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Nuclear Localization and Regulation of *erk*- and *rsk*-Encoded Protein Kinases

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We demonstrate that members of the *erk*-encoded family of mitogen-activated protein (MAP) kinases (pp44/42$^{mapk/erk}$) and members of the *rsk*-encoded protein kinases (RSKs or pp90^{rsk}) are present in the cytoplasm and nucleus of HeLa cells. Addition of growth factors to serum-deprived cells results in increased tyrosine and threonine phosphorylation and in the activation of cytosolic and nuclear MAP kinases. Activated MAP kinases then phosphorylate (serine/threonine) and activate RSKs. Concurrently, a fraction of the activated MAP kinases and RSKs enter the nucleus. In addition, a distinct growth-regulated RSK-kinase activity (an enzyme[s] that phosphorylates recombinant RSK in vitro and that may be another member of the *erk*-encoded family of MAP kinases) was found associated with a postnuclear membrane fraction. Regulation of nuclear MAP kinase and RSK activities by growth factors and phorbol ester is coordinate with immediate-early gene expression. Indeed, in vitro, MAP kinase and/or RSK phosphorylates histone H3 and the recombinant c-Fos and c-Jun polypeptides, transcription factors phosphorylated in a variety of cells in response to growth stimuli. These in vitro studies raise the possibility that the MAP kinase/RSK signal transduction pathway represents a protein-Tyr/Ser/Thr phosphorylation cascade with the spatial distribution and temporal regulation that can account for the rapid transmission of growth-regulating information from the membrane, through the cytoplasm, and to the nucleus.

The binding of growth factors to their appropriate receptors at the plasma membrane results in the generation of signals that traverse the cytoplasm and eventually reach the nucleus, initiating a genomic response that ultimately leads to cell proliferation or differentiation. Much attention has been directed towards improving the understanding of how growth factor receptors initiate signaling and towards the identification of transcriptionally activated immediate-early genes. Although it is clear that protein phosphorylation is an important component in regulating processes such as transcription (9), much about the nature of the enzymes that participate in signal transduction remains unclear. As important as transcriptional regulation is the need to modulate the translational machinery in response to the requirement for new protein synthesis in proliferating cells; again, protein phosphorylation and dephosphorylation likely play a significant role (30). Here we identify two families of mitogen- and oncogene-activated protein kinases whose spatial distribution and temporal regulation are consistent with their participation in regulating gene expression and protein synthesis. These protein kinases are part of a signaling cascade regulated by tyrosine/serine/threonine phosphorylation.

Several protein-serine/threonine (Ser/Thr) kinases that may participate in regulating cell growth and development have recently been identified (20, 37, 42). Among these are two distinct families of 40S ribosomal protein S6 Ser/Thr kinases present in somatic animal cells, pp90^{rsk} (3, 13, 53) and pp70^{S6K} (7, 33). These protein kinases are differentially regulated by a variety of mitogens via distinct signaling cascades (6, 8, 13, 52). pp90^{rsk} is maximally activated within minutes of addition of growth factors or phorbol ester to cultured somatic cells, prior to the maximal activation of pp70^{S6K}. Both enzymes are regulated by serine/threonine phosphorylation, suggesting that protein-Ser/Thr kinases exist upstream in the signaling pathway that regulate these enzymes and that may in turn be regulated by protein-tyrosine (Tyr) kinases or protein kinase C (13, 14, 52). At present, no evidence exists for the putative pp70^{S6K}-protein kinase. However, recent studies have identified mitogen-regulated, pp90^{rsk}-activating protein kinases (16, 51).

These studies showed that a 42-kDa insulin-stimulated, microtubule-associated protein 2 (MAP2) kinase could partially reactivate protein phosphatase-inactivated S6 protein kinase II (51), a *Xenopus* homolog of the pp90^{rsk} family (3, 32). By using a recombinant *rsk* gene product as a substrate, two growth factor-stimulated Swiss 3T3 RSK-protein kinases (I [44 kDa] and II [42 kDa]) have been identified (16). Under every condition tested to date, RSK-kinase activity is coordinately regulated with that of pp90^{rsk}, both RSK-kinases phosphorylate recombinant RSK in vitro at pp90^{rsk} sites phosphorylated in vivo, and both enzymes activate pp90^{rsk} S6 phosphotransferase activity in vitro (8, 15, 16). These results suggested that MAP2 kinase and the RSK-kinases were related. Recently, a 43-kDa insulin-stimulated MAP2 kinase has been purified (10) and its cDNA has been isolated (12). It has been named ERK-1, for extracellular signal-regulated kinase 1 (12). Additional members of the *erk*-encoded family have also recently been identified (11). Using polyclonal antiserum directed against two distinct peptides predicted by the *erk-1* sequence, we have found that RSK-kinases I and II are members of the *erk*-encoded family of protein kinases (16). Furthermore, immunological and biochemical evidence shows that RSK-kinases I and II are also related to a 44-kDa maturation-regulated sea star oocyte myelin basic protein (MBP) kinase (16, 49) and the insulin-activated MAP2 kinase (43, 47). RSK-kinases I and II are also likely related to two MBP kinases (2) or MAP2 kinases (4, 24, 25) recently identified in somatic cells. We

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refer to the ERKs and MAP2/MBP/RSK kinases collectively as MAP kinases or pp44/42$^{mapk/erk}$.

MAP kinases are regulated by both tyrosine and threonine phosphorylation in that both protein-Tyr phosphatases and protein-Ser/Thr phosphatases can separately inactivate these enzymes (2, 5, 16, 27). The mechanism(s) for the activation of the *erk*-encoded enzymes remains to be determined. In addition to activation of the MAP kinase/RSK phosphorylation cascade during the initiation of cell proliferation (G_0/G_1 transition), *Xenopus laevis* and sea star (*Pisaster ochraceus*) homologs are also activated at the G_2/M transition during hormone-induced meiotic maturation of oocytes to eggs (37, 42). It has not yet been determined whether this cascade is also activated at G_2/M during the mitotic cell cycle.

In the present study, we demonstrate that MAP kinases and RSKs are nuclear as well as cytoplasmic protein kinases and that the nuclear (like the cytoplasmic) MAP kinase/RSK signal transduction system is regulated by tyrosine/threonine and serine/threonine phosphorylation, respectively. The nuclear MAP kinase/RSK phosphorylation cascade is activated by mitogens and phorbol ester with kinetics that parallel the activation of immediate-early gene expression (34, 46). Immunofluorescence and biochemical studies indicate that cytoplasmic to nuclear translocation participates in the growth factor-stimulated increase in nuclear-associated MAP kinase and RSK activities. Finally, we show that activated MAP kinases and RSKs efficiently phosphorylate in vitro recombinant c-Fos, recombinant c-Jun, and histone H3, indicating the potential for modification of these nuclear phosphoproteins during the early G_1 phase of the cell cycle. These studies provide for a mechanism of transducing growth-regulating information via multiple growth-regulated protein kinases and a protein-tyrosine/serine/threonine phosphorylation cascade from the extracellular environment to the nucleus. This cascade exhibits the proper temporal regulation and spatial distribution to account for many of the rapid biological responses of cells incubated with a variety of growth factors or phorbol ester or following activation of oncogene products.

MATERIALS AND METHODS

Cell cultures. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (GIBCO) plus 5% calf serum (GIBCO) until confluence. Confluent cells were further cultured in DMEM containing 0.5% calf serum and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.35) for 36 to 48 h prior to serum addition and preparation of cell lysates.

