attain mutual exclusivity in signal transduction.

The limited difference in affinity between specific and nonspecific interaction for these domains could potentially be improved if more than one domain is involved in the binding process. Most of the proteins involved in early events in TK-mediated pathways possess more than one domain, and in some cases these have been shown to interact with at least two distinct sites on an upstream cognate ligand. This potential for a bifurcated interaction could increase the affinity between two interacting partners from the additive effect of free energy of interaction of the individual domains. This potential increase in affinity would reduce the possibility of interaction of competing proteins unless they were at significantly elevated concentrations. Despite this potential to improve the level of specificity through an increased affinity,

experimental quantification of the net binding effects of several of these interactions have failed to show large increases in avidity. In fact, in some cases the binding of two domains to two distinct sites on a cognate protein or peptide is only slightly stronger than the binding associated with the individual domains.[17,18] This is usually the result of a significant entropic penalty to the free energy of binding derived from major conformational changes required for the positioning of the respective binding sites to dock. Thus, the previously, generally accepted dogma of signaling pathways being based on a relay of simple bimolecular interactions can largely be supplanted by more complex models.

The Assembly of Multiprotein Complexes Provides Specificity. In many of the TK receptor-stimulated signaling events, it has been shown that the initial event of receptor phosphorylation results in the recruitment of a defined group of proteins (for examples, see Figure 1). These proteins interact to form a complex that is based on a well-defined order of interaction and a mutual dependency for activation. Proteins involved in the complex will typically interact with more than one component of the complex via different domains. This multivalent nature of binding serves to create a higher order of specificity, since for binding to the complex the protein will require the correct spatial juxtaposition of domains to make the appropriate interactions with the binding sites on other proteins in the complex. The formation of these complexes in early signaling provides a way around the need for high levels of specificity between individual interacting components required in the linear processing model. The specificity is instead induced by the requirement of the complete complement of proteins in the correct order (Figure 2). The mutual interdependence of proteins in the complex for a steric fit and kinase activity ensures that only the correct assembly can ultimately pass a signal to downstream effectors. Interaction with competing proteins can occur, but because their presence precludes the complete complement of proteins assembling and thus restricts the binding to the downstream effector (see below), there will be no ultimate signal generated.

The formation of these early signaling complexes is based on a distinct time course. For example, stimulation of the T cell receptor results in the assembly of an ensemble of different proteins in the first 15 s of receptor activation.[21] These proteins reach their respective maximal activity at different times, but all between 1 and 2 min after stimulation. The content of the complex also seems to rearrange in this time period. By 4 min the complex appears to start to disintegrate although several of the components seem to continue to interact in smaller complexes well after this time. The disintegration of the complex is often concomitant with endosome encapsulation (John E. Ladbury, unpublished results).

Properties of Signaling Complexes. *1. Temporal Control of Signal.* Catalytic proteins in complexes proximal to the membrane operate in a diffusion-limited regime. As a result they are prone to local depletion of substrate resulting from the turnover being faster than the diffusion of new substrate to the active site. These reductions in

