Review

Signal transduction via the MAP kinases: Proceed at your own RSK

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ABSTRACT An explosion of new information linking activation of cell surface signal initiators to changes in gene expression has recently emerged. The focus of much of this information has centered around the agonist-dependent activation of the mitogen-activated protein (MAP) kinases. Although this intracellular signal transduction pathway is extremely complex, conservation of many of its components has been observed in yeast, nematodes, *Drosophila*, and mammals. Thus, these signaling proteins may participate in the regulation of a variety of cellular processes.

How cellular regulatory information generated at the plasma membrane as changes in protein-tyrosine phosphorylation is converted into altered protein-serine/threonine phosphorylation is of great interest in the cell-cycle field. One approach used to understand this process is to start with a defined, growth-regulated phosphorylation event (e.g., the phosphorylation of the S6 protein of the 40S ribosomal subunit), identify its regulating kinase(s), and then work back to the original signal transducer. Several years ago an ≈90-kDa cell cycle-regulated S6 protein kinase (now referred to as pp90^{rsk} or RSK) was identified and shown to be regulated by serine/threonine phosphorylation (for review see refs. 1 and 2). Soon after, a mitogen-activated protein kinase [referred to as MAP kinase or extracellular signal-regulated kinase (ERK)] was identified that phosphorylated and partially reactivated dephosphorylated RSK (3). Excitement came when it was demonstrated that MAP kinases required tyrosine phosphorylation for activation (4). That MAP kinase might be the "switch kinase" responsible for tyrosine-to-serine/threonine phosphorylation was appealing and amazingly simple. However, any pathway potentially important in the regulation of cell proliferation is likely to be complex and to contain many checks and balances. A flurry of reports in the last year demonstrated just that. Here I discuss some of these studies, with their various caveats, that provide new information aiding in the further biochemical and molecular dissection of the complex signaling systems regulating MAP kinases and RSK.

The observation that protein-tyrosine kinases such as the insulin receptor and v-Src were unable to activate MAP kinase *in vitro* suggested that MAP kinases might not be the switch kinases as originally believed and that there were additional signaling molecules between MAP kinases and the receptor tyrosine kinases (for review see refs. 5–8). The purification and cloning of a growth-regulated MAP kinase/ERK-activating kinase (referred to here as MEK; see ref. 9), which exhibited dual specificity (capable of phosphorylating both tyrosine and serine/threonine), strengthened this concept (9–19). Surprisingly, MEK was shown to be regulated by serine/threonine phosphorylation (11, 14, 18). Although the identification of MEK brings us a step closer in the signaling pathway to receptor tyrosine kinases, it also indicates additional intervening steps in the molecular link between tyrosine and serine/threonine phosphorylation. The complexity of the MAP kinase/RSK signaling system became more apparent when several laboratories demonstrated that c-Ras mediates transmission of activating information from receptor tyrosine kinases to the MAP kinases (20–26). One of these studies also demonstrated that c-Raf is downstream of c-Ras (23). Evidence that c-Raf might be the kinase responsible for activation of MEK was subsequently presented (27–29). With this information, these enzymes can be arranged into a linear pathway in which an activated cytoplasmic or receptor tyrosine kinase indirectly activates c-Ras, which leads to the activation of c-Raf, then MEK, then MAP kinase, then RSK, finally resulting in increased S6 phosphorylation. Unfortunately, it is not so simple (Fig. 1 and Table 1).

RSK, MAP Kinase, and MEK. Although RSK was purified on the basis of its capacity to phosphorylate the ribosomal protein S6 *in vitro*, this protein is apparently not the physiological substrate for RSK. This job is the responsibility of the 70-kDa S6 protein kinases, which are regulated via a distinct signaling system (30–32). Indeed, MAP kinase and RSK may have roles in regulating protein phosphorylations (other than S6 phosphorylation) in the nucleus as well as in the cytoplasm (for review see refs. 33–35). RSK, although coordinately reg-

