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Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer *in vivo*

(S6 kinase/protein kinase cascade/*Xenopus* oocytes/signal transduction)

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ABSTRACT **Mitogen-activated protein kinases (MAP kinases) are active only when phosphorylated. Here we examine whether the activation of *Xenopus* p42 MAP kinase might involve changes in its association with other proteins as well as changes in its phosphorylation state. We find that when p42 MAP kinase is phosphorylated and active, it is monomeric, and that when p42 MAP kinase is nonphosphorylated and inactive, about half of it is monomeric and half is a component of a 110-kDa complex. We identify Rsk, an 82-kDa protein kinase that can be phosphorylated and partially activated by p42 MAP kinase, as being specifically associated with inactive p42 MAP kinase. It is possible that the complex of inactive p42 MAP kinase and inactive Rsk acts as a single signal reception particle and that the activation of the two kinases may be better described as a fork in a bifurcating signal transduction pathway than as successive levels in a kinase cascade.**

Mitogen-activated protein kinases (MAP kinases), or extracellular signal-regulated protein kinases (ERKs), constitute a ubiquitously expressed, evolutionarily conserved family of signal relayers. Diverse cell surface receptors, including serpentine receptors and receptor tyrosine kinases, can bring about MAP kinase activation. MAP kinase activation accompanies a wide variety of biological events, including mitogenesis in fibroblasts, maturation in oocytes, activation responses in secretory cells, and both mitogenesis and transdifferentiation in PC12 rat pheochromocytoma cells. Possible MAP kinase substrates include cytoskeletal proteins, signal transducers, transcriptional regulators, and housekeeping enzymes (reviewed in ref. 1).

The best studied MAP kinases are the Erk1 (p44) and Erk2 (p42) proteins. Homologs of these proteins have been identified in a variety of animal, fungal, and plant species (reviewed in ref. 2). Many, and possibly all, of these homologs must be phosphorylated at conserved threonine and tyrosine residues for activity (3). Although bacterially expressed MAP kinases can autophosphorylate at these two sites (4–6), it appears that a distinct family of protein kinases, termed Mek proteins or MAP kinase kinases, is responsible for carrying out the rapid increases in MAP kinase phosphorylation that occur in stimulated cells (7). A number of Mek proteins have now been identified in animals and fungi (reviewed in ref. 2).

Changes in protein phosphorylation sometimes bring about, or are brought about by, changes in protein–protein interactions. For example, phosphorylation of specific tyrosine residues in receptor tyrosine kinases allows Src homology 2 (SH2) domain-containing proteins to become associated with the kinases (8). And association of Cdc2 with a cyclin allows Cdc2 to become phosphorylated at specific regulatory sites (9).

Thus it seemed plausible that MAP kinase activation might involve changes in its association with other proteins as well as changes in its phosphorylation state. We have tested this possibility using *Xenopus* oocytes and eggs as a model system. These cells are rich sources of *Xenopus* p42 MAP kinase, and their p42 MAP kinase becomes abruptly and quantitatively phosphorylated during oocyte maturation and abruptly and quantitatively dephosphorylated after egg activation (10–15). In this report we examine whether any change in *Xenopus* p42 MAP kinase's association state accompanies these changes in its phosphorylation state.

METHODS

Isolation and Lysis of *Xenopus* Oocytes and Eggs. *Xenopus* eggs and ovarian tissue were obtained as described (11). Oocytes were defolliculated manually or by treatment with collagenase (16). Oocytes or eggs were lysed and clarified in an equal volume of ice-cold lysis buffer [50 mM glycerol 2-phosphate (pH 7.4)/5–10 mM EDTA/1 mM sodium orthovanadate/2 mM phenylmethylsulfonyl fluoride, sometimes supplemented with 100 μg of leupeptin per ml and 1% aprotinin solution], as described (11).

Gel Filtration Chromatography. Lysates were further clarified by centrifugation at 50,000 rpm for 1 hr in a Beckman TLA 100.3 rotor at 4°C followed by filtration through a 0.45-μm-pore membrane. Samples (100 μl, containing roughly 2.5 mg of total protein) were subjected to gel filtration chromatography at a flow rate of 0.4 ml/min on a Superose 12 column (HR10/30, Pharmacia) equilibrated with lysis buffer. Molecular size standards were thyroglobulin (670 kDa), γ-globulin (158 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.4 kDa).

