Distinct Mechanisms for the Activation of the RSK Kinases/MAP2 Kinase/pp90rsk and pp70-S6 Kinase Signaling Systems Are Indicated by Inhibition of Protein Synthesis

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Abstract
Previous studies demonstrated that addition of protein synthesis inhibitors to quiescent cells resulted in the stimulation of S6 kinase activity. The present characterization of several growth factor- and oncogene-regulated protein-serine/threonine kinases demonstrated that pp70-S6 protein kinase and not pp90rsk, RSK kinase, or MAP2 kinase activities were rapidly stimulated. Dose-response experiments revealed a close correlation between the extent of protein synthesis inhibition and the level of activation of pp70-S6 kinase activity. Analysis of S6 phosphorylation suggests that activation of pp90rsk, S6 phosphotransferase activity, whose Xenopus homologues appear to be responsible for S6 phosphorylation during oocyte maturation, may participate in, but is not essential for, the increase in S6 phosphorylation observed in growth-stimulated somatic animal cells. These studies provide additional evidence for the existence of two distinct, independently regulated protein phosphorylation cascades activated in the early G1 phase of the cell cycle.

Introduction
Reversible phosphorylation has been implicated in the regulation of cell growth and cellular transformation (for a review, see Refs. 1–3). To gain an understanding of the processes regulating these phosphorylation events, the relevant protein kinases and/or phosphoprotein phosphatases must be identified and characterized and the mechanisms of regulation determined. To this end, several mitogen- and oncogene-regulated protein-ser/thr kinases have recently been identified and partially characterized in somatic cells. These include the 65–70 kDa S6 protein kinases (4–8), pp90rsk (9, 10), MAP2 kinases (11, 12), RSK kinases (13), raf-1 kinases (14), and casein kinase II (15–18). We have been examining the regulation of the first four of these enzyme activities.

Our studies have shown that pp70-S6K and pp90rsk are distinct protein-ser/thr kinases that phosphorylate the 40S ribosomal protein S6 in vitro (9, 10). A protein kinase assay utilizing recombinant RSK protein as a substrate has also been used to identify growth-regulated protein-ser/thr kinases (referred to as the RSK kinases) that are distinct from pp90rsk and pp70-S6K (13). These studies have revealed the rapid sequential activation of RSK kinase (maximal at 1–5 min), pp90rsk (maximal at 2–10 min), and pp70-S6K (maximal at 10–20 min) activities by a variety of mitogens and demonstrate that hyperphosphorylation of pp90rsk, and the activation/inactivation kinetics of pp90rsk and RSK kinase activities, are tightly coordinated (13, 19). Furthermore, an insulin-stimulated MAP2 kinase has been previously shown to partially activate a pp90rsk Xenopus homologue (S6 kinase II) in vitro (20), and recent studies indicate that two RSK kinases are immunologically related to a maturation-activated sea star oocyte myelin basic protein kinase and MAP2 kinase, and both RSK kinases activate pp90rsk in vitro (21). Thus, these kinases appear to participate in a common phosphorylation cascade. We show here that prior activation of RSK kinase, pp90rsk, or MAP2 kinase activities is not required for the activation of pp70-S6K activity, providing further verification that these enzymes are regulated by distinct protein phosphorylation, signal transduction pathways (10, 22). Evidence is also provided which shows that a measurable activation of pp90rsk-S6 phosphotransferase activity is not essential for increased S6 phosphorylation in cultured somatic cells.

Results
Cycloheximide Stimulates S6 Kinase Activity but not pp90rsk, RSK Kinase, or MAP2 Kinase Activities. We have previously shown that addition of cycloheximide to quiescent chicken embryo fibroblasts results in activation of S6 phosphotransferase activity in a protein kinase C-independent manner (23). An analysis of the activation kinetics following addition of cycloheximide to quiescent

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3 The abbreviations used are: ser, serine; thr, threonine; kDa, kilodalton(s); pp90rsk, 90 kDa ribosomal S6 kinase; re-RSK, recombinant rsk gene product; pp70-S6K, 70 kDa S6 kinase; MAP2, microtubule-associated protein 2; CEF, chicken embryo fibroblasts; RSV, Rous sarcoma virus; ts-CEF, CEF infected with the temperature-sensitive transformation mutant of RSV; SOS, sodium dodecyl sulfate.
CEF revealed maximal activation by 20–30 min (30 min in the experiment shown in Fig. 1A). However, parallel measurements of pp90^{{\text{r}}},{^a} RSK kinase, or MAP2 kinase activities revealed little detectable activation during this time. Similar results have been observed with anisomycin (10 \( \mu \text{M} \)) or cycloheximide in Swiss 3T3 cells.\(^{\text{a}} \)

