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Fibroblast growth factor treatment of Swiss 3T3 cells activates a subunit S6 kinase that phosphorylates a synthetic peptide substrate

(acidic and basic fibroblast growth factor/40S ribosomal subunit S6/phorbol 12-myristate 13-acetate)

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ABSTRACT **Exposure of quiescent cultures of Swiss 3T3-D1 cells to bovine brain acidic fibroblast growth factor (FGF) enhanced phosphorylation of a 31-kDa protein tentatively identified as 40S ribosomal subunit S6 (S6). Soluble extracts from FGF-treated as compared with quiescent fibroblasts exhibited up to 3-fold higher kinase activity towards S6 in exogenously added rat liver 40S ribosomes and a synthetic peptide, RRLSSLRA. This peptide was patterned after a phosphorylation site sequence in S6 and was phosphorylated with an apparent K_m corresponding to 0.18 mM. Optimal activation of the S6 kinase with pure mitogen at 10 ng/ml occurred within 15 to 20 min exposure to FGF. Half-maximal stimulation of the FGF-induced S6 kinase was attained with FGF at 0.4 ng/ml. The S6 kinase in crude extracts utilized both [γ-^{32}P]ATP (apparent $K_m \simeq 6$–8 μM) and [γ-^{32}P]GTP (apparent $K_m \simeq 3$ μM), but the ability to utilize GTP was lost after partial purification of the kinase. The FGF-stimulated kinase had an apparent M_r of about 95,000 as determined by chromatography on Sephacryl S300 but appeared to be retarded on TSK 400 HPLC columns, since it eluted with an apparent M_r of 29,000. Treatment of Swiss 3T3 cells with the tumor promoter phorbol 12-myristate 13-acetate (PMA) activated the FGF-stimulated S6 kinase. However, protein kinase C was not required to mediate the FGF activation of the S6 kinase, as FGF still evoked a two-fold activation of the S6 kinase in phorbol ester-pretreated, protein kinase C-depleted cells.**

Phosphorylation of 40S ribosomal subunit S6 (S6) on at least five serine residues can be triggered by a variety of mitogenic stimuli including growth factors, transforming gene products, and tumor promoters (1–6). Soluble extracts from insulin, epidermal growth factor, and fetal calf serum-treated cells exhibit elevated kinase activity towards reconstituted S6 (7–11). It is unclear whether one or more S6 kinases are involved, since S6 can be phosphorylated by as many as nine distinct protein-serine kinases *in vitro* (11–18). However, ion-exchange chromatography studies with extracts from chicken embryo fibroblasts infected with Rous sarcoma virus or exposed to phorbol 12-myristate 13-acetate (PMA) or 10% calf serum have implied that a unique S6 kinase may be activated by these diverse treatments (10). The S6 kinase remains to be purified from a mammalian source. Fractionation and characterization of the stimulated S6 kinase activity in cytosol have been hampered by its strict specificity for S6 (18).

In this report, we have utilized a synthetic peptide based on a sequence corresponding to one of the phosphorylation sites of S6 as a convenient substrate for a mitogen-responsive S6 kinase (19, 20). This S6 kinase was activated when quiescent mouse fibroblasts were exposed to either the acidic or the basic form of fibroblast growth factor (FGF). Although anionic and cationic FGF are distinct polypeptides, they appear to compete for a common 165-kDa receptor on Swiss 3T3 cells with equal affinity (21). How the binding of FGF to its receptor culminates in activation of the S6 kinase remains obscure, but the cascade can proceed via protein kinase C-independent mechanisms.

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EXPERIMENTAL PROCEDURES

Materials

Acidic and basic FGF were purified to homogeneity from bovine brain (21). Swiss 3T3-D1 mouse fibroblasts were provided by Li-Chaun Huang, Department of Biochemistry, University of Washington (Seattle, WA). Rat liver 40S ribosomes were the generous gift of R. Traut, Department of Biological Chemistry, University of California (Davis, CA). The peptides, RRLSSLRA and TTYADFIASGRTGRRNAIHD, were kindly synthesized in this laboratory by John Scott. [γ-^{32}P]ATP, [γ-^{32}P]GTP, and [^{32}P]orthophosphate were purchased from New England Nuclear. Electrophoresis and gel filtration protein standards were from Bio-Rad. Most other chemicals were of at least reagent grade and obtained through Sigma.