Sucrose Gradient Centrifugation. Lysates were layered on linear 5–20% sucrose gradients (5.5 ml) made up in lysis buffer. Samples were centrifuged at 50,000 rpm in a Beckman SW50.1 rotor for 12 hr at 4°C. Sedimentation standards were γ-globulin (7.2 S) and ovalbumin (3.5 S).

Antibodies. p42 MAP kinase was detected with a polyclonal antiserum (X15) raised against a 12-amino acid peptide from the *Xenopus* p42 MAP kinase sequence (IFEETAEFQPGY) conjugated through an amino-terminal cysteine residue to bovine serum albumin. Tyrosine-phosphorylated p42 MAP kinase was detected with a polyclonal anti-phosphotyrosine antiserum (11). Rsk was detected with a polyclonal antiserum provided by E. Erikson and J. Maller (17). Mek/MAP kinase kinase was detected with a polyclonal anti-peptide antiserum provided by Y. Gotoh and E. Nishida (18) or with a similar antiserum (no. 662) raised in our laboratory at Stanford.

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Abbreviation: MAP kinase, mitogen-activated protein kinase.

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Immunoblotting. Proteins were separated in SDS/10% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), blocked with 5% nonfat dry milk or 5% bovine serum albumin, and probed with X15 (1:1000), anti-phosphotyrosine antiserum (2 μg/ml), Rsk antiserum (1:2000), or Mek antiserum (1:200). Blots were washed and probed with ^{125}I-protein A as described (11).

Immunoprecipitation. Lysates were clarified further by a second 5-min microcentrifuge spin and then precleared with protein A-agarose beads (GIBCO). The precleared lysate (100 μl) was added to packed protein A-agarose beads coated with X15 or preimmune serum (50 μl; 6 μg of IgG per μl of packed beads), and the suspensions were mixed at 4°C for 40 min. Supernatants were collected and dissolved in 1 volume of 2× Laemmli sample buffer. Pellets were washed twice in lysis buffer and resuspended in 1× Laemmli sample buffer. Equal proportions of the supernatants and pellets were loaded onto SDS/polyacrylamide gels and subjected to immunoblotting.

RESULTS

Dissociation of a p42 MAP Kinase Complex During Oocyte Maturation. We first set out to determine whether the activation of p42 MAP kinase that occurs during oocyte maturation was accompanied by a change in the association of p42 MAP kinase with other proteins. Lysates were prepared from G_2-phase *Xenopus* oocytes and from M-phase, progesterone-treated oocytes and were subjected to gel filtration chromatography on Superose 12. Fractions were analyzed by immunoblotting with anti-MAP kinase antiserum. The gel filtration profiles of G_2-phase and M-phase p42 MAP kinase differed markedly from each other (Fig. 1*A*). Phosphorylated, active, M-phase p42 MAP kinase gel-filtered as a single peak, centered around a molecular mass of about 40 kDa. Nonphosphorylated, inactive, G_2-phase p42 MAP kinase exhibited a broader size distribution, which generally could be resolved into two overlapping peaks centered at 40 kDa and 110 kDa. Typically about half of the G_2-phase p42 MAP kinase was found in each peak. Treatment of G_2-phase lysates with 4 M LiCl caused the high molecular weight p42 MAP kinase peak to disappear (Fig. 1*A*, panel 3).

Phosphorylated p42 MAP kinase can be distinguished from nonphosphorylated p42 MAP kinase by its migration in SDS/polyacrylamide gels, with the phosphorylated forms migrating slightly more slowly than the nonphosphorylated form (7). In a mixture of G_2-phase and M-phase lysates, the phosphorylated p42 MAP kinase gel-filtered as a single peak at 40 kDa, and the nonphosphorylated p42 MAP kinase was divided between 110-kDa and 40-kDa peaks (Fig. 1*A*, panel 4). Thus only nonphosphorylated p42 MAP kinase was found in the high molecular weight form, even under circumstances where phosphorylated p42 MAP kinase had access to G_2-phase cellular factors. Tyrosine-phosphorylated p42 MAP kinase was detectable in M-phase lysates, but not in G_2-phase lysates, and it gel-filtered as a single 40-kDa peak (panels 5 and 6).