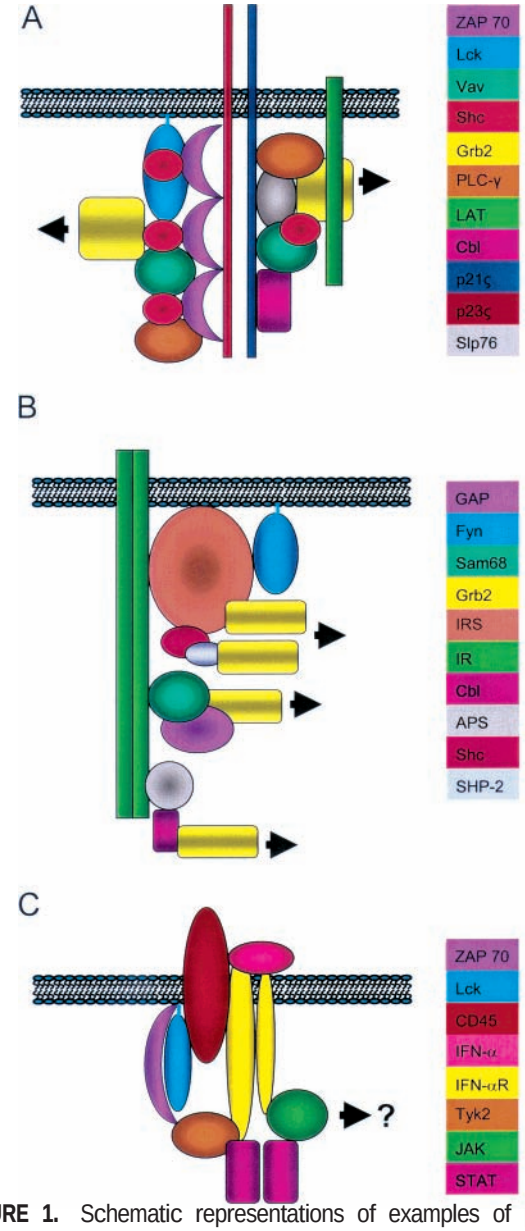

FIGURE 1. Schematic representations of examples of protein complexes formed on stimulation of TK-mediated signaling pathways. In all cases the stoichiometries of proteins are modeled based on the literature where available. Black arrows show entry to the MAP kinase pathway. (A) Complex formation on stimulation of the T cell receptor (TCR). The model is based on data gained one minute after stimulation of the receptor.[21] The stoichiometry of different components is based on ref 21 and references therein. The two isoforms of the ζ-subunit (p21ζ and p23ζ) are differentially phosphorylated. Stimulation of the TCR results in the activation of the MAP kinase pathway via the interacation of Grb2 (yellow) with SOS. (B) Complex formation on stimulation of the insulin receptor (IR) results in the assembly of a range of different proteins. The insulin receptor substrate (IRS, peach) is rapidly phosphorylated and can interact with several proteins.[42] As with the TCR, the signal from the complex feeds into the MAP kinase pathway via Grb2 (yellow). (C) Complex formation after stimulation of the interferon-α receptor (IFN-αR). The figure is based on data from ref 43 and unpublished work (JEL). The stimulation of IFN-αR has been shown to activate MAP kinase;[44] however, direct activation through the Grb2/SOS entry point has not been demonstrated.

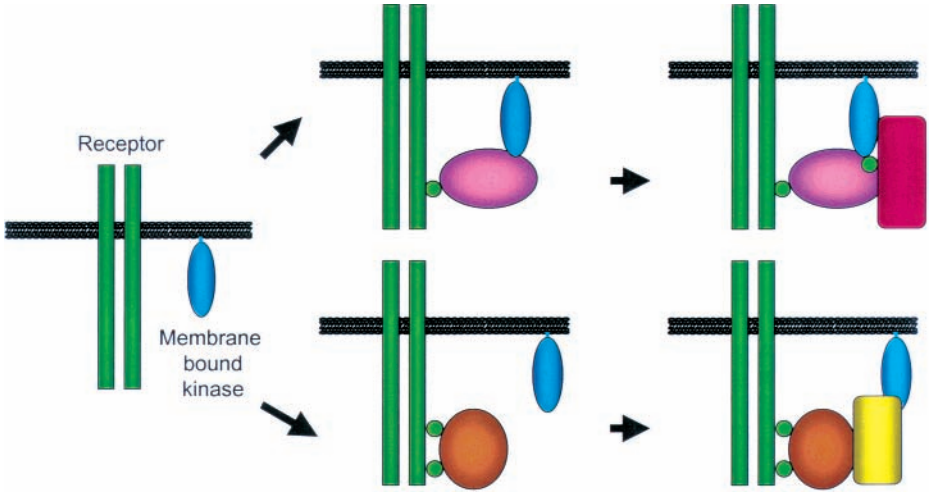

FIGURE 2. Schematic models of the interaction sequence required to ensure specificity on complex formation. Here we start with a TK, membrane-bound receptor and kinase. Stimulation of the receptor results in phosphorylation (green circle). In the top sequence, a protein binds to both the receptor (via and SH2 or PTB domain) and the kinase. This requires a specific protein with suitably juxtaposed binding sites. The kinase then phosphorylates the incoming protein, which can interact with a further protein (red) which also possesses a site for interaction with the kinase. In the lower sequence specificity for the initial protein, interaction with the receptor comes from the juxtaposition of two phosphorylation sites. A further protein builds up the complex by being able to interact with the first binding protein (orange) and the kinase (blue).