ulated and spatially distributed with MAP kinase (36), may also be phosphorylated/regulated by other protein kinases/phosphatases. In support of this, earlier studies indicated that the kinetics of RSK phosphorylation did not exactly correlate with MAP kinase activation/inactivation (37), and a growth-regulated protein kinase activity which was distinct from the MAP kinases but capable of phosphorylating recombinant RSK *in vitro* has been detected (36). Further support for the existence of additional RSK-kinase activities comes from experiments using chicken embryo fibroblasts expressing a temperature-sensitive transformation mutant of v-Src. After activation of v-Src by transfer of cells to the permissive temperature, RSK (unlike the MAP kinases) undergoes two distinct activation phases. The early, transient activation of RSK is coordinate with the activation of MAP kinases, but the prolonged, late RSK activation is coordinate with activation of a 63-kDa protein-serine kinase that phosphorylates myelin basic protein (38). Like ERKs 1 and 2 [the predominant MAP kinase isoforms in most animal cells (6, 39)], this 63-kDa enzyme may be involved in the regulation of RSK. Whether this 63-kDa enzyme is related to the as yet unidentified *erk-3* gene product (39) and/or regulated by a mechanism different from that of ERKs 1 and 2 remains to be determined.

The fact that MEK potently activates MAP kinase *in vitro* supports a direct link between MEK and the MAP kinases (10–12, 17). In contrast, MAP kinase will only partially activate RSK *in vitro* (15–30% maximal; see refs. 3 and 40). Yeast genetics also link MEK and the MAP kinases (STE7 or byr1 for MEK and FUS3/KSS1 or spk1 for MAP kinase; for review see ref. 41). However, even the relationship between MEK and MAP kinase may not be so simple. It is conceivable that several mammalian MEK homologs (as is the case for the MAP kinase and MEK homologs in yeast) exist that differentially participate in the regulation of various MAP kinases (e.g., because of differences in intracellular location). Additionally, there is limited evidence suggesting that an autokinase-enhancing factor that stimulates MAP kinase activation may exist that itself is not a kinase (42). Thus it is possible that MAP kinases
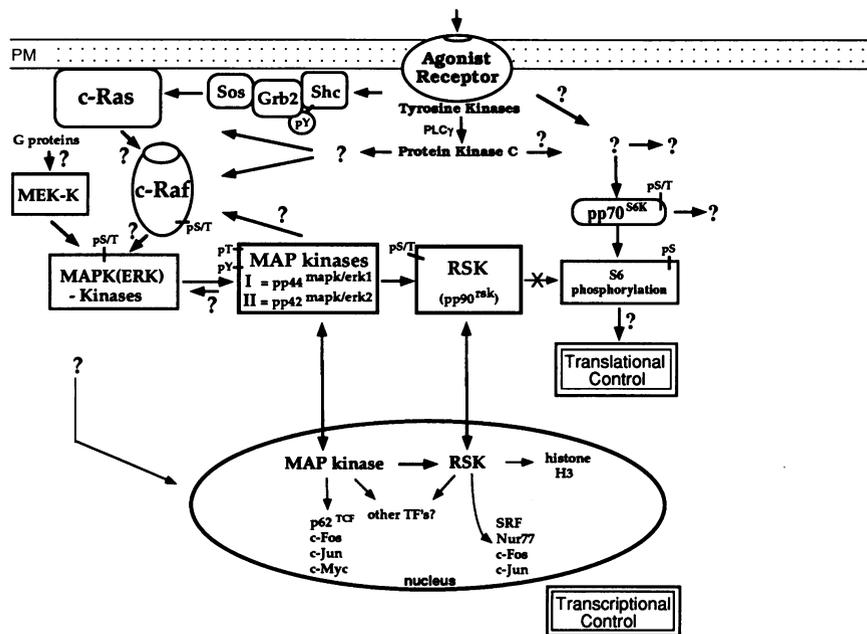
FIG. 1. Model for signal transduction pathways regulating MAP kinases and RSK. Greater detail for this model is provided in the text and in Table 1. For simplicity, some of the possible regulatory feedback loops described in the text are not shown. Briefly, the Src homology 2 (SH2)-adaptor protein Shc is tyrosine-phosphorylated in response to growth factor-activated tyrosine kinases. The tyrosine-phosphorylated (pY) Shc then interacts with Grb2 via its SH2 domain. Alternatively, Grb2 may directly interact with activated receptors. It has been proposed that Grb2/Sem-5 then modulates Ras, perhaps by recruiting Ras effector molecules such as Sos to the plasma membrane. Activated Ras then activates c-Raf (by an as yet unknown mechanism) and the serine/threonine/tyrosine phosphorylation cascade within which reside MEK, MAP kinases, and RSK. This cascade may also be regulated by heterotrimeric G proteins via activated MEK kinase. How protein kinase C participates in the activation of this cytoplasmic phosphorylation cascade is not clear. MAP kinase and RSK have been shown to be cytoplasmic and nuclear and possess the potential for directly regulating gene expression by transcription factor (TF) phosphorylation. It is not yet clear whether Raf, MEKK, or MEK can signal directly to the nucleus independently of MAP kinase and RSK. PM, plasma membrane.

could be activated by various mechanisms regulated by different stimuli and/or in different cell types.