Cell Culture and Subcellular Fractionation

Swiss 3T3-D1 mouse fibroblasts were grown to confluence in 15-cm Falcon Integrid tissue culture dishes in 10% fetal calf serum (KC Biological, Lenexa, KS) and low-glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO). Cells were rendered quiescent by 36–48 hr incubation in 0.33% fetal calf serum in DMEM. Unless stated otherwise, quiescent cells were incubated in the absence or presence of FGF at 10 ng/ml for 20 min prior to harvesting. Cells were washed twice with 10 ml of ice-cold phosphate-buffered saline, scraped into 1.5-ml plastic tubes, pelleted in a benchtop Eppendorf centrifuge for 20 sec and resuspended in 50–100 μl of buffer A (60 mM β-glycerophosphate/30 mM *p*-nitrophenyl phosphate/25 mM Mops, pH 7.2/15 mM EGTA/15 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM sodium vanadate) per dish of cells. Cells were homogenized in buffer A with 40 strokes of a Dounce homogenizer, and a 150,000 × *g* × 20 min soluble extract was obtained in a Beckman Airfuge at 5°C. Aliquots of the cytosolic extracts were immediately frozen at −70°C.

Abbreviations: FGF, fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; S6, subunit S6.

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Enzyme Assays

Unless stated otherwise, all kinase assays contained 400 nM TTYADFIASGRTGRRNAIHD peptide (22, 23) to inhibit cAMP-dependent protein kinase, 6 A_{260} units of 40S ribosomes per ml or 0.25 mM RRLSSLRA peptide, 15 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP (2 cpm/fmol for filter paper assays, 10 cpm/fmol for NaDodSO$_4$/PAGE gels), 0.04–0.12 mg of cytosolic protein/ml, and buffer B (buffer A with 5 mM instead of 15 mM EGTA) in a final volume of 25 μl. Reactions commenced upon addition of radioactive nucleotide and were of 5–30 min duration at 30°C. For termination of assays with RRLSSLRA, 20 μl aliquots were spotted on 1.5-cm^2 pieces of Whatman P81 phosphocellulose filter paper and 30 sec later washed five times for at least 2 min each wash in a solution of 10 ml phosphoric acid/liter H_2O. The wet filter papers were transferred into 6-ml plastic scintillation vials with 5 ml of Aquasol scintillation fluid and analyzed for radioactivity in a Packard counter.

Assays with 40S ribosomes were stopped upon addition of 40 μl of NaDodSO$_4$/PAGE sample buffer [0.5% NaDodSO$_4$/50 mM Tris·HCl, pH 6.8/12.5% glycerol (vol/vol)/0.04% bromophenol blue] and immediately boiled for 5 min. The samples were subjected to NaDodSO$_4$/PAGE on 16% polyacrylamide gels as described by Laemmli (24). Ribosomal proteins were visualized by Coomassie blue staining, and the band corresponding to S6 was excised and its radioactivity was analyzed in 5 ml of Aquasol. Protein was estimated by the method of Bradford (25). Details concerning electrophoresis and gel filtration are provided in the figure legends.

RESULTS AND DISCUSSION

Phosphorylation of Ribosomal S6. Ribosomal S6 undergoes multiple serine phosphorylation in quiescent fibroblasts exposed to platelet-derived growth factor, epidermal growth factor, insulin, insulin-like growth factor-1, PMA, and prostaglandin $F_{2\alpha}$ (1–5, 26). Consequently, it was relevant to explore the influence of bovine brain acidic FGF, a 17-kDa mitogen for fibroblasts. This anionic FGF elicited increased phosphorylation of a 31-kDa protein in 3T3-D1 mouse fibroblasts labeled with [^{32}P]orthophosphate (Fig. 1, lanes 1 and 2). Treatment of quiescent Swiss 3T3 cells with an impure preparation of basic FGF has recently been reported to facilitate phosphorylation of an endogenous 31-kDa protein (6).