Constant-velocity sucrose gradient centrifugation yielded similar results to those obtained by gel filtration (Fig. 1*B*). Nonphosphorylated and phosphorylated p42 MAP kinase exhibited sedimentation coefficients of roughly 6.5 S and 4.0 S, respectively (Fig. 1*B*), and sometimes the nonphosphorylated p42 MAP kinase could be seen to split into distinct 6.5S and 4.0S peaks (see, for example, Fig. 2*B*). Assuming typical partial specific volumes and frictional coefficients, these sedimentation coefficients correspond to roughly 125 kDa and 48 kDa. These values agree reasonably well with those obtained by gel filtration, ruling out the possibility that p42 MAP kinase gel-filters anomalously. The high and low molecular weight forms of p42 MAP kinase must differ in their association states or shapes, not simply in their affinities for Superose 12.

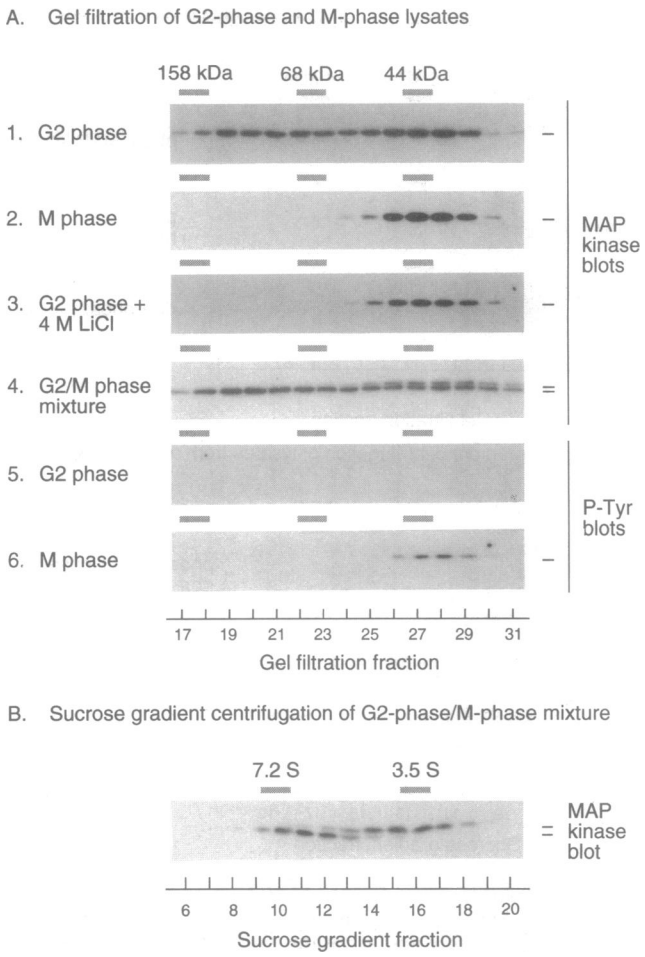

FIG. 1. Dissociation of a p42 MAP kinase complex during oocyte maturation. Fractions from Superose 12 gel filtration (*A*) and sucrose gradient centrifugation (*B*) of p42 MAP kinase in G_2-phase and M-phase *Xenopus* oocyte lysates were analyzed by immunoblotting using antiserum X15 to detect p42 MAP kinase (*A*, panels 1–4, and *B*), or anti-phosphotyrosine antiserum (*A*, panels 5 and 6) to detect tyrosine-phosphorylated p42 MAP kinase. Higher and lower molecular weight fractions were also analyzed; they contained essentially no p42 MAP kinase (data not shown). This figure is a composite of data from four experiments.

There was no change in the total mass of p42 MAP kinase at the G_2–M transition, and p42 MAP kinase was found to turn over only very slowly, as judged by immunoblotting of cycloheximide-treated oocytes (data not shown). These findings imply that the high molecular weight form of p42 MAP kinase is converted to the low molecular weight form at the G_2–M transition, rather than being replaced by it.