Subcellular fractionations. All the fractionation procedures were completed on ice. The cells were washed twice with STE (150 mM NaCl, 50 mM Tris chloride, 1 mM EDTA [pH 7.2]) and scraped into 1 ml of hypotonic lysis buffer (1 mM EGTA [ethylene glycol tetraacetic acid], 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 40 μg of phenylmethylsulfonyl fluoride per ml, and 10 μg of both pepstatin and leupeptin per ml [pH 7.2]). The cell suspension in hypotonic lysis buffer was incubated on ice for 20 to 30 min to allow swelling. The cells were then dounce-homogenized (20 to 30 strokes) with a tight-fitting pestle. The homogenate was loaded onto 1 ml of 1 M sucrose in lysis buffer and centrifuged at 1,600 × *g* for 10 min to pellet nuclei. To prepare the cytosol fraction (S150), 800 μl of the supernatant was taken above the sucrose cushion and centrifuged at 150,000 × *g* for 30 min. The sucrose cushion-purified nuclear pellet was washed by resuspension in 1 ml of 1 M sucrose in lysis buffer and centrifuged at 1,600 × *g* for 5 min to pellet nuclei. The postnuclear supernatant was combined with the supernatant from the first spin and centrifuged at 150,000 × *g* for 30 min. The two P150 fractions were combined and designated the postnuclear membrane fractions. Both nuclear and membrane pellets were solubilized in hypotonic lysis buffer containing 0.5% Nonidet P-40, 0.1% deoxycholate, and 0.1% Brij-35 and then centrifuged at 10,000 rpm for 5 min to remove insoluble material. Nonidet P-40, deoxycholate, and Brij-35 were added to the cytosol fractions, giving equal concentrations of detergents in all fractions. The purity of each fraction was monitored by measuring the cytosol marker lactate dehydrogenase activity as described before (50).

Protein kinase assays. Assays for the S6 phosphotransferase activity of pp90^{rsk} were performed in the immune complex as described previously (13). RSK kinase activity was measured with a direct cell lysate kinase assay as described before (15, 16).

To analyze the substrate specificity of pp90^{rsk} and MAP kinases, in vitro phosphorylation was performed in pp90^{rsk}-immune complexes and with partially purified pp44$^{mapk/erk1}$ and pp42$^{mapk/erk2}$, respectively. The following proteins (1 μg) or peptides (100 μM) were used in 30-μl reaction mixes: recombinant c-Fos (from T. Curran), recombinant c-Jun (from T. Curran), histones H1 and H3 (Boehringer Mannheim), 40S ribosomal protein S6, recombinant RSK, S6 peptide (Bachem), and a synthetic C-terminal Fos peptide corresponding to amino acids 357 to 367 of c-Fos, which is conserved between human, mouse, and rat and also conserved in chicken (amino acids 345 to 355). All reactions were allowed to proceed for 10 min at 30°C. For protein substrates, the reactions were terminated by adding 30 μl of 2× electrophoresis sample buffer (13) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For peptide substrates, the reactions were terminated by removal of pp90^{rsk}-immune complexes by centrifugation. One microliter of the reaction mixture was spotted onto cellulose thin-layer chromatography plates and resolved by electrophoresis in pH 3.5 buffer (pyridine-acetic acid-H_2O, 1:10:189, vol/vol/vol) for 30 min at 1.0 kV. Phosphorylation of the substrates was visualized by autoradiography and quantitated by liquid scintillation.

Affinity purification of antibodies. A recombinant RSK Affi-Gel-10 column (Bio-Rad) was prepared according to the manufacturer's instructions. The column was washed with 40 ml of PBS (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2HPO_4 [pH 7.2]). Then, 18 ml of crude antiserum was loaded onto a 5-ml column followed by a 24-h wash with PBS, a wash with 20 ml of PBS plus 1 M NaCl, and another wash with 20 ml of PBS. Affinity-purified antibodies were eluted with 16 ml of 0.2 M glycine–1 mM EGTA (pH 1.8). Fractions (2 ml) were immediately neutralized with 100 μl of 10× PBS and 100 μl of 1 M Tris (pH 11.2). The affinity-purified antibodies were further stabilized by the addition of 0.2% fish scale gelatin.

A synthetic C-terminal MAP kinase peptide made according to predicted amino acids 348 to 367 of the *erk-1* gene (12) was used to raise antiserum in rabbits. This antiserum recognizes the denatured forms of pp44$^{mapk/erk1}$ and pp42$^{mapk/erk2}$ but has an apparent higher affinity for native pp44$^{mapk/erk1}$ than native pp42$^{mapk/erk2}$ (not shown). This observation is consistent with pp44$^{mapk/erk1}$ (RSK kinase I)

being the reported *erk-1* gene product. To affinity-purify antibodies, a C-terminal MAP kinase peptide-SulfoLink Gel (Pierce) column was prepared according to the manufacturer's instructions. Two milliliters of rabbit antiserum was continuously loaded onto the 2-ml column overnight, followed by successive washes with several column volumes in 10 mM Tris (pH 7.5), RIPA buffer (10 mM Tris chloride [pH 7.2], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS), high-salt buffer (1 M NaCl, 0.1% Nonidet P-40, 10 mM Tris chloride [pH 7.2]), and 10 mM Tris (pH 7.5). Antibodies were then eluted with 10 ml of 0.2 M glycine–1 mM EGTA (pH 2.8). Fractions (1 ml) were neutralized with 40 μl of 1 M Tris chloride (pH 11.2). Fractions containing antibodies were then pooled and reloaded continuously onto the MAP kinase peptide column overnight. The column was washed with 10 mM Tris chloride (pH 7.5), 10 mM Tris–1 M NaCl, and 10 mM Tris. The antibodies were then eluted with pH 2.8 buffer and neutralized as described above. The twice-affinity-purified antibody was used in the immunofluorescence and immunoblot analyses.

Biosynthetic cell labeling, immunoprecipitation, and phosphoamino acid analysis. HeLa cells were labeled with $^{32}P_i$ (2 mCi/100-mm plate) or [^{35}S]methionine (500 μCi/100-mm plate) as described previously (13). Cell lysates were prepared and fractionated into cytosol, nuclear, and postnuclear membrane fractions as described above. Lysates were then adjusted to 1% SDS, denatured by heating to 85 to 90°C for 5 min, and diluted with 10 volumes of RIPA buffer. Ten microliters of antiserum raised against recombinant RSK protein or C-terminal MAP kinase peptide was added to one-sixth of the lysates, and 7.5 μl of affinity-purified antiserum raised against recombinant Nur77 was used for one-third of the lysates from a confluent 100-mm culture dish. Incubation proceeded for 4 to 5 h at 4°C, and immunoprecipitations were then completed as described previously (13).

For phosphoamino acid analysis, cells were labeled with 4 mCi of $^{32}P_i$ per 100-mm plate, and MAP kinase polypeptides were isolated by immunoprecipitation and further processed for phosphoamino acid analysis as described previously (17).

Immunoblot analysis. For RSK polypeptides, equal proportions (one-sixth) of each fraction (~167, 94, and 61 μg of protein from the cytosol, nuclear, and membrane fractions, respectively) were used, and immunoprecipitations were performed as described for the immune complex protein kinase assay except that after the final wash with ST buffer (13), the immune complexes were resuspended in 30 μl of 1× SDS-PAGE sample buffer and denatured by heating at 90°C for 4 min. For MAP kinase polypeptides, equal proportions (1/30) of each subcellular fraction (~30, 17, and 11 μg of protein for the cytosol, nuclear, and membrane fractions, respectively) were heat-denatured in SDS-PAGE sample buffer. Samples were resolved by SDS–7.5% (for RSK polypeptides) or 10% (for MAP kinase polypeptides) PAGE and then transferred electrophoretically to nitrocellulose membranes (55 V, overnight at 4°C). The nitrocellulose membrane was then stained with Ponceau S to visualize the protein bands. After destaining with several washes of water, the membrane was incubated twice in blocking buffer (PBS containing 0.2% Tween 20 and 2% bovine serum albumin) for 30 min each at 4°C. The nitrocellulose was then incubated for 1 h at 4°C in blocking buffer containing antiserum made against recombinant RSK (1:250 dilution) or twice-affinity-purified antiserum made against the C-terminal MAP kinase peptide (1:50 dilution). The membrane was washed three times with blocking buffer (10 min each) at room temperature and further incubated in the same buffer containing ^{125}I-protein A (0.1 μCi/ml) for another 1 h. The membrane was then washed three times with buffer, air-dried, and autoradiographed. Since the cellular volumes of the nucleus and cytoplasm have not been determined in these studies, this analysis does not reflect the actual concentrations of pp90^{rsk} and pp44/42$^{map/erk}$ in these locations. In addition, the protein level in the nuclear fractions is likely underestimated due to protein loss during nuclear isolation (41).

