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How Does the G Protein, G_{i2}, Transduce Mitogenic Signals?

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Abstract Serpentine receptors coupled to the heterotrimeric G protein, G_{i2}, are capable of stimulating DNA synthesis in a variety of cell types. A common feature of the G_{i2}-coupled stimulation of DNA synthesis is the activation of the mitogen-activated protein kinases (MAPKs). The regulation of MAPK activation by the G_{i2}-coupled thrombin and acetylcholine muscarinic M_2 receptors occurs by a sequential activation of a network of protein kinases. The MAPK kinase (MEK) which phosphorylates and activates MAPK is also activated by phosphorylation. MEK is phosphorylated and activated by either Raf or MEK kinase (MEKK). Thus, Raf and MEKK converge at MEK to regulate MAPK. G_{i2}-coupled receptors are capable of activating MEK and MAPK by Raf-dependent and Raf-independent mechanisms. Pertussis toxin catalyzed ADP-ribosylation of α_{i2} inhibits both the Raf-dependent and-independent pathways activated by G_{i2}-coupled receptors. The Raf-dependent pathway involves Ras activation, while the Raf-independent activation of MEK and MAPK does not involve Ras. The Raf-independent activation of MEK and MAPK most likely involves the activation of MEKK. The vertebrate MEKK is homologous to the Ste11 and Byr2 protein kinases in the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* respectively. The yeast Ste11 and Byr2 protein kinases are involved in signal transduction cascades initiated by pheromone receptors having a 7 membrane spanning serpentine structure coupled to G proteins. MEKK appears to be conserved in the regulation of G protein-coupled signal pathways in yeast and vertebrates. Raf represents a divergence in vertebrates from the yeast pheromone-responsive protein kinase system. Defining MEKK and Raf as a divergence in the MAPK regulatory network provides a mechanism for differential regulation of this system by G_{i2}-coupled receptors as well as other receptor systems, including the tyrosine kinases.

Key words: serpentine receptors, G_{i2}, DNA synthesis, MAPKs, MEK

Seven membrane spanning serpentine receptors coupled to the heterotrimeric G_{i2} protein are capable of stimulating DNA synthesis [Pouyssegur and Seuwen, 1992]. Three independent findings support a role of G_{i2} in the control of cell growth. First, mitogenic responses to peptides such as bombesin and thrombin, whose receptors have a seven membrane spanning structure, are inhibited by prior treatment of cells with pertussis toxin [Chambard et al., 1987; Lettorio et al., 1986]. The action of pertussis toxin involves the ADP-ribosylation of the α subunit (α_i) of G_i proteins [Ui, 1984], resulting in the inability of receptors to activate the G_i protein. Second, microinjection of anti-α_{i2} antibodies into Balb/c 3T3 cells prevented DNA synthesis in response to thrombin [Lamorte et al., 1993]. These two findings demonstrate that G_{i2} is required for thrombin receptor catalyzed stimulation of DNA synthesis. The third line of evidence is the phenotypic consequences which result from expression of GTPase-inhibited α_{i2} subunits. The constitutively activated mutant α_{i2} subunits are able to induce a transformed phenotype in Rat 1a fibroblasts [Gupta et al., 1992c; Pace et al., 1991]. Rat 1a fibroblasts expressing the GTPase-inhibited α_{i2} subunit have a decreased doubling time, a diminished requirement for serum, loss of contact inhibition, anchorage independent growth, and are capable of tumor formation in nude mice. These findings indicate a dramatic loss of growth regulation in Rat 1a cells expressing an activated α_{i2} subunit. In other fibroblast lines, such as NIH3T3 and

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Swiss 3T3, the phenotypic consequence of GTPase-inhibited α_{i2} expression is less dramatic and did not induce such a wide range of transformation characteristics [Gupta et al., 1992c]. The activated α_{i2} subunit did, however, alter the growth characteristics and shorten cell doubling times in these two cell types. Cumulatively, the findings indicate that serpentine receptors coupled to G_i proteins or expression of mutant constitutively activated α_{i2} subunits are capable of regulating or altering mitogenic responses in fibroblasts.