Reappearance of a High Molecular Weight p42 MAP Kinase Peak After Egg Activation. p42 MAP kinase is dephosphorylated and inactivated about 30 min after eggs are fertilized or parthenogenetically activated. We set out to determine whether this inactivation of p42 MAP kinase was accompanied by reappearance of the 110-kDa form of the protein. We subjected p42 MAP kinase from metaphase-arrested eggs and from activated, calcium ionophore-treated eggs to gel filtration analysis (Fig. 2*A*) and sucrose density gradient centrifugation (Fig. 2*B*). In the experiment shown in Fig. 2*A*, virtually all of the p42 MAP kinase in metaphase-arrested eggs was found to be phosphorylated, and gel-filtered as a single 40-kDa peak. Thirty minutes after ionophore treatment, about half of the p42 MAP kinase remained phosphor-

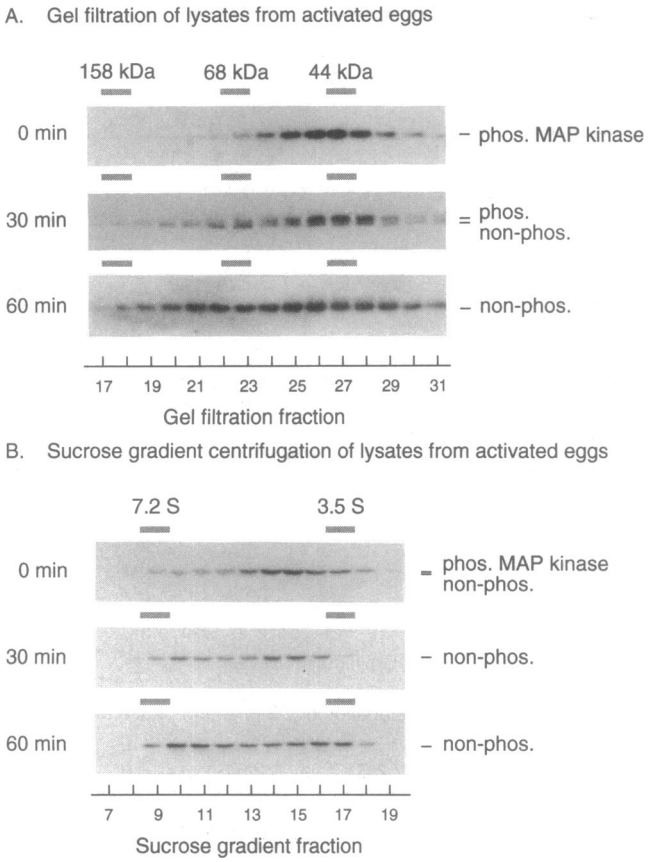

FIG. 2. Reappearance of a p42 MAP kinase complex after egg activation. (*A*) Superose 12 gel filtration analysis of p42 MAP kinase in *Xenopus* eggs before and at various times after treatment of the eggs with ionophore A23187. (*B*) Sucrose density gradient centrifugation of p42 MAP kinase from eggs before and at various times after treatment with ionophore A23187. p42 MAP kinase was detected by immunoblotting with antiserum X15. The sedimentation studies were carried out on a different batch of eggs from those used in the gel filtration studies. phos., Phosphorylated.

ylated and about half had become dephosphorylated. The phosphorylated p42 MAP kinase gel-filtered as a single 40-kDa peak, and the dephosphorylated p42 MAP kinase was distributed between a low molecular weight peak and a high molecular weight peak. By 60 min, only dephosphorylated p42 MAP kinase was seen, and it was distributed between the two peaks. Thus, after dephosphorylation *in vivo*, about half of the p42 MAP returned to the high molecular weight form.

Similar results were obtained by sucrose gradient centrifugation. p42 MAP kinase from metaphase-arrested eggs sedimented predominantly as a single 4.0S peak (Fig. 2*B*). By 30 min after activation, the p42 MAP kinase was completely dephosphorylated and was distributed about equally been 4.0S and 6.5S peaks. This finding again implies that after dephosphorylation *in vivo*, about half of the p42 MAP kinase returns to a high molecular weight form.

Conformation Change, Homocomplex, or Heterocomplex? Bacterially expressed Erk1 was found to gel-filter as a single low molecular weight peak (data not shown). If Erk1 and p42 (Erk2) MAP kinase behave similarly, then the 110-kDa form of p42 MAP kinase probably is not a homodimer, homotrimer, or highly asymmetrical monomer; more likely it is a heterocomplex.

Candidates for the p42 MAP Kinase-Associated Protein. We initially examined p42 MAP kinase immunoprecipitates from ^{35}S-labeled G_2-phase and M-phase lysates but found no obvious MAP kinase-associated protein bands (data not shown). We then examined the gel filtration profiles of two plausible candidates for MAP kinase-associated proteins: Mek, a protein kinase that can phosphorylate and activate MAP kinase; and Rsk, a protein kinase that can be phosphorylated and partially activated by MAP kinase. Both of these protein kinases are comparable in abundance to p42 MAP kinase in *Xenopus* oocytes (12, 17, 19, 20) and both of them must be able to interact at least transiently with p42 MAP kinase.