Immunofluorescence. Cells were plated onto sterilized, poly-L-lysine (2.5 μg/cm^2)-coated glass coverslips at a density of 1.5×10^4 cells per cm^2. Coverslips were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 12 min. Coverslips were then blocked with 5% goat serum in PBS for 5 min, followed by a 5-min incubation in 0.2% (vol/vol) fish scale gelatin in PBS. Cells were then incubated for 30 min with affinity-purified polyclonal antibodies at a 1:200 dilution (for RSK), 1:20 dilution (for MAP kinase), 1:300 dilution (for serum response factor [SRF]; from M. Greenberg), and 1:60 dilution (for Nur77; from L. Lau). The coverslips were then washed sequentially with 0.2% Tween 20, 5% goat serum, and 0.2% gelatin. The secondary antibody, rhodamine-conjugated goat anti-rabbit immunoglobulins (TAGO, Inc.), was used at a 1:500 dilution for 30 min.

To stain the plasma membrane, fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA) was added for 15 min after fixing the cells. To stain the Golgi apparatus, FITC-WGA was added after permeabilizing the cells with Triton X-100. In the latter procedure, the plasma membrane and nuclear envelope were also stained because the plasma membrane was not blocked with unconjugated WGA before the cells were permeabilized. The coverslips were washed in PBS and distilled water and mounted with Fluoromount-G (Fisher). For blocking experiments, affinity-purified antisera (10 μl diluted 1:200 for RSK antibodies or 10 μl diluted 1:20 for C-terminal MAP kinase peptide antibodies) were incubated overnight with 20 μg of recombinant RSK protein or 50 μg of the C-terminal MAP kinase peptide, respectively. We have also completed indirect immunofluorescence analysis of MAP kinase with a monoclonal anti-human MAP2 kinase antibody (PharMingen, 14351A) that gives results similar to those shown with our affinity-purified polyclonal antibodies (not shown). For all immunofluorescence studies, PBS buffers contained 1.47 mM $MgCl_2$ and 0.9 mM $CaCl_2$.

RESULTS

Immunolocalization of the MAP kinases and RSKs. Antibodies to recombinant RSK (α-RSK) and the predicted carboxy-terminal 20 amino acids (348 to 367) of the *erk-1*-encoded MAP kinase (α-cMAPK) were affinity purified and used to analyze the distribution of pp90^{rsk} and pp44/42$^{mapk/erk}$ in growing HeLa cells. The α-RSK stained the cytoplasm and nucleus of proliferating HeLa cells (Fig. 1B, a). This staining yielded a speckled and punctate appearance that was blocked by preabsorbing the affinity-purified antibodies with recombinant RSK protein (Fig. 1B, c). The α-cMAPK immunoprecipitates partially purified RSK kinases I (pp44$^{mapk/erk1}$) and II (pp42$^{mapk/erk2}$) (16; unpublished data), and the affinity-purified antisera also stained cytoplasmic as well as nuclear pp44/42$^{mapk/erk}$ (Fig. 1A, a). Peptide-preabsorbed serum did not reveal any staining (Fig. 1A, c). In addition, both MAP kinase and RSK staining was ex-

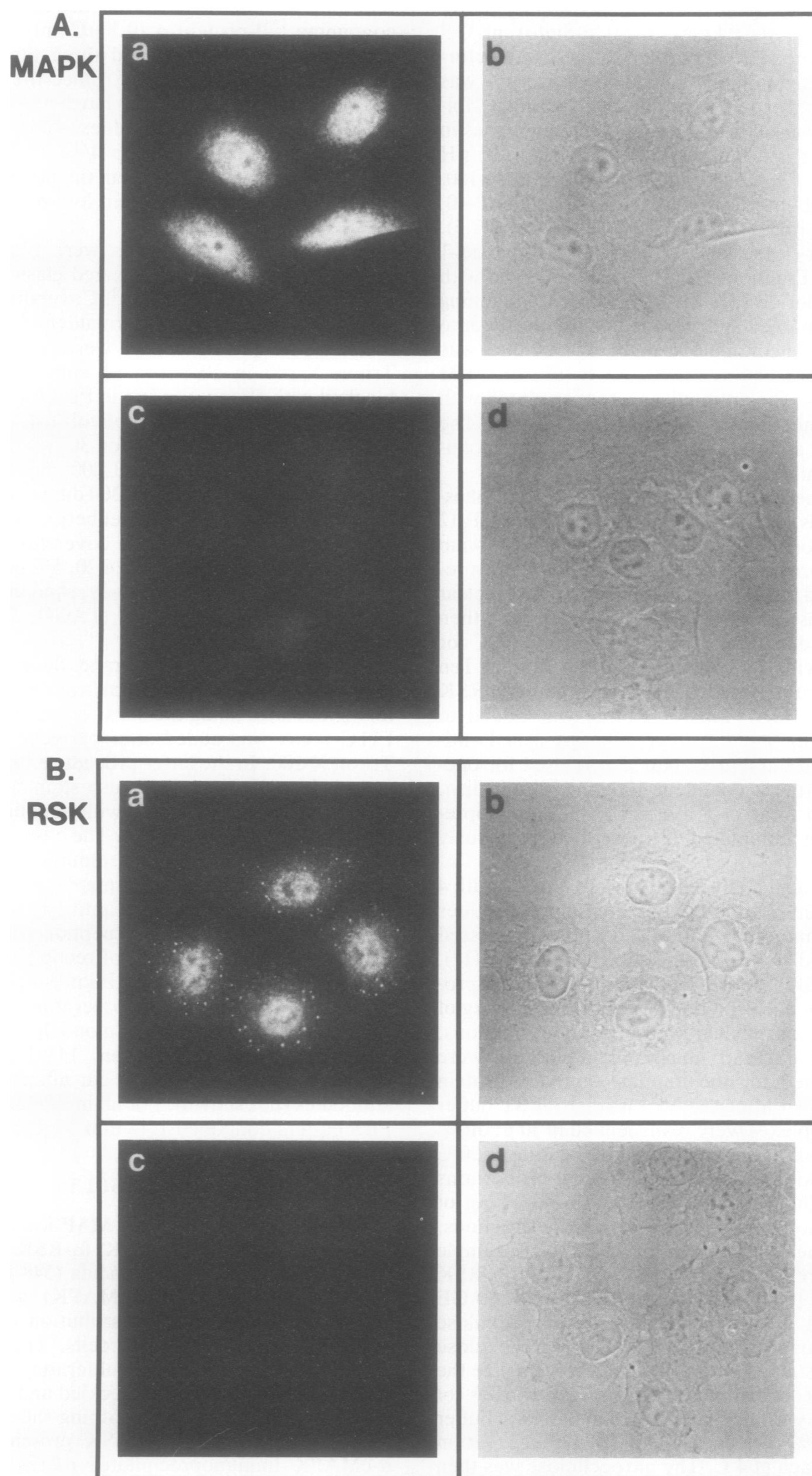

FIG. 1. Immunofluorescence staining of MAP kinases and RSKs in proliferating HeLa cells. HeLa cells were fixed in paraformaldehyde and incubated with affinity-purified antiserum made against C-terminal MAP kinase peptide (panel A, a) or recombinant RSK protein (panel B, a). (Panel A, c) Antiserum preincubated with C-terminal MAP kinase peptide. (Panel B, c) Antiserum preincubated with recombinant RSK protein. The corresponding phase contrast pictures are shown to the right of each immunofluorescence photograph (panels b and d).