Incubation of cultured cells with protein synthesis inhibitors followed by addition of various growth factors results in the superinduction of several growth-regulated genes to a level severalfold greater than with mitogen alone (24–27). Therefore, we analyzed the effect of cycloheximide plus expression of pp60^{{\text{src}}} protein-tyrosine kinase activity on the regulation of S6 phosphotransferase activity (Fig. 2). As shown, cycloheximide (1 h, Lane 3) stimulated S6 kinase activity measured in direct assays to the level observed following activation of pp60^{{\text{src}}} protein-tyrosine kinase activity (by transfecting ts-CEF to the permissive temperature, 35°C, for 1 h) (Lane 2). Addition of cycloheximide to quiescent ts-CEF plus activation of pp60^{{\text{src}}} increased S6 kinase activity above that observed with either agent added alone (Lane 4). Similar results were obtained with puromycin (Lanes 5 and 6). This effect was also observed when cycloheximide or anisomycin was added with serum (10% v/v) to CEF or with phorbol myristate acetate, fibroblast growth factor, or epidermal growth factor to Swiss 3T3 cells (data not shown). It is currently not known whether this less-than-additive, further stimulation of S6 kinase activity in growth-stimulated cells incubated with cycloheximide is related to the superinduction phenomena of immediate-early genes (24–27).

Cycloheximide, Puromycin, Sodium Vanadate, and Serum Activate Chromatographically Related S6 Protein Kinases. We have previously observed the stimulation of related 65–70 kDa S6 kinase activities (pp70-S6K) by serum, phorbol ester, or expression of pp60^{{\text{src}}} based on similar sequential chromatography elution profiles (single comigrating peaks) on Pharmacia cation- and anion-exchange columns (Mono S and Mono Q, respectively) and by gel filtration (28). As shown in Fig. 3, the single major peak of S6 protein kinase activity stimulated by cycloheximide, puromycin, or sodium vanadate for 1 h coeluted with the serum-stimulated S6 phosphotransferase activity (stimulated for 1 h) on Pharmacia Mono S (Fig. 3A, fractions 18–20) and Mono Q resins (Fig. 3B, fraction 22). The ability of pp70-S6K to bind to Mono S and Mono Q resins using the same buffer conditions is characteristic of this enzyme (5, 28). At this time (1 h after serum stimulation), pp90^{{\text{r}}},{^a} S6 phosphotransferase activity contributes only a minor fraction of the total activity measured after DEAE chromatography (10, 22) and was not detected with this analysis where cation-exchange Mono S chromatography was used as the first column. These partially purified enzyme preparations were highly specific for S6 in 40S subunits, did not require Ca^{2+} for activity, were not inhibited by the heat-stable inhibitor of cyclic AMP-dependent protein kinase, and yielded an autophosphorylating 65 kDa protein kinase that comigrated with the highly purified serum-stimulated CEF and chicken embryo S6 kinases previously described (Ref. 5 and data not shown).

Inhibition of Protein Synthesis by Cycloheximide Correlates with Activation of S6 Kinase Activity. Shown in Fig. 4 are the results obtained when the ability of cycloheximide to stimulate S6 kinase activity was compared to its ability to inhibit cellular protein synthesis. Concentrations of cycloheximide strongly inhibiting protein synthesis correlated with activation of S6 kinase activity (Fig. 4A). Inhibition of protein synthesis by \(~70\%\) resulted in

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\(^{\text{a}}\) J. Chung, unpublished observations.
near maximal pp70-S6K activation under these experimental conditions. Like cycloheximide, sodium vanadate can, at high concentrations, strongly inhibit protein synthesis (29, 30). Thus, both agents could potentially stimulate S6 kinase activity by signals generated as a result of inhibition of protein synthesis. Alternatively, inhibition of protein synthesis by cycloheximide could result in the turnover of S6 kinase-regulatory proteins (e.g., S6 protein kinase-phosphatases), which are also inhibited by low concentrations of sodium vanadate (31, 32), thus leading to stimulation of S6 kinase activity. Sodium vanadate stimulated S6 kinase activity at 10–100 μM, concentrations not affecting protein synthesis (Fig. 4B). Addition of sodium vanadate to 1 mM resulted in maximal stimulation of S6 kinase activity but also resulted in partial inhibition of protein synthesis (70%; Fig. 4B).