MAP Kinase Kinase Kinases: The Role of Raf and MEK Kinase. Although c-Raf has been shown to activate MEK in vitro (27–29), it may not be the growth factor-regulated activator of MEK in all circumstances. Evidence that c-Raf mediates signaling from c-Ras to MEK/MAP kinase includes *Drosophila* genetic studies (43, 44), in vitro reactivation experiments (27–29), and baculovirus expression studies in insect cells (45). However, other results indicate that c-Raf may not directly phosphorylate and/or be the sole activator of MEK. First, in rat PC12 pheochromocytoma cells, inducible expression of activated Raf does not result in significant activation of MAP kinase and RSK, whereas the same experiment using inducible expression of activated Ras does (23). Second, v-Raf-transformed Rat-1 fibroblasts do not exhibit activated MAP kinase, whereas v-Raf-transformed NIH 3T3 mouse cells do (46). Third, c-Raf hyperphosphorylation (frequently employed to indicate c-Raf activation) does not correlate with the

rapid phosphorylation/dephosphorylation (activation/inactivation) of MAP kinases in PC12 cells (23), and although c-Raf is phosphorylated and activated in murine interleukin-2-dependent T cells (47, 48), MAP kinase and RSK are not (49, 50). Finally, although the MAP kinase/MEK connection is recapitulated in yeast, c-Raf yeast homologs have not been identified (41). Does this mean that c-Raf is not upstream of MEK? The answer is still not clear. Extrapolation of the *Drosophila* results in combination with the in vitro and in vivo work described above provides convincing evidence for the existence of such a relationship. However, these apparently conflicting results indicate that the connection between c-Raf and MEK is certainly not simple.

How can we account for these discrepancies? Perhaps inhibitors of signal transduction between c-Raf, MEK, and MAP kinase are expressed in a cell type-specific manner. For example, an immediate-early gene (*3CH134*) product recently identified as a protein phosphatase has been shown to exhibit specificity toward the phosphorylated tyrosine re-

quired for MAP kinase activation as opposed to other tyrosine-phosphorylated substrates (51). It is noteworthy that 3CH134 protein expression is coordinate with inactivation of MAP kinase activity following mitogen stimulation in 3T3 cells. In *Schizosaccharomyces pombe*, a cellular inhibitor (the *mei3*[+] gene product) regulates the ran1[+] kinase, which mediates signal transduction from ras1 to byr1 (52). Differential expression of mammalian homologs of mei3[+] or 3CH134 could account for the apparent block in c-Raf-to-MAP kinase signaling seen in some cell types. Additionally, other upstream, MEK-activating kinases may exist that at physiological concentrations act in concert with Raf to fully activate MEK. On the basis of this hypothesis, knocking out one coactivator would block downstream signaling (and would account for the genetic data) or overexpressing one might compensate for the other with regard to activation of the pathway (see ref. 35 for a discussion of the potential effects of signal synergism). Indeed, in addition to c-Raf, a novel MAP kinase kinase kinase or MEK kinase (MEKK) has been identified with sequence homology to *S. cerevisiae* STE11 (70). It has been proposed that each of these MAP kinase kinase kinases (c-Raf and MEKK) can individually phosphorylate and activate MEK in response to various extracellular stimuli in different cell types. It is also possible that under certain conditions both enzymes may contribute to and be necessary for maximal activation of the MAP kinase signaling system.

MAP kinases have been shown to phosphorylate c-Raf at a subset of sites phosphorylated in vivo (53, 54) and to also phosphorylate MEK in vitro (71). Although phosphorylation of these sites is not regulatory in vitro, they may have an important physiological role that, for example, provides for a cyclic relationship (generating amplification or inhibitory feedback loops) among c-Raf, MEK, and MAP kinase (Fig. 1). Phosphorylation and activation of Raf may also require multiple input signals, both Ras-dependent and Ras-independent (23, 35, 55). Much work is still needed at the cellular, molecular, and biochemical levels to further define the relationship between these protein kinases during the signaling process.