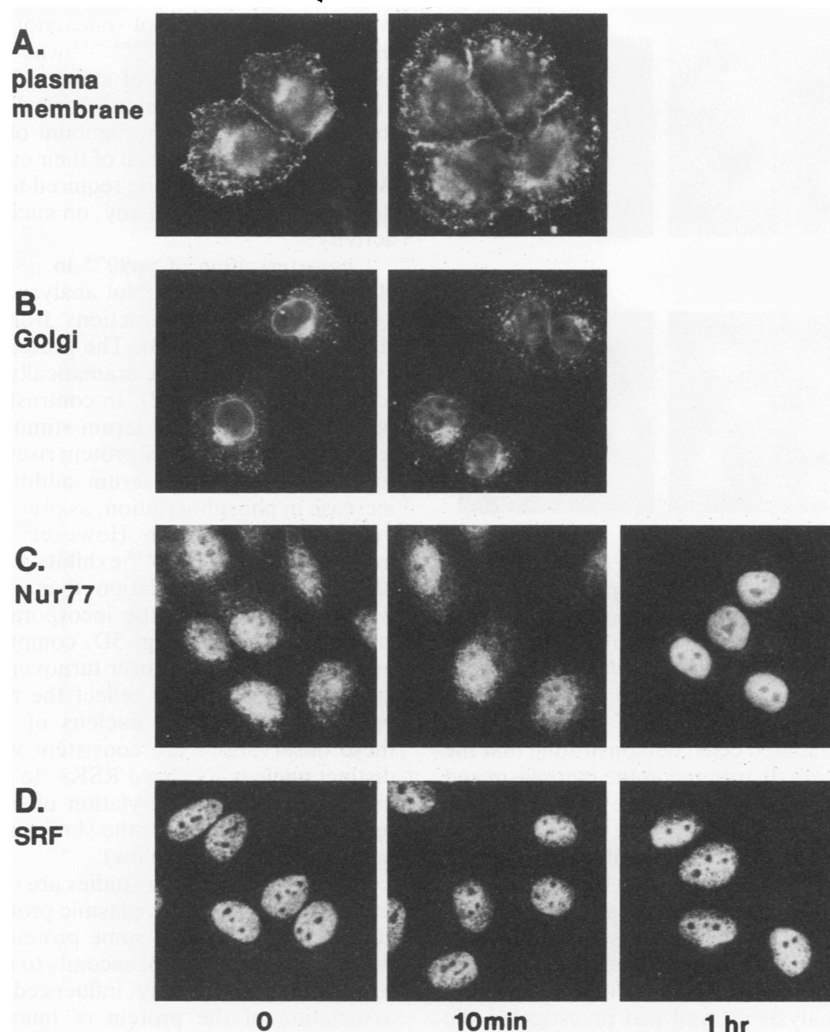

FIG. 2. Fluorescent staining of proteins localized in various compartments of the cells. Serum-starved HeLa cells or cells stimulated with serum for 10 min were processed for plasma membrane (A) or Golgi (B) staining with FITC-conjugated WGA. Affinity-purified antiserum made against Nur77 (C) or SRF (D) was also used to examine the staining pattern of these proteins. For Nur77 staining, the exposure time for 1-h serum-stimulated cells is about one-sixth of that for serum-deprived and 10-min serum-stimulated cells, reflecting the large increase in Nur77 synthesis (21, 28).

cluded from the nucleoli, which is consistent with nucleoplasmic staining and not staining of the nuclear periphery.

Immunofluorescence staining of a variety of other cellular proteins was used as a marker to confirm the nuclear localization of the MAP kinases and RSKs. Immunofluorescence staining with antibodies to SRF (Fig. 2D) or Nur77 (Fig. 2C) exhibited predominantly nuclear staining. Like the MAP kinases and RSKs, nuclear staining was excluded from the nucleoli. In addition, FITC-conjugated WGA was used to stain the plasma membranes of fixed but nonpermeabilized cells and predominantly the Golgi and nuclear envelope of permeabilized cells, which yielded clearly distinct fluorescence patterns. We do occasionally observe some perinuclear MAP kinase staining in addition to the nuclear staining, which may indicate some association with the endoplasmic reticulum and/or nuclear envelope. The addition of serum growth factors to quiescent cells for 10 min does not result in the redistribution of SRF or Nur77, as is observed with the MAP kinases and RSKs (see below).

MAP kinases and RSKs exhibit nuclear accumulation during the G_0 to G_1 transition. Indirect immunofluorescence analysis was also used to examine the distribution of MAP kinases and RSKs in quiescent cells and cells stimulated to reenter the cell cycle (Fig. 3). In quiescent cells, the MAP kinases were distributed evenly throughout the cell. After 5 min of serum stimulation, MAP kinases appear to be concentrating in the nucleus. At 1 to 2 h after serum stimulation, MAP kinases remain concentrated in the nucleus. Similarly, RSK immunofluorescence staining also reveals a detectable redistribution or concentration of pp90^{rsk} in the nucleus following serum addition to quiescent cells. As with the MAP kinases, the RSKs remain concentrated in the nucleus well into G_1, after their apparent recombinant RSK and S6 phosphotransferase activities, respectively, have begun to return to basal levels (see below).

Confocal microscopy was used to confirm the nuclear localization of the MAP kinases and RSKs as well as to demonstrate that the increased nuclear staining was not the result of cell shape changes following the reinitiation of cell proliferation. Shown in Fig. 4 are two optical sections from

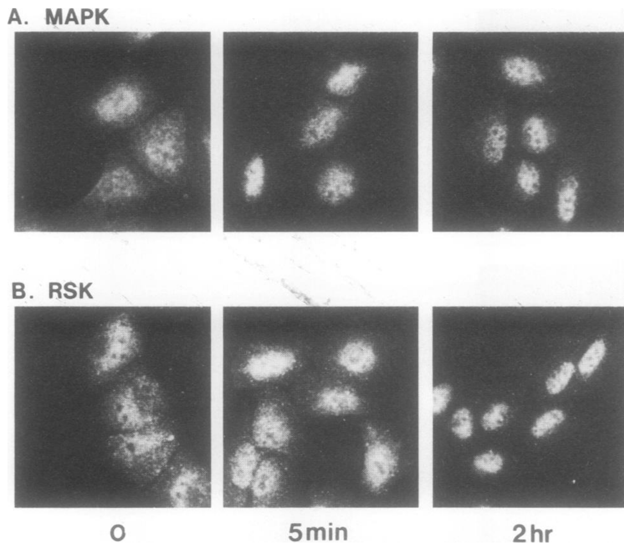

FIG. 3. Distribution of MAP kinases and RSKs in quiescent and serum-stimulated HeLa cells. Serum-deprived HeLa cells or cells stimulated with serum for 5 min or 2 h were fixed and incubated with affinity-purified antiserum made against C-terminal MAP kinase peptide (upper panel) or recombinant RSK protein (lower panel).

quiescent and serum-stimulated cells, demonstrating that the MAP kinases and RSKs are distributed in the cytoplasm and nucleus of quiescent cells and accumulate in the nucleus after serum addition.

Characterization of the subcellular distribution of pp44/42$^{mapk/erk}$ in quiescent and proliferating HeLa cells by cell fractionation. Having demonstrated a nuclear and cytoplasmic distribution for the MAP kinase/RSK signaling system by indirect and confocal immunofluorescence studies, we examined the distribution of MAP kinase and RSK molecules by immunoblot analysis as well and investigated the regulation of protein phosphorylation of these enzymes in these fractions. Immunoblot analysis with the affinity-purified αc-MAPK peptide antiserum shows that after cellular fractionation, pp44/42$^{mapk/erk}$ appears to be largely cytosolic in quiescent cells (Fig. 5). Following serum growth factor addition, both enzymes in the cytoplasm migrate with a slower mobility (indicating altered phosphorylation), and a small but reproducible increase in these slower-migrating forms now appears in the nuclear fraction (Fig. 5A, compare lanes 3 and 4).