What role do G_i proteins play in controlling mitogenesis? Defined signal functions of G_{i2} include inhibition of adenylyl cyclase and regulation of selected K^+ channels. Both of these functions are dissociable from the signal transduction pathways controlled by G_i proteins involved in mitogenesis [Gupta et al., 1992a]. Current thinking and experimental evidence is that G_i proteins directly regulate unique effectors by possibly including tyrosine kinases that are involved in committing a cell to mitogenesis [Seuwen et al., 1990]. These specific effectors have not been identified, but clues are available based on the membrane and cytoplasmic signaling networks regulated by G_i-coupled serpentine receptors and GTPase-inhibited α_{i2} subunits.

Figure 1 shows signal transduction systems that are regulated by the heterotrimeric G_{i2} protein. Following activation by GTP binding, the $\beta\gamma$ subunit complex has the potential to differentially regulate the isoforms of adenylyl cyclase. $\beta\gamma$ subunits directly inhibit the calmodulin-activated type 1 adenylyl cyclase but may contribute to the stimulation of the type 2 and 4 adenylyl cyclase isoforms [Tang and Gilman, 1991, 1992]. The $\beta\gamma$ subunits from G_i proteins may also regulate the PLCβ2 or β3 enzymes contributing to the generation of inositol trisphosphates, calcium mobilization and increased diacyglycerol production. The activated α_{i2} GTP complex inhibits adenylyl cyclase and modulates specific K^+ channels [Freissmuth et al., 1989; Gerhardt and Neubig, 1991; Yatani et al., 1988]. The inhibition of adenylyl cyclase and cAMP synthesis can be dissociated from G_i regulation of mitogenesis. In Rat 1a cells overexpression of α_{i2} results in transformation but not an inhibition of cAMP synthesis (Gupta and Johnson, unpublished observations). In Swiss 3T3 cells, cAMP enhances insulin-stimulated DNA synthesis even though pertussis toxin inhibits DNA synthesis in response to bombesin, thrombin, and serum [Lettorio et al., 1986; Murayama and Ui, 1987; Seuwen et al., 1990; Zachary et al., 1990]. The K^+ channels regulated by α_i subunits are restricted in specific cell types and are not found in fibroblasts.

The α_i polypeptides are myristoylated at their NH_2-terminus [Buss et al., 1987], which is required for their ability to signal in cells [Gallego et al., 1992]. Nonmyristoylated GTPase-deficient mutant α_{i2} polypeptides when stably expressed in Rat 1a cells are membrane associated, but fail to alter growth regulation and induce transformation [Gallego et al., 1992]. This finding indicates that myristoylation is most likely required for interaction of α_{i2} polypeptides with effector enzymes that initiate second messenger responses which regulate growth.

A telling observation in defining mitogenic signal pathways regulated by G_i proteins was that mitogen activated protein kinases (MAPKs) are activated by the thrombin receptor in addition to tyrosine kinase-encoded receptors (i.e., EGF or PDGF receptors). The activation of MAPK occurred in cell types where thrombin behaved as a mitogen. This is a direct demonstration of the convergence of G_{i2}- and tyrosine kinase-linked signal transduction networks. Thrombin receptor activation of MAPK is pertussis toxin-sensitive [Chambard et al., 1987], while tyrosine kinase activation of MAPK is pertussis toxin-insensitive [L'Allemain et al., 1991]. MAPKs are serine-threonine protein kinases found in the cytoplasm, and upon activation a fraction of the MAPK enzyme is translocated to the nucleus [Meloche et al., 1992]. Several sub-

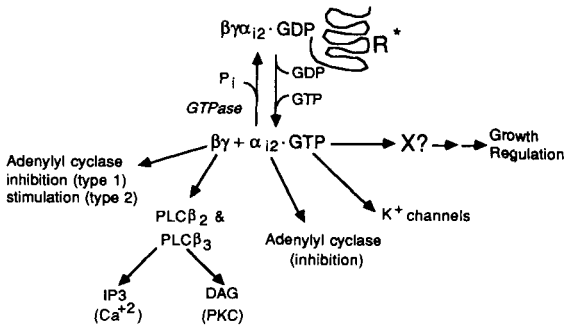

Fig. 1. G_{i2} regulation of specific effector enzymes and ion channels. G_{i2}-coupled receptors (i.e., thrombin and M_2AChR receptors) activate signal transduction by catalyzing GDP/GTP exchange resulting in the dissociation of α_{i2}. GTP from the $\beta\gamma$ subunit complex. Both $\beta\gamma$ and α_{i2}. GTP are capable of regulating specific effectors. The effector(s) directly coupled to α_{i2} involved in transducing mitogenic signals is not defined (see text for discussion).