substrate concentration result in reduced overall reaction rate. Thus, the relative balance of diffusion and reaction rates determine the spatial distribution of signaling components, the complex lifetime, and the rate of signal transfer.[22] The phosphorylation of tyrosine is the key to downstream signaling but will always be prone to dephosphorylation. Thus, in the case of TK-mediated signaling, the balance of diffusion and reaction rates is further complicated by the competition between kinase and phosphatase activity. The binding of an SH2 domain to pY restricts phosphatase access, but this is limited by the on and off rates of the interaction and local phosphatase concentrations. The formation of a complex results in a package of proteins that protect the phosphorylated sites, sterically hindering access by other proteins. Thus, for example, the initial phosphorylation of tyrosine residues in the ITAM motif of the ζ-subunit of the T cell receptor by the Src kinase, Lck, results in recruitment of ZAP70.[23] The SH2 domains of ZAP70 bind to these ITAM motifs and essentially preclude normal phosphatase activity. As other members of the assembly are recruited, a stable complex is formed further limiting phosphatase activity. This phosphatase inhibition allows some control of the period over which the signaling machinery is in place. As a result signals can be propagated over distinct periods of time on the basis of the complex size and the kinetics of interaction of its components. These are less prone to local fluctuations in protein concentration. The assembly of proteins in this way in early signaling can be considered as a checkpoint or gate for the subsequent downstream response.

2. Amplification of Signal. Catalytic activity of a protein increases the concentration of downstream signaling proteins based on the rate of substrate turnover. If this occurs sequentially down the pathway, a "cascade" of increased activity results.[24,25] There are numerous, elegant theoretical approaches describing signaling on the basis of bimolecular cycles demonstrating how linear pathways can amplify a signal. However, the ultimate gain in signal through Hill coefficient multiplicity is unlikely to apply to the early events involving the signaling complexes. The relative on and off rates of proteins in the complex are significantly affected by the "locking" of the complex state. This means that the proteins are not involved in increasing signal sensitivity. Although some pathways ultimately result in a dramatic increase in secondary messenger concentration or gross up-regulation of downstream effectors, amplification is limited in the early events in TK-mediated signal transduction.

3. Membrane Localization. All of the early signaling complexes are attached to the cell membrane. This raises the question of why TK signaling complexes involve membrane-bound proteins. Membrane localization of proteins involved in the signal transduction process has been considered to be important to increase their rate of encounter after receptor activation. This would lead to a stronger signal based on the cascade effect described above. Calculations have suggested, however, that the encounter rates of membrane-linked, compared to cytosol-located proteins, were actually very similar. The increase in first encounter rate by virtue of membrane localization is too small to be responsible for truly enhanced signal transduction.[26] This observation suggests that the function of membrane localization is to increase the number (or average lifetime) of complexes. The effect of membrane localization is to provide an effective increased local concentration of interacting partners and this gives rise to an increase in apparent affinity. This enhancement in association has been calculated to be as much as 1000-fold.[27] This also reduces the number of interacting proteins per cell required to achieve the same extent of association.

4. The Importance of Promiscuity. The complex potentially overcomes the lack of specificity in individual protein−protein interactions (see above), actually allowing for some promiscuity. Promiscuity could be an evolutionarily important benefit if, as is the case in many tyrosine-kinase mediated signaling pathways, the signal is fundamental to the organisms survival. For a highly specific interaction, a mutation in one protein in a linear pathway could present a life-death outcome. Thus, the provision of a level of promiscuity in the binding provides a buffering effect ensuring that existence is less tenuous. From the mass of data acquired for the Src SH2 domain interactions only two amino acid substitutions have been seen to seriously compromise binding.[14,28] This lack of potency in the amino acid context of the binding site corroborates the idea of promiscuity providing an evolutionary advantage in that most mutational events that may have occurred will have remained functionally silent. This may explain why very few diseases appear to be solely linked to mutations in SH2 domains, although these domains are central to many signal transduction pathways in higher eukaryotes.[14]