Biosynthetic labeling with $^{32}P_i$ followed by immunoprecipitation shows that there is an increase in the phosphorylation of cytosolic and nuclear pp44/42$^{mapk/erk}$ following growth factor addition (Fig. 5B). Phosphoamino acid analysis reveals an increase in tyrosine and threonine phosphorylation in both MAP kinase molecules and in both the cytosolic and nuclear fractions (Fig. 6). Interestingly, the nuclear MAP kinase proteins have more phosphoserine relative to phosphothreonine and phosphotyrosine than their cytoplasmic counterparts. This does not change significantly following serum stimulation; however, this observation is consistent with the detection of a distinct population of nuclear enzyme. In addition, HeLa pp42$^{mapk/erk2}$ appears to be the predominant MAP kinase detected in both the cytosolic and nuclear fractions, as determined by immunoblot analysis. Immunoprecipitation of ^{32}P-labeled protein from serum-stimulated HeLa cells consistently revealed more labeling of nuclear pp42$^{mapk/erk2}$ than nuclear pp44$^{mapk/erk1}$ as well, whereas the extent of phosphorylation of cytoplasmic pp44$^{mapk/erk1}$ and pp42$^{mapk/erk2}$ was nearly the same (Fig. 5A and B). A comparison of staining by Western immunoblot analysis and incorporation of ^{32}P during biosynthetic labeling may indicate a higher amount of phosphorylation of the nuclear MAP kinases than of their cytoplasmic counterparts. Additional studies will be required to verify this as well as to determine the effects, if any, on nuclear MAP kinase specific activity.

Characterization of pp90^{rsk} in quiescent and proliferating HeLa cells. By immunoblot analysis, pp90^{rsk} is found in the cytosol and nuclear fractions from serum-deprived cells (Fig. 5C, lanes 1 and 3). The protein level of pp90^{rsk} in the cytosol does not change dramatically after serum stimulation (compare lanes 1 and 2). In contrast, the amount of nuclear pp90^{rsk} increases after serum stimulation. The relative molecular mass of the RSK protein rises from 80 to 84 kDa to 85 to 90 kDa following serum addition, consistent with an increase in phosphorylation, as previously observed with the cytosolic enzyme (13). However, in quiescent cells, the inactive cytosolic pp90^{rsk} exhibits a much greater amount of basal-level phosphorylation than its nuclear counterpart, which exhibits very little incorporation of ^{32}P under these labeling conditions (Fig. 5D, compare lanes 1 and 3). This may be the result of slower turnover of phosphate in pp90^{rsk} in the nucleus or may reflect the reduced levels of active pp44/42$^{mapk/erk}$ in the nucleus of quiescent cells. Again, these observations are consistent with the fractionation of distinct nucleus-localized RSKs. In addition, the differences in basal-level phosphorylation of cytoplasmic and nuclear pp90^{rsk} may account for the slower activation kinetics of the nuclear pp90^{rsk} (see below).

Cellular fractionation studies are complicated by the problem of leakage of nucleoplasmic proteins during the fractionation process. Indeed, some proteins leak from nuclei at a rate ($t_{1/2}$) on the order of seconds to minutes (41). The rate of leakage can be greatly influenced by the nature of the association of the protein of interest with other nuclear proteins or structures. Nur77 is a nuclear transcription factor and member of the steroid/thyroid hormone receptor superfamily (28, 29). As reported previously (28) and confirmed here (Fig. 2C), immunofluorescence staining demonstrates that Nur77, like the MAP kinases and RSKs, is predominantly nuclear, whereas the reported distribution of Nur77 as determined by cell fractionation studies is largely cytoplasmic (28). We wished to determine whether, during cell fractionation, the distribution of the MAP kinases and RSKs was similar to that of Nur77. To do this, quiescent and serum-stimulated cells were labeled with [^{35}S]methionine and fractionated as described in the legend to Fig. 5, and three immunoprecipitations were carried out in parallel with antibodies to Nur77, MAP kinases, and RSKs (Fig. 7). The distribution of the MAP kinases and RSKs under these conditions was similar to that detected with immunoblotting. In particular, the increase in nuclear pp44$^{mapk/erk1}$, pp42$^{mapk/erk2}$, and pp90^{rsk} by 10 min following serum growth factor addition is clearly evident. The fractionation of Nur77 was similar to that of the MAP kinases and RSKs, i.e., largely cytoplasmic. However, unlike MAP kinases and RSKs at 10 min, no increase in nuclear protein was observed. We do detect an apparent modest increase in the ratio of nuclear to cytoplasmic Nur77 at 30 min to 1 h after serum addition, perhaps reflecting additional Nur77 protein synthesis as well as extensive posttranslational modification at this time (28).

Regulation of cytosolic and nuclear MAP kinase and RSK activities and identification of potential nuclear substrates.

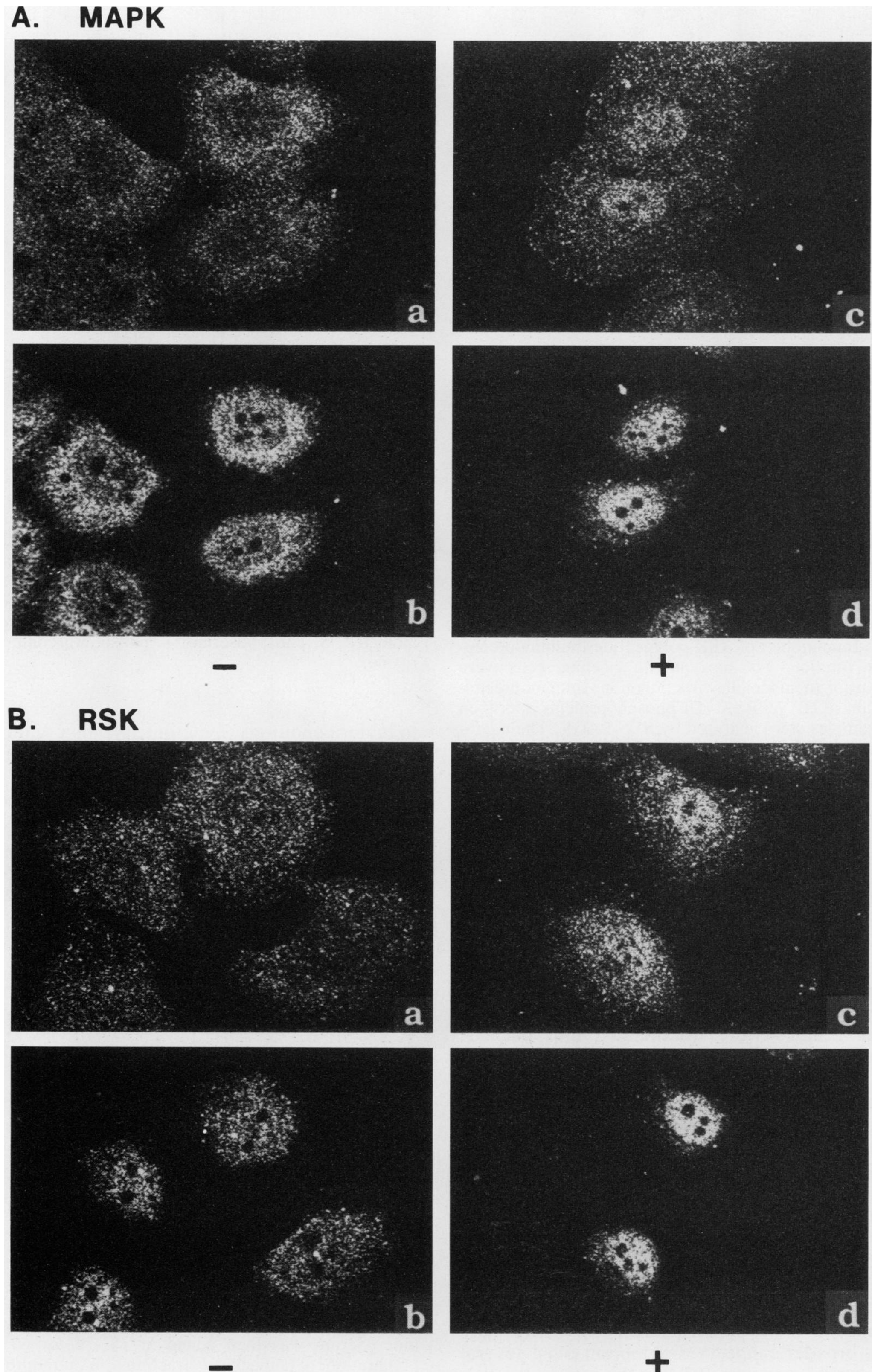

FIG. 4. Confocal laser scanning microscopy. Immunofluorescence staining of MAP kinases (A) and RSKs (B) was performed as described in the legend to Fig. 1 with serum-deprived HeLa cells (− [panels and b]) or cells stimulated with serum for 10 min (+ [panels c and d]). A confocal laser scan microscope was used to photograph optical sections at 1.1 μm (panels a and c) or 2.5 μm (panels b and d) from the bottom of the cells.