strates have been identified for MAPKs, including the EGF receptor [Northwood et al., 1991], c-Myc [Seth et al., 1991], c-Jun [Pulverer et al., 1991], RSK90 [Wood et al., 1992] and $cPLA_2$ [Lin et al., 1993; Nemenoff et al., 1993]. All of these proteins are involved in regulating mitogenesis, and the activation of MAPK activity appears pivotal in initiating DNA synthesis in response to G_i-coupled receptors.

Several laboratories have elegantly demonstrated that MAPKs are themselves activated by phosphorylation [Ahn et al., 1990; Gomez and Cohen, 1991; L'Allemain et al., 1991; Ray and Sturgill 1987]. Activation of MAPK requires phosphorylation of the MAPK protein on both tyrosine and threonine [Boulton et al., 1991; Gotoh et al., 1991; Ray and Sturgill, 1988; Robbins and Cobb, 1992; Robbins et al., 1993; Seger et al., 1991; Wu et al., 1991] a reaction catalyzed by the protein kinase referred to as MAPK kinase (MAPKK) or MEK (*M*AP/*E*RK *k*inase] [Ahn et al., 1991; Crews and Erikson, 1992; Gomez and Cohen, 1991; Matsuda et al., 1992; Nakeilny et al., 1992; Rossomando et al., 1992; Seger et al., 1992a,b]. MEK is a dual recognition kinase capable of phosphorylating a tyrosine and threonine on MAPK both of which are required for activation. MEK is itself activated by phosphorylation on serine and threonine residues [Crews et al., 1992; Crews and Erikson, 1992; Kosako et al., 1992, 1993; Wu et al., 1993], effectively establishing a protein kinase cascade whose activity is controlled by tyrosine kinases and G protein-linked effectors.

The pertussis toxin sensitivity of thrombin receptor stimulation of the MAPK system implicated G_i proteins in mediating this response. This prediction was confirmed by demonstrating that expression of GTPase-inhibited α_{i2} constitutively activated MAPK activity in Rat 1a fibroblasts [Gallego et al., 1992; Gupta et al., 1992b]. Interestingly, expression of v-Src also constitutively activated MAPK [Gallego et al., 1992]. These results reinforce the hypothesis that tyrosine kinase and G protein-coupled signal transduction networks converge in the cytoplasm to regulate the MAPK system. The ability of GTPase-inhibited α_{i2} and v-Src to constitutively activate MAPK was not simply a result of transformation because v-Ras expression in Rat 1a cells did not sustain a similar level of MAPK activation, even though Rat 1a cell transformation appeared greater with v-Ras than with either v-Src or GTPase-inhibited α_{i2} [Gallego et al., 1992; Gupta et al., 1992b].

The regulation of MAPK activity by EGF and thrombin involves a common MEK activation [Gardner et al., 1993]. To date we have not observed activation of MAPK without a concomitant MEK activation. This tight linkage between MEK and MAPK activation was also observed with expression of v-Src and GTPase-inhibited α_{i2} in Rat 1a cells. In v-Src and GTPase-inhibited α_{i2} transformed cells the constitutive activation of MEK accounts for most if not all of the increased MAPK activity.