Assignment of the 31-kDa phosphoprotein as S6 was supported by reconstitution studies with rat liver 40S ribosomes. Soluble extracts from avian and mammalian fibroblasts treated with 10% fetal calf serum, EGF, or PMA retain an enhanced ability to phosphorylate S6 when phosphatase inhibitors such as β-glycerophosphate are present (7–9, 18). Cytosolic extracts prepared from acidic FGF-treated cells in the presence of added 40S ribosomes and [γ-^{32}P]ATP displayed a 3.1 ± 0.9 (SD; n = 4)-fold increase in exogenous S6 phosphorylation (Fig. 1, lanes 3 and 4). The FGF-stimulated S6 kinase activity was also supported by [γ-^{32}P]GTP [2.4 ± 0.5 (SD; n = 4)-fold activation compared with extracts from quiescent non-treated cells; data not shown]. Inhibition of endogenous cAMP-dependent protein kinase-catalytic subunit activity with a synthetic peptide (TTYADFIASGRTGRRNAIHD) corresponding to the N-terminal third of the heat stable cAMP-dependent protein kinase inhibitor (22, 23) did not appreciably reduce the extent of the FGF-stimulated S6 phosphorylation with either nucleotide triphosphate (Fig. 1, lanes 5, 6, and not shown). S6 phosphorylation by cell-free extracts of FGF-treated fibroblasts was unaffected by heparin at 1 μg/ml but was abolished with heparin at 30 μg/ml as well as by 50 mM NaF, 10 mM *N*-ethylmalemide, and 2.5 mM $MnCl_2$.

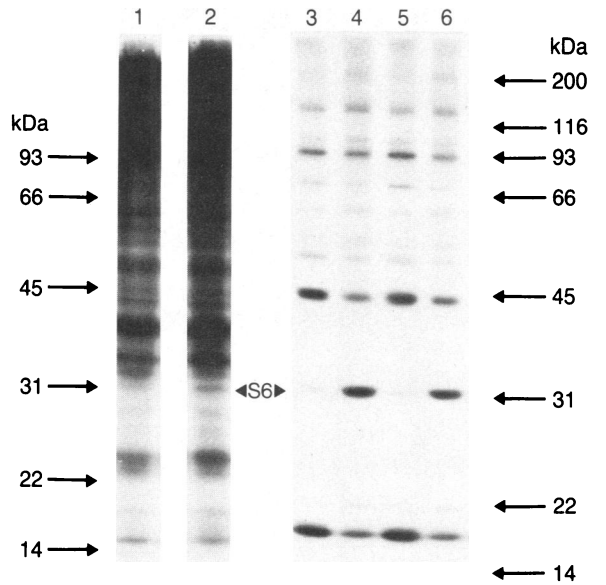

FIG. 1. Autoradiography of FGF stimulation of S6 phosphorylation in intact mouse fibroblast cells and cell-free extracts. Confluent and quiescent 3T3-D1 cells in 15-cm dishes were incubated for 3 hr in 10 ml of DMEM without phosphate but reconstituted with 200 μCi of [^{32}P]orthophosphate (1 Ci = 37 GBq). During the final 20 min, acidic FGF at 10 ng/ml was added to the labeling medium of some dishes. Cells were subsequently washed 6 times with 15 ml of ice-cold phosphate-buffered saline (PBS) and scraped into 1.5 ml of PBS. Cells were pelleted in an Eppendorf benchtop centrifuge (20 sec), resuspended in NaDodSO$_4$/PAGE sample buffer, and boiled for 5 min. Equivalent to one-third of a dish of untreated (lane 1) and FGF-treated (lane 2) cells were applied to 16% polyacrylamide gels and electrophoresed as described by Laemmli (24), 25 mA per gel. In other experiments, cytosolic extracts from control quiescent cells (lanes 3 and 5) and cells pretreated for 20 min with acidic FGF at 10 ng/ml (lanes 4 and 6) were prepared as described in *Experimental Procedures*. Extracts (0.4 mg of protein/ml) were incubated at 30°C for 15 min with 15 μM [γ-^{32}P]ATP (lanes 3–6) (10 cpm/fmol), 6 A_{260} units of rat liver 40S ribosomes per ml, and buffer B, in the absence (lanes 3 and 4) and presence (lanes 5 and 6) of 400 nM protein kinase inhibitor-derived peptide in a final volume of 25 μl. Reactions were terminated upon addition of 40 μl of NaDodSO$_4$/PAGE sample buffer, immediately boiled for 5 min, and electrophoresed as above. Positions of the marker proteins, myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme are indicated (lanes 1 and 2, left; lanes 3–6, right). Coomassie-blue stained gels were dried and exposed to Kodak X-Omat XRP-1 film with Dupont Hi plus Cronex intensifying screens.