G Protein/Ras Signaling Studies. The role of Ras in mediating signal transmission from several receptor tyrosine kinases to the serine/threonine kinases (Raf, MEK, MAP kinase, and RSK) has been examined by using dominant interfering Ser[17] → Asn mutants of Ras [(S17N)Ras; refs. 20–23] as well as by the expression of activated Ras (24, 25). However, activation of MAP kinases may not be entirely Ras-dependent and,

Table 1. A partial list of the proteins, with some of their known properties, that participate in the $G_0 \to G_1$ transition as shown in Fig. 1

Signaling molecule(s)	Activity	Function/location
Shc	Src homology 2 (SH2)-adaptor proteins	Transmit signal from agonist receptor to c-Ras
Grb2/Sem-5	No known catalytic activity	Overexpression leads to transformation
Sos (Son-of-sevenless)	Guanine nucleotide exchange factor	Regulates c-Ras following recruitment to plasma membrane
c-Ras	GDP/GTP binding GTPase	Mediates signal transduction from receptors to Ser phosphorylation cascade; cellular homolog of v-Ras
c-Raf	Ser/Thr kinase	Growth-dependent regulation of Ser/Thr phosphorylation; cellular homolog of v-Raf
MEK kinase (MEKK or MAP kinase kinase kinase)	Ser/Thr kinase	Heterotrimeric-G-protein-regulated protein kinase
MEK (MAP kinase/ERK-activating kinase) or MAP kinase kinase	Dual-specificity kinase (Ser/Thr/Tyr kinase)	Growth-dependent regulation of Ser/Thr/Tyr phosphorylation
MAP kinase (mitogen-activated protein kinase) or ERK (extracellular signal-regulated kinase)	Dual-specificity kinase	Growth-dependent regulation of Ser/Thr phosphorylation; autophosphorylates on Tyr at regulatory site; cytoplasmic and nuclear
RSK (family of 85- to 92-kDa ribosomal S6 kinases)	Ser/Thr kinase	Growth-dependent regulation of Ser/Thr phosphorylation; cytoplasmic and nuclear
SRF (serum response factor)	Transcription factor*	Regulates serum response element (SRE)-mediated transcription
p62TCF (ternary complex factor)	Transcription factor*	Regulates SRE-mediated transcription
c-Fos	Transcription factor*	Regulates AP-1 and SRE-mediated transcription; cellular homolog of v-Fos

*These transcription factors represent a partial list of potential nuclear targets of this Ser/Thr phosphorylation cascade.

like Raf, may exhibit some cell-type specificity. For example, in rat PC12 cells activation of MAP kinases via phorbol ester-dependent activation of protein kinase C was blocked by (S17N)Ras (22, 23) or by overexpression of GTPase-activating protein (26). However, protein kinase C-dependent activation of MAP kinase was not antagonized by (S17N)Ras in Rat-1 fibroblasts (20). Similarly, blocking Ras activation does not effect G protein (AlF$_4^-$)-mediated activation of MAP kinase (21, 26). However, signal transduction by a Pertussis toxin-sensitive heterotrimeric G_i protein is mediated by Ras (46, 56), and Ras activation by G_i requires activation of an intermediate protein-tyrosine kinase (56). Thus, even G protein-mediated signaling to the MAP kinases can occur through Ras-dependent and -independent pathways. These data are also consistent with the possibility that MEKK and c-Raf can work independently or together to activate MEK by various agonists.

Because of the importance of Ras in normal cell proliferation, differentiation, and tumorigenesis, the mechanism by which it is activated and then transmits information is the subject of intense study. In addition to yeast and Drosophila genetics, the use of Xenopus oocyte cell-free extracts to study signaling to MAP kinase may provide yet another system for biochemically teasing out the Ras regulators and effectors. Cell-free extracts from G_2/M-arrested oocytes retain the capacity to respond to cell-cycle activators, including activated Ras, cyclin A, and cyclin B, as assessed by activation of MAP kinase activity (57–59). Such a system can be used to monitor