Gel Filtration of Mek. *Xenopus* Mek has a predicted molecular mass of 44 kDa (21), and migrates at about 44 kDa in SDS/polyacrylamide gels. We found that both G_2-phase and M-phase Mek gel-filtered as single peaks at about 68 kDa (Fig. 3). Essentially no Mek was found in fractions 17–19, which contained substantial amounts of G_2-phase p42 MAP kinase. Immunoprecipitation with anti-MAP kinase antiserum (X15) under mild conditions brought down no detectable Mek, and immunoprecipitation with anti-Mek antiserum (no. 662) brought down no detectable p42 MAP kinase (data not shown). Thus, there is no evidence that a Mek/MAP kinase heterodimer accounts for all or part of the high molecular weight MAP kinase peak, and it is not clear why the 44-kDa Mek protein gel-filters at 68 kDa.

Gel Filtration of Rsk. G_2-phase Rsk gel-filtered as a single peak, overlapping the high molecular weight G_2-phase p42 MAP kinase peak (Fig. 3*A*). The apparent molecular mass of the G_2-phase Rsk peak (110–130 kDa) is in reasonable agreement

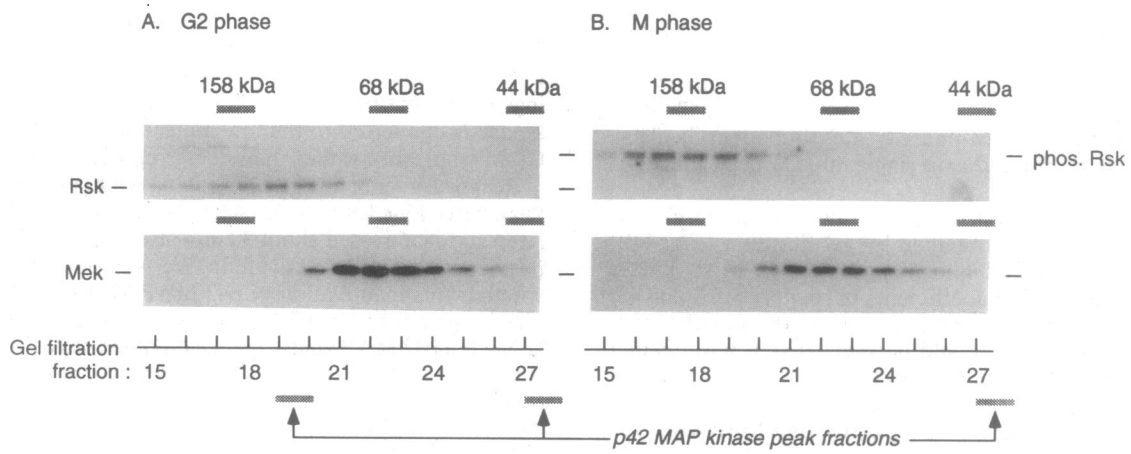

FIG. 3. Superose 12 gel filtration analysis of Rsk and Mek in G_2-phase (*A*) and M-phase (*B*) lysates. Fractions were analyzed by immunoblotting with anti-Rsk (*Upper*) or anti-Mek (*Lower*) antibodies. The peak fractions for G_2-phase and M-phase p42 MAP kinase are shown schematically for comparison.

with the predicted molecular mass of a complex composed of one molecule of Rsk (82 kDa) and one molecule of p42 MAP kinase (42 kDa). These observations suggest that Rsk might be the MAP kinase-associated protein. M-phase Rsk was found to migrate at around 90 kDa in SDS/polyacrylamide gels and gel-filtered with an apparent molecular mass of about 150 kDa (Fig. 3*B*), in agreement with previous reports of the molecular mass of purified, active Rsk (17).