Analysis of S6 Phosphorylation. The extent of S6 phosphorylation in 32P-labeled CEF incubated with cycloheximide (100 μM) was examined by two-dimensional alkaline- and acid-urea gel electrophoresis (Fig. 5). This gel system provides for the separation of unphosphorylated 40S ribosomal protein S6 from its five phosphorylated derivatives (a–e) and provides information concerning the extent of S6 phosphorylation (33–35). Fig. 5 shows data analyzing the extent of S6 phosphorylation in quiescent CEF (Fig. 5A) and CEF treated for 2 h with 10% (v/v) calf serum (Fig. 5B), 100 μM cycloheximide (Fig. 5C), and 1 mM sodium vanadate (Fig. 5D). The increase in overall phosphorylation and shift to the highly phosphorylated forms of S6 (forms c–e) correlates with a shift of Coomassie blue-stainable S6 from unphosphorylated S6 (A, the predominant form in Fig. 5A, not shown) to the more highly phosphorylated forms (a–e). Although an increase in S6 phosphorylation in response to protein synthesis inhibitors was reproducible, obtaining maximal phosphorylation of S6 with 4.5 mol phosphate/mol S6 was observed less often with cycloheximide than was observed with serum or sodium vanadate in parallel experiments. This difference may be the result of secondary effects of protein synthesis inhibition, differences in the kinetics of S6 phosphorylation, or the requirement for activation of other S6 kinases (e.g., pp90S6K) for maximal in vivo phosphorylation. The overall increase in S6 phosphorylation with CEF supports the results reported by others showing increased S6 phosphorylation in rat livers in response to cycloheximide or puromycin (36, 37), HeLa cells treated with cycloheximide (38), or Swiss 3T3 cells incubated with sodium vanadate (39).

**Discussion**

Previous studies have shown that pp90S6K and pp70-S6K activities are differentially regulated (10, 19, 22) and that serum stimulation of HeLa cells provides evidence that pp90S6K (activated ~10-fold) and pp70-S6K (activated less than 2-fold) lie on distinct signaling pathways (10), whereas pp90S6K and the RSK kinases/MEK2 kinases lie on the same signaling pathway (13, 20, 21). Supporting these observations, a recent study has shown differential
distinct cycloheximide synthesis suggests that the increased MAP2 kinase activity observed in puromycin-treated cells is due to a decrease in the amount of S6 kinase present in the cell. This decrease is likely due to the inhibition of S6 kinase activity by cycloheximide, which is a known inhibitor of S6 kinase activity. The effect of cycloheximide on S6 phosphorylation in quiescent CEF is shown in Figure 5, where A depicts the autoradiogram of cycloheximide-treated and untreated cells, and B-D show the autoradiograms of cycloheximide-treated cells with varying concentrations of cycloheximide.

Low the basal levels measured in quiescent cells may result in the activation of a feedback pathway, stimulating an S6 kinase activity (in an attempt to stimulate protein synthesis). Although at this time we cannot distinguish between these and other possibilities, we have utilized this response to demonstrate that nonoverlapping signaling pathways exist that regulate distinct mitogen- and oncogen-activated protein-ser/thr kinases and that a significant increase in S6 phosphorylation can occur without activation of RSK kinase and pp90S6 kinases in CEF.

Recent studies have demonstrated that mitogen-stimulated and vanadate-stimulated S6 kinases are biochemically identical (44). Similarly, S6 kinases have been purified from the livers of rats given injections of cycloheximide and are biochemically related to mitogen-activated S6 kinases (45, 46). Here, we present evidence that pp90S6 kinase phosphorylation activity is not significantly stimulated under the described conditions, whereas the S6 kinase that is activated in cultured CEF following inhibition of protein synthesis is biochemically related to the CEF serum- and src-activated 65-70 kDa kinase. The latter enzyme has also been shown to be related to an activated S6 protein kinase isolated from developing chicken embryos (5).