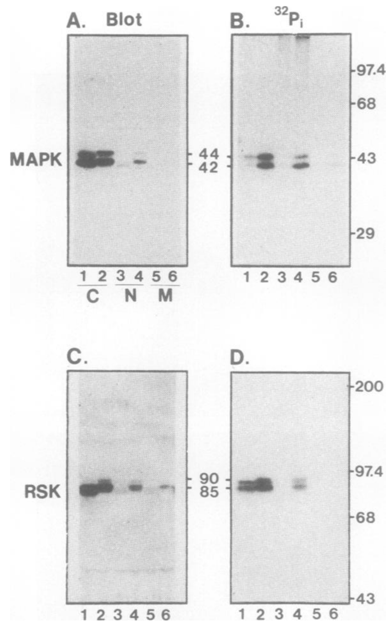

FIG. 5. Immunoblot analysis and phosphorylation of MAP kinase and RSK polypeptides in subcellular fractions. Cytosol (lanes 1 and 2), nuclear (lanes 3 and 4), and membrane (lanes 5 and 6) fractions were prepared from serum-deprived HeLa cells (odd-numbered lanes) or cells stimulated with serum for 5 min (even-numbered lanes). (A) Immunoblot of MAP kinase polypeptides in each fraction. (C) Immunoblot of RSK polypeptides immunoprecipitated from each fraction. For biosynthetic labeling, cells were labeled with $^{32}P_i$ prior to subcellular fractionation. Immunoprecipitation of MAP kinase (B) or RSK (D) phosphoproteins was then completed, and samples were analyzed by SDS-PAGE. The migration of molecular size standards (kilodaltons) is indicated to the right. The position of MAP kinase or RSK polypeptides from serum-stimulated cells is indicated.

Having demonstrated by indirect immunofluorescence and confocal microscopy, and by immunoblotting and immunoprecipitation, the cytoplasmic and nuclear localization of the MAP kinases and RSKs, we believed it was equally important to demonstrate that the nuclear enzymes were active and growth responsive. This is of importance if these enzymes are to participate in nuclear phosphorylation events. HeLa cell homogenates were fractionated into nuclear, postnuclear membrane, and cytosol fractions, and the kinase activities were measured. As shown in Fig. 8B, the majority of the serum-stimulated pp90^{rsk} activity is measured in the cytosol fraction (~65 to 70% over several experiments; lane C, inset), ~20 to 30% of the activity is localized in the nuclear fraction (lane N), whereas very little activity is associated with the membrane fraction (lane M). This subcellular distribution of pp90^{rsk} activity is quantitatively and reproducibly found with a variety of other subcellular fractionation procedures (data not shown). MAP kinase activity (Fig. 8A) was measured by using recombinant RSK as a substrate as described previously (15, 16) and appears to be more equally distributed in these fractions, with ~30 to 50% (we obtained a broader range in distribution over several experiments than observed with RSK) in the cytosol fraction (lane C), ~30 to 45% in the nuclear fraction (lane N), and ~15 to 30% in the postnuclear membrane fraction (lane M). The distribution of the MAP (recombinant RSK) kinase activity in the nuclear fraction appeared to be more sensitive

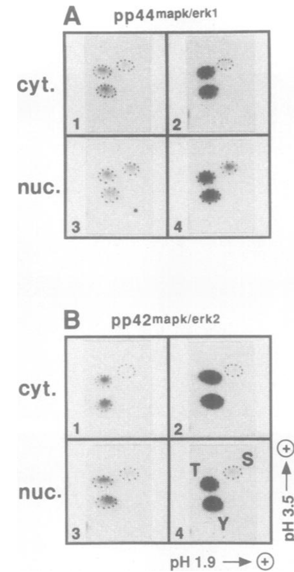

FIG. 6. Phosphoamino acid analysis of MAP kinase polypeptides from quiescent and serum-stimulated cells. Biosynthetically ^{32}P-labeled MAP kinase polypeptides were isolated by immunoprecipitation from cytosol (1 and 2) or nuclear (3 and 4) fractions of serum-deprived HeLa cells (odd-numbered panels) or cells stimulated with serum for 5 min (even-numbered panels). Isolated polypeptides were then processed for phosphoamino acid analysis. (A) pp44$^{mapk/erk1}$; (B) pp42$^{mapk/erk2}$. The directions of electrophoresis are indicated. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

to buffer conditions (hypotonicity) than that of pp90^{rsk}. This may indicate that MAP kinases are associated with other nuclear proteins and that this association is sensitive to buffer conditions; however, this remains to be tested. Furthermore, pp90^{rsk} in the cytosol and nucleus and the MAP (recombinant RSK) kinase activities in the cytosol, nucleus, and membrane fractions are growth regulated, as shown by the increase in pp90^{rsk} activity (5- to 20-fold) and MAP (recombinant RSK) kinase activities (4- to 12-fold) following serum stimulation (see insets). Our analysis with affinity-purified α-cMAPK did not reveal significant staining of the plasma membrane (Fig. 1, 3, and 4), nor did these antibodies detect significant levels of pp44/42$^{mapk/erk}$ in membrane preparations by immunoblot analysis (Fig. 5), indicating that the growth-regulated RSK kinase activity detected in the

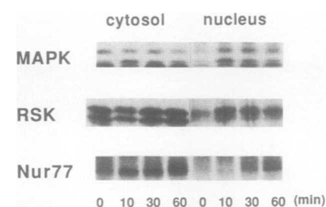

FIG. 7. Subcellular fractionation of MAP kinases, RSKs, and Nur77. Serum-deprived HeLa cells were labeled with [^{35}S]methionine. Serum was then added at 10, 30, or 60 min before the preparation of subcellular fractions. Immunoprecipitations were then performed with antiserum made against MAP kinase C-terminal peptide (top panel), recombinant RSK (middle panel), or recombinant Nur77 (bottom panel). The exposure time of the fluorograph is 10 and 30 h for cytosol and nuclear MAP kinases and 12 and 24 h for cytosol and nuclear RSKs, respectively.

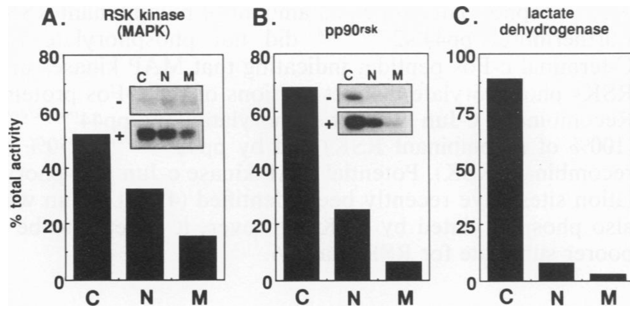

FIG. 8. Subcellular distribution of MAP kinase and pp90^{rsk} activities. Serum-deprived HeLa cells were stimulated with serum for 5 min. Cell homogenates were prepared in hypotonic lysis buffer and then fractionated into cytosol (lane C), nuclear (lane N), and postnuclear membrane (lane M) fractions. MAP kinase activities (measured by using recombinant RSK as the substrate, panel A), pp90^{rsk} activity (panel B), or the cytosol marker enzyme lactate dehydrogenase activity (panel C) in each fraction was measured and presented as a percentage of the total activity in all fractions. (Insets) Autoradiography of in vitro phosphorylation of recombinant RSK protein (A) or 40S ribosomal protein S6 (B). —, serum-deprived cells; +, serum-stimulated cells.

membrane fractions by our assays may be another member of the *erk*-encoded MAP kinase family. However, additional studies will be needed to confirm this possibility. The nuclear localization of MAP kinase and pp90^{rsk} activities is not due to contamination with cytosolic enzymes, since the assays for the cytosol marker enzyme lactate dehydrogenase (Fig. 8C) show that less than 9% of the total lactate dehydrogenase activity is present in the nuclear fractions and less than 3% of the activity is present in the membrane fractions in the experiment shown in Fig. 8. Similar subcellular distribution of MAP kinase and pp90^{rsk} activities was also found in mouse Swiss 3T3 cells and chicken embryo fibroblasts (data not shown).