Phosphorylation of the Synthetic Peptide RRLSSLRA. Rat hepatic ribosomal protein S6 has been partially sequenced for a region that contains the major phosphorylation sites for cAMP-dependent protein kinase and one of the insulin-stimulated phosphorylation sites (19). A synthetic peptide, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (RRLSSLRA), based upon this sequence has been shown to be a substrate for cAMP-dependent protein kinase and a trypsin-activated kinase (20). The latter may be the Ca^{2+}-dependent phospholipid-stimulated protein kinase C, since this kinase also phosphorylates the peptide (27). While the first serine residue is the preferred site for cAMP-dependent protein kinase, insulin appears to modulate the phosphorylation of the adjacent serine (19). Hence, we were interested in the possibility that RRLSSLRA might serve as a convenient substrate for the FGF-activated S6 kinase. The peptide TTYADFIASGRTGRRNAIHD was used to inhibit the endogenous cAMP-dependent kinase activity towards RRLSSLRA, while the presence of EGTA prevented activation of protein kinase C.

In the presence of these inhibitors, cytosolic extracts from acidic FGF-treated cells were discovered to contain enhanced RRLSSLRA kinase activity. Although RRLSSLRA appeared to be phosphorylated with both high- and low-affinity kinetics (apparent K_ms of approximately 0.18 mM and 1 mM, irrespective of ATP or GTP as the second substrate), the high-affinity kinase activity seemed to be more sensitive to FGF regulation (Fig. 2 and not illustrated). The low-affinity kinase activity may have corresponded to a different kinase that was not subject to mitogenic control. Consequently, 0.25 mM RRLSSLRA was used in subsequent studies to minimize the contribution of the low-affinity kinase and accentuate the effect of the growth factor. Even though this concentration of the peptide was only slightly greater than the putative K_m, reaction rates were linear with respect to time for at least 10 min. Most peptide assays were of 5-min duration.

The apparent V_{max} of RRLSSLRA phosphorylation was accelerated in response to FGF with no alterations in the relative affinities for the peptide, ATP, or GTP (Figs. 2 and 3). The apparent K_m values for ATP and GTP (8 and 3 μM, respectively) with RRLSSLRA were comparable to apparent K_m values derived for ATP and GTP with S6 in 40S ribosomes (6 and 3 μM, respectively, not illustrated). With 0.25 mM RRLSSLRA and 15 μM [γ-^{32}P]ATP, extracts from FGF-treated cells had 2.5 ± 0.5 (SD; n = 6)-fold more kinase activity than detected in quiescent cell extracts. This kinase activation was 2.2 ± 1.2 (SD; n = 6)-fold with [γ-^{32}P]GTP. Under these assay conditions the S6 kinase activity was determined in extracts from 3T3 cells exposed to acidic FGF as a function of time and mitogen concentration (Fig. 4). Maximal stimulation of RRLSSLRA phosphorylation with [γ-^{32}P]ATP by 10 ng/ml of the growth factor was evident within 15 to 20 min exposure to FGF (Fig. 4*A*). Half maximal activation of peptide phosphorylation was achieved with acidic FGF at 0.4 ng/ml (Fig. 4*C*). Identical results were obtained when [γ-^{32}P]GTP was substituted for [γ-^{32}P]ATP (not shown), or when S6 in 40S ribosomes served as the protein substrate (Fig. 4*B* and *D*). The rate at which FGF evoked optimal stimulation was comparable to the reported time course of activation of the S6 kinase in 3T3 cells by epidermal growth factor (8).