The serine-threonine kinase Raf is capable of phosphorylating and activating MEK [Dent et al., 1992; Howe et al., 1992; Kizaka-Kondoh et al., 1992; Kyriakis et al., 1992; Wood et al., 1992]. Our current findings indicate that c-Raf, immunoprecipitated with an antibody recognizing the C-terminus of Raf, is robustly activated by tyrosine kinase encoded receptors such as those for EGF and PDGF [Gardner et al., 1993]. Raf activation is determined by measuring the ability of the immunoprecipitated Raf to phosphorylate a mutant kinase-inactive MEK [Gardner et al., 1993]. Interestingly, the thrombin receptor endogenously expressed in Rat 1a cells activates MEK and MAPK in a pertussis toxin-sensitive fashion but does not measurably activate Raf (Winitz and Johnson, manuscript in preparation). This finding suggests that Raf-dependent and Raf-independent mechanisms exist for the activation of MEK and MAPK by different growth factors.

The suggestion that a Raf-independent pathway may exist for regulation of MEK and MAPK was evident in studies defining the pheromone response pathway in yeast. Complementation analysis of the pheromone-controlled signal cascade has defined a protein kinase system that regulates the yeast homologs of MAPK. As shown in Figure 2, Spk1 and Fus3-Kss1 are the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* homologs of vertebrate MAPK [Cairns et al., 1992; Nadin-Davis and Nasim, 1988; Wang et al., 1991] Ste7 is the upstream kinase in *S. cerevisiae* that regulates Fus3-Kss1 activity; Byr1 is the *S. pombe* homolog of Ste7. MEK represents the vertebrate homolog of Ste7 and Byr1 [Crews et al., 1992; Seger et al., 1992a,b]. The upstream regulators of Ste7 and Byr1 are Ste11 and Byr2 in *S. cerevisiae* and *S. pombe,* respectively. Ste11 and Byr2 are similar in sequence to one another in their kinase do-

mains. The mammalian serine-threonine protein kinase Raf, which phosphorylates and activates MEK, is unrelated in sequence to Ste11 and Byr2. No yeast homolog of Raf has been identified. Thus, Raf represents a divergence in mammalian cells from the yeast pheromone-responsive protein kinase system.

We have recently identified the mouse homolog of Byr2 and Ste11, denoted MEK kinase (MEKK) [Lange-Carter et al., 1993]. MEKK is a 672 amino acid protein (73 kDa). The primary sequence of MEKK suggests two domains, an NH_2-terminal serine-threonine rich region and a COOH-terminal protein kinase catalytic domain (Fig. 3A). Figure 3B shows the homology of the MEKK COOH-terminal kinase region with the corresponding regions of Ste11 and Byr2. The catalytic domain of MEKK shows approximately 75% similarity and 35% identity with the kinase catalytic domains of Byr2 and Ste11. The NH_2-terminal moieties of MEKK, Ste11, and Byr2 show little similarity in sequence, although the three kinases are of similar size. Of notable interest is the sequence ^{211}PPPSS^{215} in MEKK which is conserved in Ste11. Mutation of the second proline to a serine in the Ste11 kinase results in constitutive activation, presumably because of loss of a negative regulatory function encoded in this region of the protein [Stevenson et al., 1992]. Conservation of this regulatory sequence in MEKK suggests a similar regulatory function, but this idea has not been tested as yet.

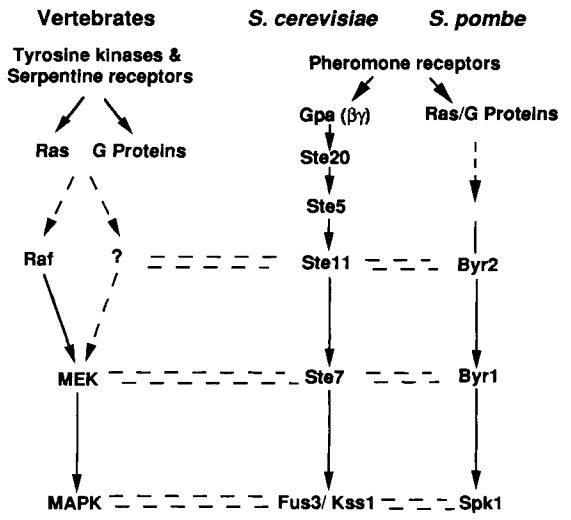

Fig. 2. Comparison of the vertebrate MAPK network to the pheromone-induced mating pathways in yeast. Double dashed lines indicate related protein kinases. MEK, Ste7, and Byr1 are related in sequence and function, while Ste11 and Byr2 are related and regulate Ste7 and Byr1, respectively. The *S. cerevisiae* mating pathway requires the yeast G protein/pheromone receptor, whereas the *S. pombe* mating pathway requires Ras/G protein and pheromone receptor. Raf is unrelated in sequence to either Byr2 or Ste11. Ste20 and Ste5 appear to be downstream of the G protein but upstream of Ste11 in the *S. cerevisiae* mating response pathway.