The Complex and the MAP Kinase Pathway. The formation of the protein complex as the primary event in the signal transduction process acts as the initial gate prior to the cell being committed to a course of action. As a result this assembly of proteins has to be rigorously controlled against error. Furthermore, the complex has to produce a downstream response that can be recognized as a distinct signal, quite different from any other. The importance of these points is dramatically emphasized when it is considered that many of these TK-mediated early signaling events feed directly into the mitogen-activated protein (MAP) kinase pathway. Here we focus on trying to understand how TK-mediated signaling complexes might affect this important pathway in TK-mediated signaling. Generically this pathway is made up of the sequential progression of serine/threonine and tyrosine phosphorylation events on a sequence of substrate molecules (for recent reviews[29−31]). The pathway is entered through an adapter protein from the early signaling complex (e.g. Grb2) binding to the son-of-sevenless (SOS) protein. This triggers the turnover of GTP by the Ras protein and the phosphorylation of the first MAP kinase. The resulting activation of this first protein in the MAP series (MAPKKK, also known as Raf or MEKK) stimulates it to phosphorylate its substrate (MAPKK, also known as MEK). This activated protein then activates its downstream phosphorylation target (MAPK, also known as ERK). Ultimately the signal is passed into the cell nucleus via protein translocation and results in gene transcription. There are several families of the MAP kinases existing across different cell types that can receive signals from many TK-mediated complexes. Thus, there exists a fundamental question of how this machinery is able to decipher what signal is being received. In other words, what is it about the signal coming in through the common Sos-Ras entry point that effects a distinct response from the MAPK pathway? This question is exemplified when one considers that all three, mammalian isoforms of the MAPKKK, Raf, share a common upstream Ras, (and MEK is the only commonly accepted downstream substrate).

In light of the properties described, we, among others, hypothesize that the assembly of protein complexes as the initial cytoplasmic signaling event provide a way in which commitment to a given downstream response could be regulated by temporal effects. As described above, the formation of a tight complex of interacting proteins provides a well-protected environment from which the disruptive activity of phosphatases is largely banished. This will allow the control of the longevity of a signal in a reproducible and more reliable way than in a bimolecular binding event that is more prone to the fluctuating concentrations of specific phosphatases. Thus, the length of a given impulse via the Sos/Ras entry point will affect the time of activation of the GTPase. This will then influence the sequential kinase temporal activity of the MAPs and ultimately modulate the MAP kinase signal. For example, an extended signal time will ultimately lead to an increase in concentration of the MAP kinases which can have the following effects: (1) increased transcription directly through MAPK interaction, (2) an increase in phosphorylation of transcription factors via interaction with MAPK,[32] (3) increased time for interaction with scaffold proteins (see below), (4) increased potential to interact with other molecules, e.g., through parallel processing (see below), and (5) increased possibility of proofreading of the signal via other protein interactions.[33] One major role of the protein assembly at the receptor, therefore, is to propagate a signal of the required length to allow the MAP kinase pathway to commit to a given downstream effect.

There is a growing body of evidence that supports the idea that temporal control of activation of the MAP kinase pathway at the point of entry is important in dictating cellular response. After the entry into the pathway, there are a number of potential variations in protein interactions that could modulate the response. For example, there are a number of different isoforms of Raf. The differential activation of Raf isoenzymes by Ras family proteins has been demonstrated in PC12 cells. Neuronal differentiation of these cells is triggered by neurotropic factors, such as nerve growth factor (NGF), which can support the long-lasting activation of the ERK pathway. In contrast, factors such as EGF that elicit transient ERK activity are mitogenic. Both EGF and NGF induce transient ERK activation via Ras and Raf-1, but the latter can invoke the sustained activation of the ERK pathway via the activation of B-Raf by Rap1.[34] In addition there is the potential for interaction with scaffold or subsidiary proteins such as KSR[35−37] and MP1[38] that are hypothesised to be able to exert control over the activity of the MAP kinase components. However, all of these variations have to be dictated by the input signal at the point of entry through SOS/Ras and, as such, need to be able to determine which signal is being received from the early signaling event (i.e., complex formation).

