

Extracellular Signals and Reversible Protein Phosphorylation: What to Mek of It All

Minireview

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The signal transduction pathways that utilize mitogen-activated protein (MAP) kinases (*erk* gene products) have been highly conserved throughout evolution and are critical for the conversion of diverse extracellular signals to biochemical events that are regulated by serine/threonine phosphorylation (Crews et al., 1992). Early studies demonstrated that phosphorylation of both tyrosine and threonine residues is required for full MAP kinase/Erk activity, leading to the suggestion that these enzymes integrate multiple signaling events (Anderson et al., 1990). More recent data show, however, that MAP kinases are phosphorylated on both tyrosine and threonine by a single enzyme(s) termed Mek (previously called MAP kinase kinase) (Ahn et al., 1992). Thus, research into the regulation of MAP kinase is now more appropriately directed at the regulation of Mek activity.

Ras-Raf Interaction

Mek apparently acts as an integration point for at least two different G protein-mediated signal transduction pathways in animal cells; one involves Mek kinase (Lange-Carter et al., 1993), whereas the other pathway utilizes two extensively studied proto-oncogene products, Raf and Ras (see Figure 1). The first clue regarding upstream activators of Mek came from Raf- and Ras-transformed cells, in which MAP kinases are constitutively active. Several laboratories ascertained that in the case of Raf-transformed cells, this constitutive activity is due to the ability of Raf to phosphorylate and activate Mek directly (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992). The use of a dominant negative Raf mutant further delineated the hierarchical relationship between Raf and Ras. Overexpression of such a mutant blocks the mitogenic stimulus from an activated Ras protein, thus demonstrating that Raf is downstream of Ras in this signaling hierarchy (Kolch et al., 1991). How Raf mediates a signal from Ras remains unknown, but recent work suggests several possible mechanisms.

The interaction between Ras and Raf may be direct, i.e., a complex may form between the two molecules, according to work from several laboratories. Three groups have now exploited the yeast two hybrid system and in vitro binding assays to uncover aspects of this interaction. Van Aelst et al. (1993) detected such protein-protein interaction in vivo. They showed that the noncatalytic N-terminus of Raf is sufficient for complex formation with Ras and that mutations affecting the guanine nucleotide-binding or the effector loop domain of Ras disrupt interaction with Raf. Vojtek et al. (1993) have just described a major advance in the two hybrid approach utilizing a cDNA library prepared by random priming and selection for inserts between 350 and 700 bp in length. Thus, only limited

regions of a protein the size of Raf are expressed. When this library was screened for proteins that would interact with Ras, 9 of 19 clones yielding positive signals expressed N-terminal portions of Raf. A region of 81 amino acids is common to all the isolates and extends from amino acid 51 to 131 of c-Raf. As measured by a quantitative β -galactosidase assay, this domain of Raf shows significantly greater interaction than full-length c-Raf. The strong preference for a limited domain of Raf in these assays suggests that Ras-Raf interaction in vivo requires an additional factor(s) to facilitate access of Ras for this region of Raf. The interaction requires Ras-GTP in vivo and in vitro, and Raf is unable to interact with a Ras effector mutant.

In studies utilizing both in vitro binding and the two hybrid system, Zhang et al. (1993) further emphasized that the Ras effector domain is critical for interaction with the N-terminal Raf domain residues (1–257). Mutations in the Ras effector domain reduce Ras-Raf interaction in yeast to about the same extent as they reduce Ras transforming efficiency. Moreover, Ras-Raf interaction greatly reduces the capacity of Ras-GAP to stimulate Ras GTPase, consistent with interaction of GAP or Ras at the same site. Zhang et al. determined that a c-Raf mutant (C168S), previously shown to be defective in responding to Ras, fails to interact as strongly with Ras as wild-type c-Raf (1–257) with in vitro or two hybrid assays. This residue, which lies in a cysteine finger, thus contributes, along with the sequence 51 to 131 mentioned above, to the binding of Ras to Raf. This work has resulted in a dramatic leap in the understanding of Ras downstream signaling and the potential role of Raf in cell proliferation. It will be of great interest if the two hybrid system is able to reveal new components of this pathway.

Stable Raf-Ras interaction has not been demonstrated previously in animal cell extracts; however, Moodie et al. (1993) have demonstrated an association between Ras and Raf in a crude brain extract. They used immobilized Ras as an affinity matrix and showed that Raf, as well as Mek and MAP kinase, are retained on the matrix. This

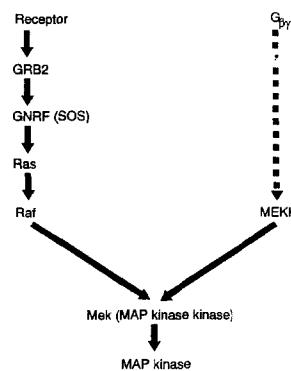


Figure 1. Schematic Representation of the Two Pathways Leading to Mek Activation.

complex formation is GTP dependent and, as found in the two hybrid system, is not detected when a Ras effector domain mutant is used.