We next examined the temporal regulation of the nuclear MAP kinase and RSK phosphotransferase activities to determine whether it was similar to that reported for their cytoplasmic counterparts (14, 15). Following serum addition, both the nuclear and cytosolic MAP kinase and RSK activities rise rapidly. The cytosolic MAP kinase and RSK activities reach maximal stimulation between 2 and 5 min and begin to decline after 10 to 20 min. At 40 min, approximately 50% of the maximal activity remains (Fig. 9A). The nuclear MAP kinase activity changes with kinetics similar to that in the cytosolic fraction, whereas the nuclear RSK activity exhibits slightly slower activation kinetics than the cytosolic counterpart (Fig. 9B). We occasionally observe a more rapid rate of inactivation of the nuclear pp90^{rsk} activity than of its cytosolic counterpart. Thus, it appears that the spatial distribution of these growth-modulated enzymes is also an important component of their regulation. Neither the significance nor the mechanism responsible for the delayed activation of nuclear RSK compared with its cytosolic

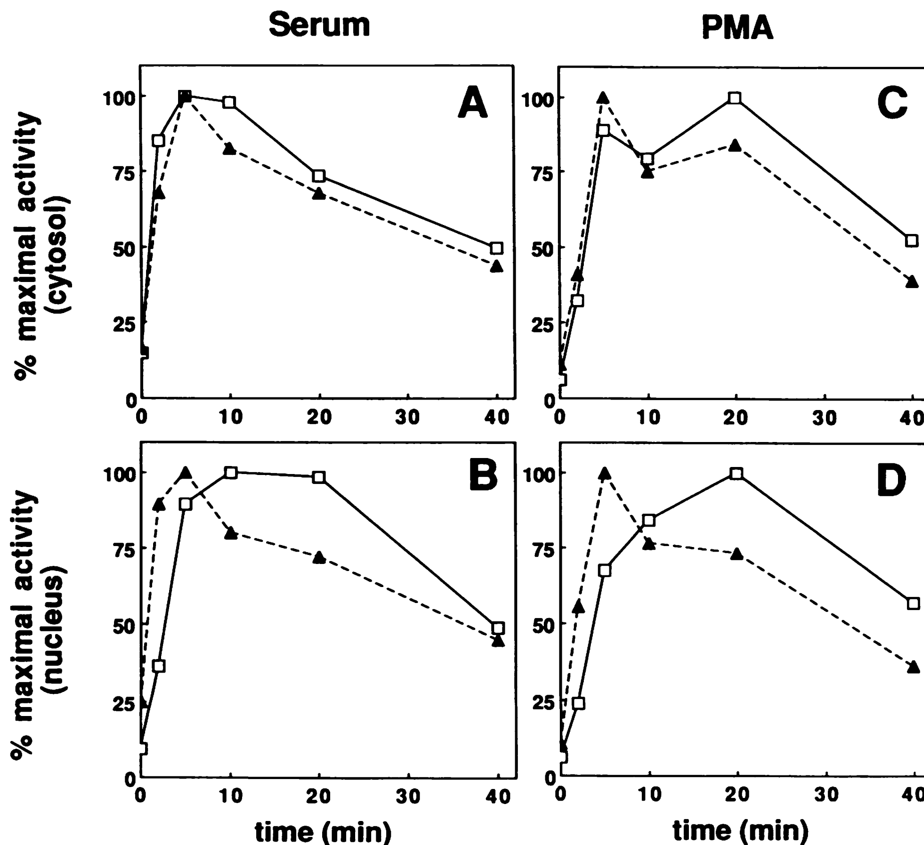

FIG. 9. Regulation of cytosolic and nuclear MAP kinase and pp90^{rsk} activities following serum or phorbol ester addition to quiescent cells. Serum-deprived HeLa cells were incubated with serum (A and B) or phorbol myristate acetate (C and D) for the indicated times (2 to 40 min). Cell lysates were then prepared, and MAP kinase (▲) and pp90^{rsk} (□) activities in the cytosol (A and C) and nuclear (B and D) fractions were measured. The maximal activity during this time course is designated as 100%.

TABLE 1. Phosphorylation of nuclear proteins by RSK and MAP kinases[a]

Substrate	% Activity		
	RSK	MAP kinase I	MAP kinase II
S6	100	0	0
Recombinant RSK	22	100	100
c-Fos	293	655	720
c-Jun	4	110	50
H3	19	ND[b]	ND
H1	0	ND	ND
S6 peptide (RRLSSLRA)	100	0	0
c-Fos peptide (AHRKGSSSNEP)	300	0	0

[a] In vitro phosphorylation of various proteins (1 μg) or peptides (100 μM) was performed in $pp90^{rsk}$-immune complexes or with partially purified $pp44^{mapk/erk1}$ or $pp42^{mapk/erk2}$. Their enzymatic activities were normalized to S6 (for RSK) or recombinant RSK (for MAP kinase) phosphotransferase activities and are presented as a percentage of these activities. For peptide substrates, phosphorylation of c-Fos peptide was compared with that of S6 peptide. The values are the average of two independent experiments.
[b] ND, not determined.

counterpart has been established. However, this observation is of added importance, as it once again demonstrates that the nuclear enzymes (as detected by cell fractionation) are distinct from the cytosolic enzymes. Thus, we appear to be truly measuring the activity of the nuclear enzymes. It should be noted that we have not determined the contribution of nuclear enzymes (due to leakage) to the activity measured in the cytosolic fractions. It is also worth noting that even at 4 h, when these activities are at 20 to 40% of their peak activity, they are still severalfold more active than measured in quiescent cells. Finally, tumor-promoting phorbol myristate acetate also rapidly and transiently activates the nuclear MAP kinase and RSK activities (Fig. 9D). Thus, protein kinase C-mediated signaling to the nucleus also occurs at least in part via the MAP kinase/RSK phosphorylation cascade. Recent studies indicate that certain isoforms of protein kinase C may translocate to the nucleus (31, 38), where they may participate in the regulation of these enzymes.

Only a small number of substrates for both MAP kinases and RSK have been identified to date (19, 23, 37, 42, 45, 48). Having established their nuclear as well as cytoplasmic distribution, we wished to determine whether any established nuclear phosphoproteins might also be in vitro substrates for these enzymes. In Table 1 we present data examining the phosphorylation of potential nuclear targets c-Fos, c-Jun, histone H1, and histone H3 and a comparison of their phosphorylation with substrates used to identify and/or characterize the MAP kinases (recombinant RSK [15, 16]) and RSKs (40S ribosomal protein S6). RSK in the immune complex assay phosphorylated all the substrates shown except histone H1. Interestingly, phosphorylation of the full-length recombinant c-Fos by RSK was three times greater than that of the ribosomal protein S6. Similar results were obtained when comparing the RSK phosphorylation of a c-Fos C-terminal peptide comprising amino acids 357 to 367 (containing previously identified potential phosphorylation sites [40]) with the phosphorylation of the S6 peptide (RRLSSLRA). $pp44^{mapk/erk1}$ and $pp42^{mapk/erk2}$, partially purified as described previously (16), did not significantly phosphorylate S6 or histone H1, but both MAP kinases did phosphorylate recombinant c-Fos seven times better than they phosphorylated an equal amount of recombinant RSK. Furthermore, $pp44/42^{mapk/erk}$ did not phosphorylate the C-terminal c-Fos peptide, indicating that MAP kinases and RSKs phosphorylate distinct portions of the c-Fos protein. Recombinant c-Jun was phosphorylated by $pp44^{mapk/erk1}$ (100% of recombinant RSK) and by $pp42^{mapk/erk2}$ (50% of recombinant RSK). Potential MAP kinase c-Jun phosphorylation sites have recently been identified (4, 44). c-Jun was also phosphorylated by RSK; however, it appears to be a poorer substrate for RSK than S6.