Mitogen-stimulated pp70-S6K is phosphorylated on serine and threonine residues (47), and treatment of highly purified pp70-S6K with phosphoprotein phosphatases results in loss of activity (10, 48). It is possible that cofactor-independent pp70-S6K protein kinases may be detected in cells incubated with cycloheximide. In this regard, a novel 54 kDa MAP2 kinase has been identified...
in the livers of cycloheximide-treated rats (49). However, a mitogen-regulated MAP2 protein-threonine kinase with similar characteristics (e.g., with the ability to phosphorylate MAP2 but not myelin basic protein in vitro) has not been identified, and the 54 kDa MAP2 kinase, as well as the 42 kDa MAP2 kinase, apparently does not activate pp70-S6K in vitro (49). Furthermore, as shown in Fig. 1, C and D, we do not detect stimulation of RSK kinase or MAP2 protein kinase activities by cycloheximide, whereas we do detect serum- and sodium vanadate-stimulated RSK kinase and MAP2 kinase activities in cultured cells. Thus, the mechanism of pp70-S6K activation by cycloheximide remains unclear.

These data also show that S6 phosphorylation following addition of cycloheximide to cultured cells is not dependent upon activation of pp90^rsk but is apparently due to the stimulation of pp70-S6K kinase activity. It cannot be determined by the experiments described here, however, to what extent pp90^rsk participates in physiological S6 phosphorylation. However, current evidence suggests that both may play a role. During Xenopus oocyte maturation, pp90^rsk homologues are the only S6 kinases that have been identified to date that are coordinately regulated with S6 phosphorylation. Furthermore, in serum-deprived HeLa cells, serum stimulates pp90^rsk activity greater than 10-fold while only stimulating pp70-S6K activity by 50% or less (10). RSK kinases and MAP2 kinase activities are also activated under these conditions, and phosphorylation of the 40S ribosomal protein S6 is greatly increased.

Thus, it is likely that in growth-stimulated somatic cells, pp90^rsk participates in early S6 phosphorylation and works together with pp70-S6K to yield maximal phosphorylation. In addition, although few potential physiological substrates have been identified for pp70-S6K, a limited but significant number of substrates for pp90^rsk have been identified (50). These observations imply that pp90^rsk has the potential to modulate other processes regulating cell proliferation. Finally, in addition to characterizing the multiple signaling pathways regulating S6 kinase activity, it may now be possible to more easily analyze signal transduction downstream of pp70-S6 kinase for substrates other than S6, since under the experimental conditions used here, cycloheximide rapidly activates S6 kinase activity without altering the activity of several other mitogen-regulated protein-serine/threonine kinases. Continued biochemical descriptions of these two highly conserved protein kinase pathways will provide valuable information addressing questions related to normal and cancerous cell growth.

Materials and Methods

Cell Cultures and Protein Kinase Assays. CEF and CEF infected with NY72-4, a temperature-sensitive transformation mutant of RSV, were prepared and cultured as described (10, 28). For preparation of cell-free lysates, quiescent cells or cells incubated with various agents for the indicated times were washed in STE (150 mM NaCl, 50 mM Tris-Cl, pH 7.2), scraped in 0.8 ml lysis buffer (10 mM KPO4, 1 mM EDTA, 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N′,N′′-tetraacetic acid, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM diithiothreitol, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 40 μg/ml phenylmethylsulfonyl fluoride, pH 7.2). Dounce homogenized, and clarified by centrifugation at 100,000 × g for 30 min. Assays for pp70-S6K were performed as described (5). Assays for pp90^rsk were completed in the immune complex as reported previously, using rabbit antiserum prepared against recombinant chicken rsk gene product (10). Assays for RSK kinase activity were completed as follows. Briefly, 10 μl of cell lysate (generally, 5–10 μg protein, as determined by the method of Bradford (51)) were incubated at 30°C for 15 min (linear reaction conditions) in a reaction mixture containing 2 μg gel-purified recombinant chicken rsk gene product (re-RSK polypeptide), 10 mM MgCl2, 50 mM ATP (10 μCi [γ-32P]ATP), 20 mM (4-2-hydroxyethyl)1-piperazineethanesulfonic acid, pH 7.2, 0.1 mg/ml bovine serum albumin, 0.05 μg heat-stable cyclic AMP-dependent protein kinase inhibitor (Sigma), 0.05% Triton X-100, and 0.05% Brij-35, in a total volume of 50 μl. Assays for MAP2 kinase activity were similarly completed using 2 μg of pEV-vrfi (a gift from Dr. K. Yphantis, Harvard Medical School) in place of re-RSK polypeptide. The reactions were terminated by adding 12.5 μl of 5X SDS-polyacrylamide gel electrophoresis sample buffer, followed by heating at 90°C for 4 min. The protein synthesis inhibitors used in this study had little effect on the in vitro S6 phosphotransferase assay.