MEKK was shown to phosphorylate and activate MEK [Lange-Carter et al., 1993]. MEKK activation of MEK is Raf independent, suggesting that MEK is a convergence point immediately upstream of MAPK for various signals initiated at the cell surface. Both Raf and MEKK are capable of phosphorylating and activating MEK. It is clear that tyrosine kinases such as the EGF receptor can activate Raf; it is presently unknown if MEKK is activated by G protein-coupled or tyrosine kinase initiated signals. The conservation of the MAPK system in yeast and mammals demonstrated by the identification of MEKK strongly argues for a G protein role in MEKK regulation. This does not exclude the ability of tyrosine kinases to regulate both MEKK and Raf.

Defining MEKK and Raf as a divergence in the MAPK network provides a mechanism for differential regulation of the system. For example, as proposed in Figure 4, Raf and MEKK both regulate MEK leading to activation of MAPK. However, Raf and MEKK most likely also recognize other protein substrates in the cell, and the recognition of these substrates could be different for Raf and MEKK. Thus, the differential regulation of Raf and MEKK would result in the common activation of MAPK but allow selective regulation of other substrates for the two kinases.

At present there is limited information regarding the differential regulation of Raf and MEKK. Our results suggest that in both Rat 1a and NIH3T3 cells EGF robustly activates Raf, MEK, and MAPK [Gardner et al., 1993]. Thrombin activates MEK and MAPK, albeit 25–50% of that observed with EGF in both cell types, but no Raf activation can be detected in response to thrombin. This result strongly indicates that in a pertussis toxin-sensitive G_i-dependent manner, the thrombin receptor is able to activate MEK and MAPK independent of Raf. Appropriate antisera for immunoprecipitating MEKK and measuring its activity in response to thrombin and EGF are not yet available. It is also unknown if the Raf-independent activation of MEK and MAPK results from MEKK activation. This is obviously a major area of interest for our laboratory.