G_2-Phase-Specific Coimmunoprecipitation of p42 MAP Kinase and Rsk. We subjected lysates from G_2-phase and M-phase oocytes to immunoprecipitation with an antiserum to p42 MAP kinase (X15) and used immunoblotting to determine whether Rsk coprecipitated with p42 MAP kinase. The X15 antiserum precipitated p42 MAP kinase equally efficiently from G_2-phase lysates and M-phase lysates (Fig. 4*A*). X15 also precipitated Rsk from G_2-phase lysates, but not from M-phase lysates. Neither protein was precipitated by preimmune serum. Antiserum X15 did not recognize G_2-phase or M-phase Rsk directly in immunoblots (data not shown), and Rsk shows no sequence similarity to the peptide against which X15 was raised. These findings argue that G_2-phase p42 MAP kinase is associated with G_2-phase Rsk.

We carried out similar experiments with a cell-free oocyte extract system (14, 15, 22–24), using okadaic acid to bring about activation of p42 MAP kinase and Rsk. The results were similar to those found in intact oocytes: Rsk coprecipitated with nonphosphorylated p42 MAP kinase from untreated extracts and did not coprecipitate with phosphorylated p42 MAP kinase from okadaic-treated extracts (data not shown). This finding indicates that all components needed to cause the complex to dissociate are present in cytoplasm.

X15 was as effective at clearing Rsk from G_2-phase lysate as it was at clearing p42 MAP kinase, with about 60% of each protein pelleting in each cycle of immunodepletion (Fig. 4*B*). Three rounds of sequential immunodepletion with X15 removed >90% of the Rsk from G_2-phase lysates (Fig. 4*B*), implying that much or all of the cell's Rsk is complexed with p42 MAP kinase.

DISCUSSION

We have assessed the association state of *Xenopus* p42 MAP kinase during oocyte maturation and after egg activation. In agreement with several previous studies (12, 25, 26), we find that active p42 MAP kinase is monomeric. In contrast, roughly half of the inactive p42 MAP kinase is a component of a complex. Another component of the complex appears to be Rsk, and much or all of the cell's Rsk can be coimmunoprecipitated with p42 MAP kinase. If Rsk and p42 MAP kinase are about equal in abundance, as is suggested by purification data (12, 17, 19, 27), then much of the observed complex may be accounted for by Rsk/p42 MAP kinase heterodimers. The combined molecular masses of p42 MAP kinase (42 kDa) and Rsk (82 kDa) are sufficient to account for the observed molecular mass of the complex (roughly 110 kDa).

During oocyte maturation, p42 MAP kinase becomes phosphorylated and activated, and these events are tightly correlated with the release of p42 MAP kinase from the complex. After egg activation, p42 MAP kinase is dephosphorylated and inactivated, and these events are accompanied by its return to the complex. The complex observed here consists of wild-type proteins expressed at natural levels.

Preliminary evidence indicates that mouse p42 (Erk2) MAP kinase is a component of a similar-sized complex in quiescent NIH 3T3 cells and becomes monomeric when phosphorylated (S.-y.C., unpublished data). This finding contrasts with those of Scimeca and coworkers, who have reported that a small proportion of the p44 Erk1 present in PC12 cells can be crosslinked to Rsk and that in response to NGF, which brings about Erk1 activation, the proportion of crosslinked Erk1 increases (28). The discrepancies between the two studies could be due to differences in the MAP kinases or in the cell types or methods used.

Our work demonstrates that the phosphorylation of p42 MAP kinase by Mek is only one step in a multistep process that accompanies activation. A number of events occur roughly concomitantly: phosphorylation of p42 MAP kinase and Rsk, activation of p42 MAP kinase and Rsk, dissociation of the p42 MAP kinase/Rsk complex, and, at least in some cell types, redistribution of p42 MAP kinase and Rsk from the cytoplasm to the nucleus (29, 30) (Fig. 5). Dimerization of Rsk may also accompany its release from the p42 MAP kinase complex, since both active Rsk in crude lysates (the present study) and purified active Rsk (17) gel-filter with apparent molecular masses of about 150 kDa. It should be possible now to determine the order of and causal relationships among the steps in the activation of p42 MAP kinase and Rsk.