A further example of the importance of temporal control of the MAP kinase pathway is demonstrated in the activation of ERK and RSK in fibroblasts by exposure for 5−10 min to platelet-derived growth factor (PDGF) or epidermal growth factor (EGF). Despite the kinetics and amplitude of activity of ERK and RSK being almost identical, PDGF-treated cells entered S phase, whereas this did not occur with EGF exposure. The rates of inactivation of ERK/RSK were faster in cells treated with EGF (240 min for PDGF and 30−45 min for EGF).[39] Longevity of a signaling pathway and the requirement for some control over this is further exemplified in the immune system where efficient T-lymphocyte response the expression of IL2 requires signaling to be sustained for several hours.[40]

An increasing number of studies have highlighted the possibility of parallel or a neural network-based processing of signals as ways of overcoming lack of specificity in individual linear pathways. So for example the signal coming from the Sos-Ras entry point through the MAP kinase pathway could be modulated by the interaction of a protein from another signaling pathway effecting a distinct downstream nuclear response. Indeed, there are some examples where the decision to commit to a given cellular response is dependent on proteins which are not evoked in the MAP kinase sequence. For example, although the MAP kinase pathway is a major effector of Raf, accumulating evidence suggests that it is not the only one and that Raf-1 is capable of signaling to different downstream pathways. This evidence is based mainly on observations of Raf triggering biological effect in the absence of ERK activation. Activated Raf-1, but not activated MEK mutants, can drive differentiation of rat hippocampal neurons.[41] In addition a number of novel Raf substrates have been inferred, although MEK remains the only widely accepted substrate at present. Additional evidence of parallel processing of signals can be inferred in the growing number of examples of redundancy of pathways. The deletion or mutation of one or more components of a signaling pathway has been shown not to completely knockout the downstream response.

Invoking parallel processing, or neural networking, requires a higher level of complexity in the signaling process. This would require that two or more pathways are stimulated with sufficient control to ensure that they are destined to meet at a "junction point" proteins within a given time interval. Although this is a difficult area to probe experimentally, there is little direct evidence for an extracellular stimulant to a TKR being shown to activate two different pathways that can ultimately meet at the MAP kinase pathway junction point. Nonetheless, the potential for controlled temporal activation of the pathway makes the formation of early signaling complexes in the TK-mediated pathways compatible with both parallel and neural network-based signaling.

Conclusions

Eukaryotic cells have evolved several methods to sustain the active dialogue between cells and their environment, and intracellular signal transduction is a very diverse and, to a large extent, poorly understood field. As a result it is very difficult to give a general description to encompass a type of pathway. In this article we have highlighted the evidence for the appearance of protein complexes as early events in TK-mediated signaling. This does not mean that these complexes are ubiquitous to TK-based events, or that complexes do not appear in other signaling pathways. We have also tried to analyse why this assembly of proteins might have evolved and demonstrate how it has features required in the downstream processing of signals. We suggest that the formation of complexes of proteins as an early signaling event can, via temporal control based on the number and type of proteins associated, be at least partly responsible for the discrimination of signals by the downstream effectors (i.e., by the MAP kinase pathway). The challenge is now to clarify this temporal effect experimentally.

The formation of a complex as an early event in TK-mediated signal transduction presents a major conundrum for the development of drugs to inhibit aberrant pathways. Despite massive investment by the pharmaceutical industry, there has been very limited success in developing inhibitors to early signaling interactions. This can be attributed to several factors. First, the observation of promiscuity in the interactions of the protein domains presents problems for drug binding. The binding surfaces of these domains present very similar topologies,[13] and drug specificity is difficult to achieve. This is further complicated in the SH2 domain interaction by such a significant part of the free energy being attributable to the pY residue. Attempts to replace this with other moieties have to date largely been met with failure.[12] Second, the formation of a complex potentially will effect inhibitor binding by preventing access to sterically hindered target sites. Third, there may well be issues of toxicity associated with the stimulation of different pathways based on the affect that inhibitors may have on temporal activation. Thus, the greater understanding of the role of complexes in the specificity of signal transduction is essential for future pharmaceutical intervention.

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