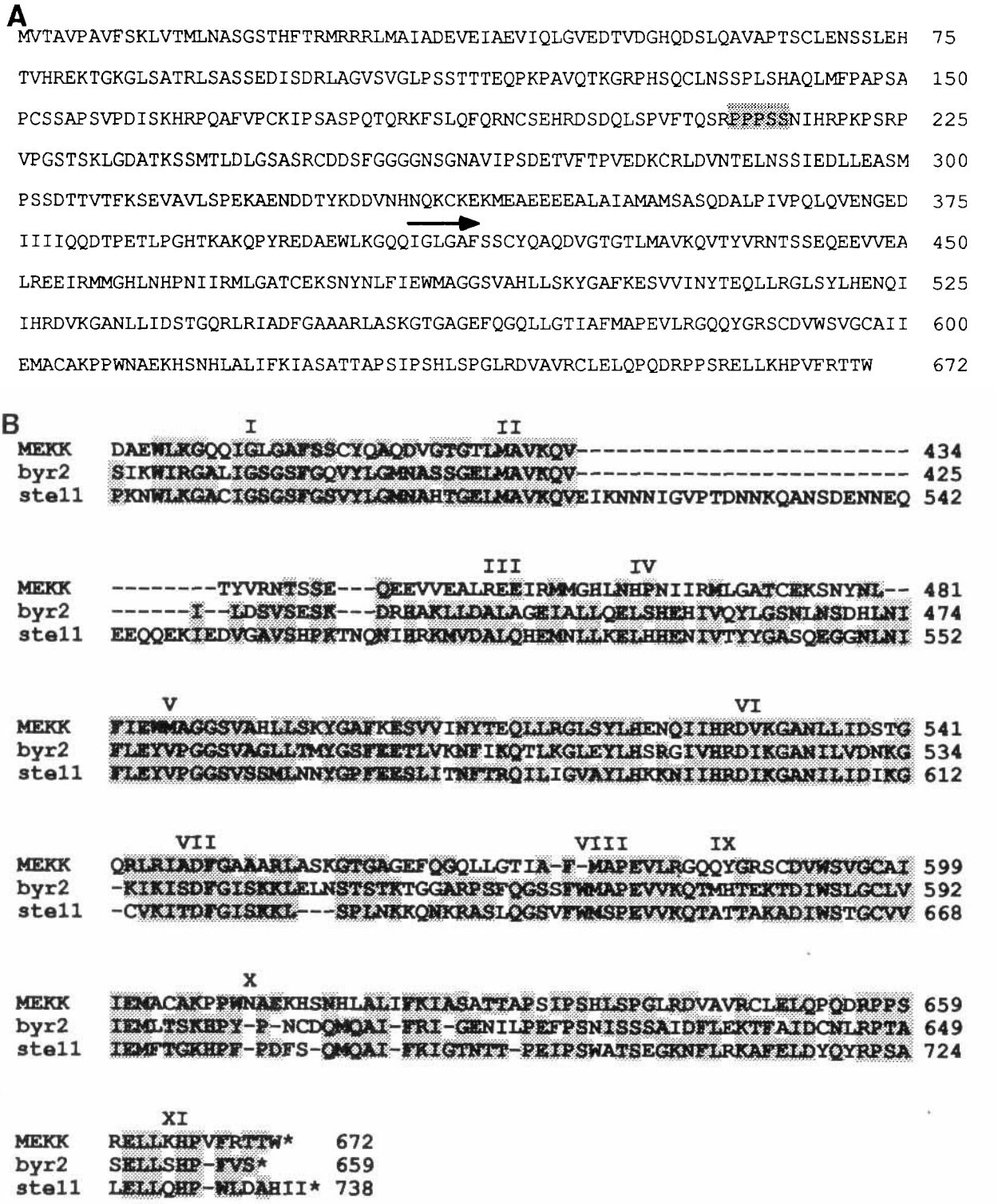

Fig. 3. Amino acid sequence of MEKK and comparison to the kinase domains of Ste11 and Byr2. **A:** MEKK encodes a 672 amino acid protein (73 kDa). The NH_2-terminal half of MEKK is moderately serine/threonine rich (20%). The COOH-terminus encodes the kinase domain (beginning at the arrow). The shaded sequence PPPSS (residues 211–215) is conserved in Ste11. Mutation of the second proline in this sequence to serine in the Ste11 protein kinase results in constitutive activation. **B:** Sequence homology of MEKK, Ste11, and Byr2 kinase domains. COOH-terminal kinase domains of MEKK, Ste11, and Byr2 are approximately 35% identical and 80% homologous.

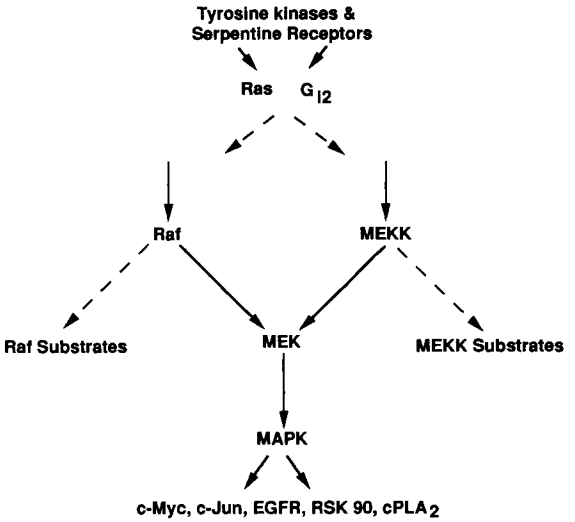

Fig. 4. Model depicting the convergence of Raf and MEKK regulating MEK activity. MEK is a dual function kinase that uniquely phosphorylates both tyrosine and threonine residues on MAPK required for MAPK activation. Raf and MEKK are serine/threonine protein kinases that are capable of phosphorylating MEK but are predicted to also regulate other substrates. This model predicts a common regulation of MEK and MAPK by Raf and MEKK but allows specific regulation of additional substrates for the two kinases.