The signal relayers upstream and downstream from MAP kinase are often regarded as a cascade. Each sequential level of the MAP kinase cascade could provide signal amplification or be responsible for a discrete temporal or spatial wave of the signal. This model does not fit well with what is now

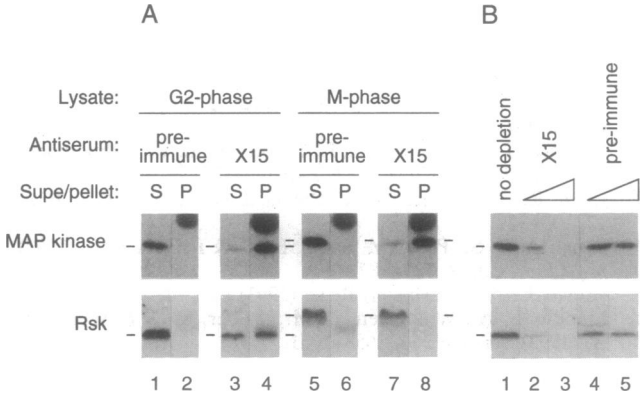

FIG. 4. Coimmunoprecipitation of p42 MAP kinase and Rsk from G_2-phase, but not M-phase, *Xenopus* oocyte lysates. (*A*) G_2-phase or M-phase lysates were immunoprecipitated with p42 MAP kinase antiserum (X15) or preimmune serum. Supernatants (S) and pellets (P) were analyzed by immunoblotting with X15 or anti-Rsk antiserum. (*B*) Exhaustive immunodepletion with antiserum X15, with analysis for the presence of p42 MAP kinase (*Upper*) and Rsk (*Lower*). Lane 1 represents the starting G_2-phase lysate. Lane 2 represents the lysate supernatant after one cycle of immunodepletion with X15. Lane 3 represents the lysate supernatant after three cycles of immunodepletion with X15. Lanes 4 and 5 represent one and three cycles of immunodepletion with preimmune serum.

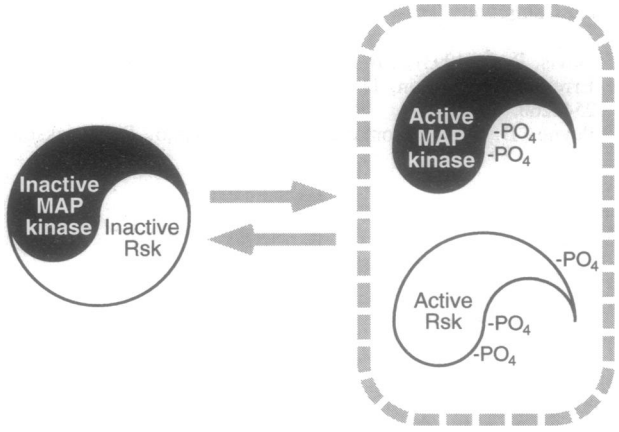

FIG. 5. Schematic depiction of the various events that accompany the activation of p42 MAP kinase and Rsk.

known of the signal transmission between p42 MAP kinase and Rsk: the two proteins may be about equal in abundance, are activated concomitantly, and are concentrated in the nucleus upon activation.

Instead, it may be that the complex of p42 MAP kinase and Rsk acts as a single signal reception particle. Within the complex the inactive proteins could regulate each other in some respect—for example, by subduing each other's autophosphorylation activity, inhibiting each other's access to activators, or regulating each other's localization—or the complex could simply facilitate transmission of a signal from one component to the other. Since the activated kinases undoubtedly interact with distinct target molecules, the activation of p42 MAP kinase and Rsk constitutes a fork in the signal transduction pathway. The scenario we describe here bears some resemblance to the activation of heterotrimeric guanine nucleotide-binding proteins; in both cases, an inactive complex dissociates to yield active subunits with distinct effector functions.

A great deal of evidence has been presented recently showing that various proteins upstream from MAP kinase can interact physically with each other, at least *in vitro* or when overexpressed. Raf, Mek, and MAP kinase all bind to a GTP–Ras column, and GTP–Ras binds to a Raf column; interactions between Raf and Ras and between Raf and Mek can be detected by two-hybrid screening; and coexpression of Raf and Mek in insect cells yields complexes of the two proteins (reviewed in ref. 31). There are indications that some of these interactions may be disrupted by phosphorylation (32), as is the interaction between p42 MAP kinase and Rsk we describe here. It will be of interest to see whether the dissociation of inactive complexes into active molecules is a common theme in this ancient signal transduction system.

Note Added in Proof. We have recently found that an antiserum raised against the C terminus of Rsk (Upstate Biotechnology, Lake Placid, NY) brings down no MAP kinase beyond the low levels seen in preimmune serum immunoprecipitates. It is possible that the antiserum does not recognize the complexed form of Rsk, or that Rsk is substantially less abundant than is suggested by the purification data cited above.

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