the ability of other putative growth-regulating molecules to modulate the MAP kinase signaling pathways. For example, genetic and biochemical evidence supports the notion that CDC25-like guanine nucleotide exchange factors (similar to the product of the Drosophila gene Son-of-sevenless, Sos) are important in signal transduction from tyrosine kinases to Ras (60, 61). Using other approaches, several laboratories have now provided evidence that a mammalian Sos does indeed regulate Ras signaling (72). Similarly, SH-PTP2 (an SH2-containing protein-tyrosine phosphatase similar to the product of the Drosophila gene corkscrew) may act in concert with Raf in signal transduction by receptor tyrosine kinases (62, 63). The ability of these and other proteins (like the SH2/SH3-adaptor proteins discussed below) to regulate MAP kinase activity may be tested and/or confirmed in the Xenopus cell-free system by simply pipeting in the activated proteins. Similarly, the need for Ras, Raf, or other signaling proteins in the transduction system can be monitored with the addition of dominant interfering mutant proteins or with affinity-purified neutralizing antibodies. Further, a variety of pharmacological reagents may be useful to dissect these pathways in in vitro and in vivo systems. The cell-free extract system may also be amenable for use as an assay system for the purification of novel participants (both upstream and downstream of Ras) in the pathway(s) regulating MAP kinase. A preliminary study using this assay system has provided evidence for a novel protein factor for p21^{ras}-dependent activation of MAP kinase (59).

SH2 Domains. Protein-tyrosine phosphorylation is known to be involved in modulating the activity of some target enzymes as well as in generating specific protein complex networks involved in signal transduction via various SH2 and SH3 domains (for review see refs. 64–66). With regard to the mechanism of signal transduction from tyrosine kinases to c-Raf, MEK, MAP kinase, and RSK, the evidence supports the requirement for tyrosine phosphorylation and specific protein complex formation, as opposed to tyrosine phosphorylation and activation of a "switch" protein-serine/threonine kinase. For example, the tyrosine phosphorylation of a SH2-containing protein (p46^{shc}, p52^{shc}) which has no known enzymatic function catalyzes complex formation with a SH2-containing nonphosphorylated polypeptide, Grb2/Sem-5 (67). The grb2/sem-5 gene products are implicated in activation of the Ras pathway by tyrosine kinases in both Caenorhabditis elegans and mammalian cells (68, 69). Overexpression of Shc proteins in PC12 cells leads to neurite outgrowth, and this response is blocked by expression of the (S17N)Ras dominant interfering mutant (67). Thus, the Ras-mediated activation of MAP kinases, Raf, and RSK by receptor tyrosine kinases can apparently be mediated by the tyrosine phosphorylation of Shc, followed by its binding to the SH2 domain of Grb2. Rozakis-Adcock et al. (67) hypothesized that a non-SH2 region of Grb2 may regulate Ras via some interaction with guanine nucleotide exchange factors. Indeed, recent evidence from several laboratories supports this hypothesis (for review see ref. 72). The mammalian ho-

molog of the *Drosophila* Ras activator, Sos, has now been shown to bind to the SH3 domains of Grb2. Grb2, by binding to tyrosine-phosphorylated Shc or activated receptors, effectively concentrates Sos in the vicinity of the plasma membrane-associated c-Ras. Sos converts the inactive, Ras·GDP form to the active Ras·GTP form and, by a mechanism which is still unclear, activates the cytoplasmic protein phosphorylation cascade described above.

As indicated by the title of this review, there is great risk in trying to describe the ever-expanding, complex signaling network involved in the regulation of MAP kinases. Fig. 1 is one attempt to represent this pathway schematically and has been referred to throughout the text and in Table 1. Additional studies have recently identified several substrates for MAP kinase and RSK where phosphorylation appears to modulate function. Space limitations prevent description of these results here. Further characterization of the tyrosine kinase/Ras-mediated activation pathways regulating the MAP kinases and RSK is awaited with interest— and yes, the map will become even more complex.

When writing a review with space and reference limitations, one is at RSK of ERKing many of one's colleagues. I have cited many recent reviews which I am sure contain your references and I apologize for those that I have overlooked. After submission of this review, no fewer than eight (and probably more by now) papers describing the role of Sos in Ras signaling have been published. These studies are briefly summaried here and are referenced in the review cited (72). I thank members of my laboratory and Drs. Raymond L. Erikson and Malcolm Whitman for their comments and suggestions and Dr. David E. Levin for providing ref. 41 prior to publication.

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