The activation of Raf is not exclusive for tyrosine kinase-encoded receptors. A G_i-dependent activation of Raf can be demonstrated with the M_2 muscarinic AChR (M_2AChR) when sufficient receptor numbers are expressed in Rat 1a cells. With M_2AChR transfected Rat 1a clones expressing 1.5–2 $\times$ 10^5 receptors per cell, carbachol activates Ras, Raf, MEK, and MAPK [Winitz et al., 1993]. This response is completely inhibited by pertussis toxin. The M_1 muscarinic acetylcholine receptor (M_1AChR) does not activate this pathway in Rat 1a cells, even though the M_1AChR robustly activates phospholipase C activity. The M_1AChR is preferentially coupled to G_q, in contrast to the M_2AChR which is preferentially coupled to G_{i2}. The difference between M_2AChR and thrombin receptor regulation of the MAPK regulatory network may be related to recruitment of different signal transduction components and receptor number in Rat 1a cells. At modest G_i-coupled receptor numbers the results suggest MEK and MAPK are activated in a Raf-independent pathway. At high receptor number the G_i-linked receptor may recruit additional G proteins or other coupling molecules that are capable of activating the Raf regulatory network. This hypothesis is currently being tested with fibroblasts expressing different numbers of G_i-coupled receptors. If this hypothesis is correct, then increased expression of the thrombin receptor in Rat 1a and NIH3T3 cells may recruit and activate the Raf pathway. Some evidence for this scenario is apparent since the thrombin receptor regulates MAPK in all cell types where it has been examined, while thrombin receptor activation of Ras occurs in only a subset of cell types [van Corven et al., 1993]. In cell types where Ras is activated in response to thrombin it is likely that Raf will also be activated. The availability of recombinant MEK as a substrate for Raf and MEKK allows for the first time the ability to directly assay these regulatory networks.

The effectors directly coupled to G_{i2} and regulated by the α_{i2} subunit that initiate activation of the MAPK regulatory system are presently unknown. Several groups have proposed that one α_{i2} regulated effector is likely to be a tyrosine kinase. Indirect experimental findings support the hypothesis of an α_{i2}-coupled tyrosine kinase. First, in cell types demonstrating a thrombin or lysophosphatidic acid (LPA) stimulated Ras activation, this response is inhibited by the tyrosine kinase inhibitor, genestein [van Corven et al., 1993]. Second, in Rat 1a cells the M_2AChR activates PI3-kinase activity that is immunoprecipitable by anti-phosphotyrosine antibodies (Russell and Johnson, unpublished observations). Third, tyrosine kinase activity can be detected in anti-phosphotyrosine immunoprecipitates from extracts prepared from cells stimulated with neuropeptide mitogens such as bombesin and vasopressin [Zachary et al., 1992]. The receptors for these peptides have a seven membrane spanning serpentine structure and are coupled to G_i and G_q proteins. It remains, of course, possible that the G_i-linked effector is not a tyrosine kinase and that regulation of tyrosine kinase activity is indirect and involves an intermediate second messenger.

The G protein-coupled effector in *S. cerevisiae* that initiates the pheromone-induced activation of the MAPK activation pathway has not been defined. Yeast have not evolved the growth factor-regulated membrane-associated tyrosine kinases including the *src* kinase family. This suggests that it is very unlikely that the yeast G protein-linked effector encodes a tyrosine kinase. The identification of the mouse MEKK enzyme substantiates the conservation in regulation of the MAPK system in mammals and yeast. Thus, it is possible that the G_{i2}-linked effector activating the MAPK system via a Raf-independent pathway in mammals is not a tyro-

sine kinase. The search for unique G_{i2}-regulated effectors may provide the unexpected, such as a serine-threonine kinase or even a phosphatase directly linked and regulated by G_i proteins. Identification of the G_i-coupled effector that initiates activation of the MAPK system will allow us to define the mitogenic response network controlled by G proteins.

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