DISCUSSION

The MAP kinase/RSK signaling cascade provides a mechanism for transmitting growth-regulating information via protein-tyrosine phosphorylation through protein-Ser/Thr kinases from the cytoplasm to the nucleus. We demonstrate that members of the *erk*-encoded family of protein kinases (MAP2 kinase, MBP kinase, RSK kinases I and II; referred to collectively as the MAP kinases or $pp44/42^{mapk/erk}$) and the *rsk*-encoded family of protein kinases (referred to collectively as RSKs or $pp90^{rsk}$) are localized in the nucleus as well as in the cytoplasm. It is likely that members of the *erk*-encoded family of protein kinases are in equilibrium between the plasma membrane and the nucleus, and while at the membrane or in the cytoplasm they are activated by tyrosine/threonine phosphorylation. They then phosphorylate other protein targets throughout the cell, including $pp90^{rsk}$, which in turn phosphorylates and modulates the structure and function of downstream targets of this cytoplasmic and nuclear protein-Tyr/Ser/Thr signaling cascade.

In these studies, it is difficult to make a direct correlation between the indirect immunofluorescence and cell fractionation results. Cell fractionation experiments are complicated by leakage of nuclear proteins at a rate which is dependent on many factors, such as protein size, charge, and the nature of association of proteins of interest with other nuclear molecules. For example, the nuclear transcription factor Nur77, like many other members of the steroid/thyroid hormone receptor superfamily, has been shown to be localized in the nucleus by immunofluorescence but appears to be predominantly cytoplasmic by subcellular fractionation studies, likely the result of leakage during the fractionation process (28). We have shown that Nur77, $pp44/42^{mapk/erk}$, and $pp90^{rsk}$ exhibit similar cellular distributions by immunofluorescence staining or by cell fractionation. Indirect immunofluorescence of nuclear proteins can be affected by cell shape changes as well as accessibility of antibodies to antigen. It is likely that cell shape changes cannot account for the redistribution of MAP kinases or RSKs, as in parallel experiments, no change in the distribution of Nur77 or SRF was observed by 10 min following serum addition to quiescent cells. Furthermore, confocal microscopy both confirms the nuclear localization of the MAP kinases and RSKs and supports the indirect immunofluorescence studies showing the growth factor-stimulated redistribution of these enzymes. Immunofluorescence studies can also be affected by fixation methods, antibody affinity and accessibility, and other parameters. For this reason, we have analyzed the cellular distribution of MAP kinases and RSKs by several methods, all of which consistently show that these growth-regulated enzymes are nuclear and cytoplasmic.

Cytoplasmic and nuclear MAP kinases and RSKs are rapidly and coordinately regulated, with the cytoplasmic $pp90^{rsk}$ activated immediately prior to its nuclear counterpart. Part of this lag may be due to the slower translocation

of activated $pp90^{rsk}$ into the nucleus or to the translocation of the activated *erk*-encoded kinases into the nucleus, where they can phosphorylate and activate unstimulated $pp90^{rsk}$, or to the lower extent of nuclear $pp90^{rsk}$ basal phosphorylation in quiescent cells. Although a reproducible lag in nuclear $pp90^{rsk}$ activity is measured, almost no lag in the activation of nuclear pp44/42 MAP (RSK) kinase activity is observed, supporting the latter possibility. There is immunodetectable $pp44/42^{mapk/erk}$ in the nucleus of quiescent cells that may also be activated by an upstream protein-Tyr kinase. Therefore, these enzymes may also be in the nucleus or translocate in response to growth stimuli. The cellular homolog of the Abelson protein-Tyr kinase (c-*abl*) and a second protein-tyrosine kinase, referred to as FER, have been shown to be nuclear enzymes (26, 55), making these candidate MAP kinase-activating kinases.

Unlike other growth-modulated, broad-specificity protein kinases, like cyclic AMP-dependent protein kinase, protein kinase C, and cdc2/H1 protein kinase, MAP kinases and RSKs have been found to have a limited number of substrates (19, 23, 45, 48). These include several phosphoproteins that participate in the regulation of cell proliferation. For example, in addition to phosphorylating and activating $pp90^{rsk}$ (16, 51) MAP2 kinases have been shown to affect microtubule dynamics in *Xenopus* interphase extracts, indicating that this enzyme participates in the reorganization of microtubules during the transition from interphase to mitosis (23). A MAP2 kinase has also been shown to phosphorylate the epidermal growth factor (EGF) receptor at threonine 669 in 3T3-L1 preadipocytes in response to EGF, which may be of importance with regard to protein kinase C-independent signaling by EGF and downregulation of the EGF receptor (39, 54).

Activated $pp90^{rsk}$ and its homologs have also been shown to phosphorylate lamin C (56), troponin I and glycogen synthase (19), and MAP2 (15). A rabbit skeletal insulin-activated protein kinase, which is a member of the *rsk* family (35), that phosphorylates the regulatory subunit of a type 1 protein phosphatase, resulting in an activated protein phosphatase 1 (18), has recently been identified. The existence of homologous systems in various cell types could provide explanations for mitogen-stimulated dephosphorylation of a variety of proteins as well as the transient activation of many growth-associated changes in enzyme activities and gene expression.

Protein phosphorylation plays an important role in transcriptional regulation (9). Several lines of evidence suggest that MAP kinase/RSK signaling participates in these processes. First, the MAP kinase/RSK phosphorylation cascade is cytoplasmic and nuclear. It should be noted that transcription factor phosphorylation may occur in both locations. Second, these enzymes are regulated with the correct kinetics to account for the modulation of immediate-early gene transcription by growth factors and tumor-promoting phorbol esters. Third, biochemical evidence indicates that these enzymes could associate with or be DNA- or RNA-binding proteins. For example, MAP kinases and RSKs bind to heparin-agarose resins, an anionic polymer like RNA or DNA that interacts with a wide variety of nucleic acid-binding proteins. In addition, RSK substrates like S6 are very basic and are found associated with nucleic acids. Indeed, nuclear S6 has been described as a chromatin-associated protein that undergoes growth-modulated changes in phosphorylation (22). Fourth, several potential nuclear substrates for the MAP kinases and RSKs have been identified. For example, another chromatin-associated protein, histone H3, is also an in vitro substrate for RSK (Table 1). Since the proposed histone H3 phosphorylation sites (36) overlap the recognition motif of RSK (19), $pp90^{rsk}$ may participate in the nuclear phosphorylation of H3 in early G_1. Additional studies will be necessary to confirm this hypothesis. Other DNA-binding proteins with basic domains are also candidate targets for RSKs. The transcription factor Nur77 (NGF1-B/N10/TIS1, a member of the seroid/thyroid hormone receptor superfamily) has been shown to be phosphorylated in situ (21, 28), and recombinant Nur77 is phosphorylated by $pp90^{rsk}$ in vitro at common sites (8b). Additionally, SRF is phosphorylated in vitro by RSK at sites phosphorylated in vivo (8a). Experiments are now under way to determine the effects of Nur77 and SRF phosphorylation on DNA binding, ligand-binding activity, protein-protein interactions, stabilization, and localization. We have also shown that full-length recombinant c-Fos and c-Jun are in vitro targets for $pp90^{rsk}$, $pp44^{mapk/erk1}$, and $pp42^{mapk/erk2}$ (Table 1). MAP kinases have recently been shown to phosphorylate c-Jun and c-Myc in vitro (4, 44). Furthermore, RSKs but not the MAP kinases will phosphorylate a C-terminal c-Fos peptide containing serine phosphorylation sites believed to be required for transcriptional *trans* repression of the c-*fos* promoter (40). It should be noted that a variety of other protein-Ser/Thr kinases have been shown or have the potential to phosphorylate these substrates in vitro (1). We are adding to that list, noting that the MAP kinases and RSKs are both nuclear and regulated coordinately with immediate-early gene expression. These in vitro experiments will provide the basis for future studies examining the phosphorylation sites and the physiological effects of transcription factor phosphorylation. Thus, these studies implicate the MAP kinase/RSK signal transduction cascade in the transmission of growth-modulating signals or information (via protein phosphorylation) from the cytoplasm into the nucleus during the initiation of cell proliferation.

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