Although Ras and Raf interact to form a complex, it is unclear how Raf is activated. Entrance of Ras into the N-terminal domain of Raf may simply expose the catalytic domain as do N-terminal deletions, but none of the studies mentioned above addressed the question of Raf kinase activity. It will be critical to determine if binding of Ras, and perhaps additional factors, increases the capacity of Raf to phosphorylate Mek. It is also possible that Raf binding to activated Ras may simply serve as a mechanism to translocate Raf to the membrane where it can then act as a substrate for an upstream kinase and/or phosphorylate downstream targets. This model of G protein-mediated kinase translocation is not without precedent. The kinase responsible for β -adrenergic receptor down-regulation (β ARK) is activated by binding of the $\beta\gamma$ subunit of the heterotrimeric G protein that is responsible for adrenergic signal transduction (Inglese et al., 1993). In this way, upon agonist addition the $G\alpha$ subunit releases the $\beta\gamma$ subunits to activate its effector molecules, while the isoprenylated $\beta\gamma$ subunit complex binds β ARK, mediating its translocation and thus initiating a negative feedback loop. This analogy is supported by the presence of Mek, a substrate of Raf, in membrane fractions (Crews and Erikson, 1992).

Phosphorylation site mapping of Raf supports the idea that Ras binding alone is insufficient for Raf activation. Morrison et al. (1993) have mapped the mitogen-induced phosphorylation sites of Raf to three serine residues. One of these sites, Ser-621, appears to be critical for Raf activation. Previous reports showed that triple infection of insect cells with baculovirus encoding v-Ras, v-Src, and Raf results in Raf activation (Williams et al., 1992). Morrison et al. (1993) showed, however, that mutation of Ser-621 to alanine abrogates the ability of v-Src and v-Ras to activate Raf in this system, suggesting a role for protein phosphorylation in mitogenic Raf activation.

Raf-Mek Interaction

Unlike most kinase-substrate interactions, which are transient, Raf stably binds to its substrate, Mek, as evidenced in both in vitro and in vivo systems. Van Aelst et al. (1993) found that Mek interacts with the carboxyl catalytic domain of Raf in the yeast two hybrid system. Furthermore, since Ras can interact with the N-terminal domain of Raf, the authors proposed that Raf can act as a bridge to allow Ras and Mek to complex. This hypothesis appears to be valid, at least in this system, since Ras and Mek can interact only when full-length Raf is also expressed. Raf and Mek can also form a tight complex in vitro. Huang et al. (1993) showed that the purification of epitope-tagged Mek from insect cells doubly infected with Raf and Mek recombinant baculovirus yields Mek complexed with Raf. Such a complex can also be formed with immunoprecipitated Raf and purified Mek and cannot be disrupted with 3 M NaCl, 70% ethylene glycol, or 3% n-octyl glucoside. Neither Raf nor Mek catalytic function is required for this tight binding.

In another in vitro system, Moodie et al. (1993) showed that Mek activity, in addition to Raf and MAP kinase, is

selected from a brain extract by immobilized Ras. Furthermore, this association is GTP dependent, and interestingly, both MAP kinase and Mek activities dissociate from the complex upon incubation with ATP. These authors also reported that the removal of Raf from the lysates, by clearing with Raf antibodies beforehand, did not change the ability of the Mek activity to associate with the immobilized Ras. Given the two hybrid system data, in which no interaction between Mek and Ras was detected, these in vitro results suggest the presence of additional proteins in the complex mediating the binding of Mek activity to the immobilized Ras.

Trimeric G Protein Activation and Mek

Although it has been convincingly demonstrated that activation of Ras can stimulate the MAP kinase pathway in some cell types, recent data suggest the existence of a parallel alternative cell type-specific G protein link to this pathway (see Johnson et al., 1993). For example, Gardner et al. (1993) have demonstrated that the heterotrimeric G protein-linked thrombin receptor and the GTPase-inhibited G_i2 mutant, $Gip2$, both activate Mek in a Raf-independent manner in vivo. They propose that the protein mediating the heterotrimeric G protein signal is the recently identified serine/threonine kinase, Mek kinase (MEKK; previously called MAP kinase kinase kinase) (Lange-Carter et al., 1993). Mek kinase was cloned based on sequence similarity with the putative yeast homologs of Mek activators, STE11 and Byr2. Whether there is a direct interaction between one of the subunits of heterotrimeric G proteins and Mek kinase, as described above for Ras and Raf, remains to be determined.

To date, a number of the major players involved in transducing signals to serine/threonine protein kinases have been identified, although description of the links between G proteins and these protein kinases is still incomplete. While the data discussed here suggest that our understanding of G protein-protein kinase links is improving, it should be noted that protein interactions may occur in vitro that do not occur in vivo. There is also a concern that overexpression of normal or mutated proteins in animal cells, a frequently used technique, may engender global changes in the cell, resulting in the alteration of more than one pathway. For example, such experiments may promote interactions that do not normally occur and do not take into account the fact that enzyme:substrate ratios are greatly altered, thus resulting in the aberrant substrate phosphorylation. The multiplicity of pathways in yeast that use Mek and MAP kinase is becoming more evident. Several yeast proteins (i.e., STE5 and STE20) have been genetically identified as effectors of G proteins, but remain to be identified in mammalian cells. Identification and analysis of these proteins should aid in our characterization of the pathways converging on Mek. In both yeast and higher eukaryotes, the redundancy at each level in this pathway further complicates investigation. Placing Mek1, Mek2 (Zheng and Guan, 1993), Erk1, Erk2, and other members of the cast in the appropriate pathway will require in animal cells, as it does in yeast, a combination

of gene deletion, expression of the appropriate mutants of the enzymes, and biochemistry.

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