Purification of Recombinant RSK Polypeptide. A complementary DNA fragment encoding amino acids 53–752 of chicken rsk was inserted into the EcoRI site of the vector pEV-vrfi, and Escherichia coli RR1 were transformed with this construct as described (52). The recombinant chicken RSK polypeptide produced in bacteria was isolated as an insoluble complex (inclusion bodies) from lysed bacteria, and the RSK polypeptide was further purified by preparative SDS-polyacrylamide gel electrophoresis and elution from gel slices. The gel-purified re-RSK polypeptide was used directly in the in vitro RSK kinase assays described above.

Chromatographic Fractionation of S6 Kinase Activity. Cell-free lysates from 5–7 × 10^7 CEF were prepared 1 h after the addition of cell serum (10% v/v), cycloheximide (100 μM), puromycin (500 μM), or sodium orthovanadate (1 mM), clarified by centrifugation at 100,000 × g, filtered through a 0.22 μm filter, and fractionated by cation-exchange chromatography (Mono S, Pharmacia FPLC system) followed by anion-exchange chromatography (Mono Q, Pharmacia), as previously described (5, 28). Partially purified S6 kinase activity from all sources was concentrated by dialysis and stored at –20°C.

Gel Electrophoresis. 40S ribosomal proteins used in S6 protein kinase assays, recombinant RSK used for RSK protein kinase assays, and MAP2 used for MAP2 protein kinase assays were resolved by sodium dodecyl sulfate-12%, -7.5%, and -6% polyacrylamide gels, respectively, as described (53). For qualitative analysis of in vitro S6 phosphorylation, Coomassie blue-stained gels were dried and exposed to Dupont Cronex-4 film or Kodak XAR-5 X-Omat film with Dupont Lightning Plus intensifying screens. For quantitation, radiolabeled substrates were identified and excised, and associated radioactivity was determined by liquid scintillation spectrometry.

5 J. Blenis and J. Chung, unpublished observations.

6 R. H. Chen and J. Blenis, unpublished observations.
Ribosomes were isolated from 14C-labeled cultures as described (34). Briefly, CEF were seeded at 0.5–1 × 10^7 cells/150-mm Falcon culture dish and allowed to reach confluence. The medium was then changed to serum-free Dulbecco’s modified Eagle’s medium containing 15% normal phosphate levels. After 12 h, 32P (100 μCi/ml) was added for an additional 12 h. Cultures were then incubated with serum (10% v/v), cycloheximide (100 μg/ml), or sodium vanadate (1 mM) for 2 h. Ribosomes were isolated and analyzed by two-dimensional alkaline- and acid-urea polyacrylamide gel electrophoresis (33–35).

**Protein Synthesis.** CEF were grown to confluence in 60-mm Corning culture dishes and then incubated for an additional 12–24 h in Dulbecco’s modified Eagle’s medium containing 25% normal methionine levels. To measure the rate of protein synthesis, [35S]methionine (5–50 μCi/ml) was added to all cultures followed by addition of buffer containing various concentrations of cycloheximide or sodium vanadate for 1 h. The plates were then rinsed in ice-cold phosphate-buffered saline, scraped in 0.01 M NaOH-0.05% SDS, boiled, and sonicated briefly. Aliquots were removed for determination of [35S]methionine incorporation into 15% trichloroacetic acid-precipitable protein as well as total protein concentration by the Bradford dye-binding assay (51). The percentage inhibition of protein synthesis was calculated based on the decreased incorporation of 35S cpm/μg protein compared to untreated control cells.

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**References**