Fractionation of S6 Kinase Activity by Gel Filtration. Since RRLSSLRA proved to be a reliable probe for the FGF-activated S6 kinase in 150,000 × g supernatants of cells, we employed this substrate to detect the kinase in fractionated

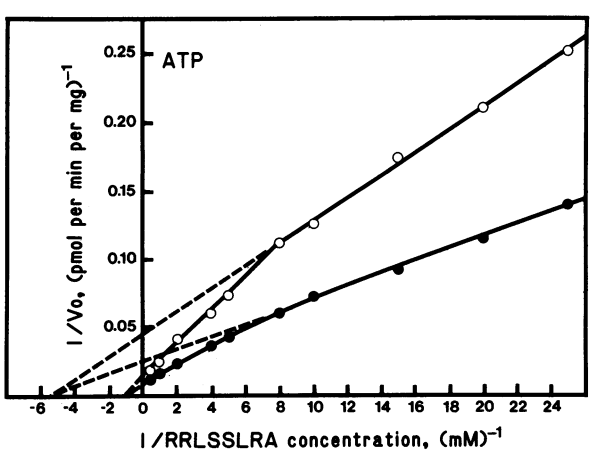

FIG. 2. Concentration dependence of RRLSSLRA phosphorylation. Cytosolic extracts from acidic FGF (10 ng/ml)-treated (●) and untreated (○) mouse fibroblasts were assayed for kinase activity with 0.04–2 mM concentrations of the synthetic peptide RRLSSLRA and 15 μM [γ-^{32}P]ATP. Similar results were obtained in three independent experiments.

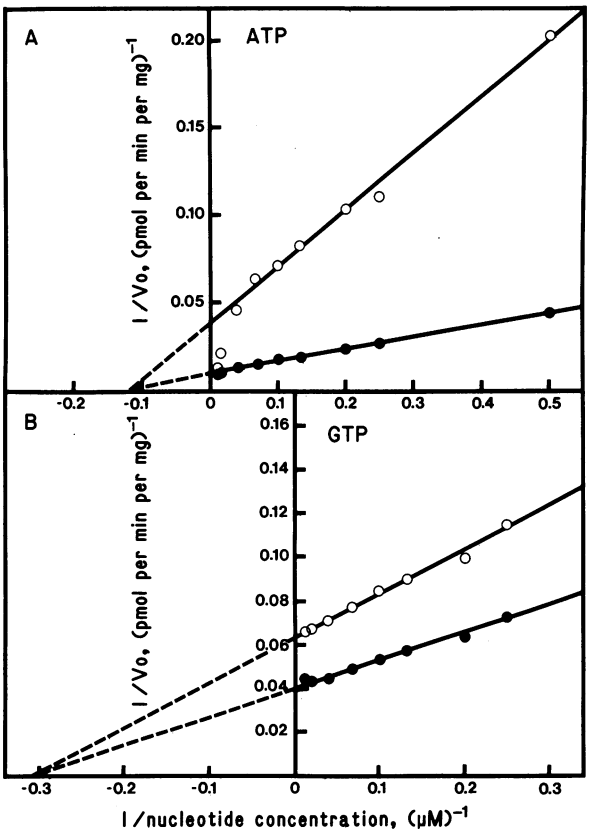

FIG. 3. Concentration dependence of ATP and GTP phosphorylation of RRLSSLRA. Cytosolic extracts from acidic FGF (10 ng/ml)-treated (●) and untreated (○) 3T3-D1 cells were assayed for kinase activity with 0.25 mM RRLSSLRA peptide and 1–75 μM [γ-^{32}P]ATP (*A*) or [γ-^{32}P]GTP (*B*). Similar results were obtained in three independent experiments.

extracts with [γ-^{32}P]ATP. A FGF-induced RRLSSLRA phosphorylating activity eluted with an apparent M_r of 95,000 ± 8,000 (SD; n = 3) from Sephacryl S300 columns (Fig. 5*A*). By contrast, this same kinase emerged from TSK-400 HPLC columns with an apparent size corresponding to 29 ± 3 (SD; n = 4) kDa (Fig. 5*B*). Some of the RRLSSLRA kinase activity from fractionated quiescent cell extracts could be accounted for by the 3–5% residual cAMP-dependent protein kinase-catalytic subunit activity that was not completely abolished by the inhibitory peptide and coeluted with the FGF-induced RRLSSLRA kinase from TSK-400 columns. No FGF-stimulated RRLSSLRA phosphorylating activity with [γ-^{32}P]GTP as the nucleoside triphosphate substrate could be demonstrated with cytosolic extracts fractionated on Sephacryl S300 or TSK-400 columns (not illustrated). Thus it would appear that peptide phosphorylation by cytosol with [γ-^{32}P]GTP may have involved an intermediate reaction. As only one FGF-regulated kinase peak could be resolved by the two gel filtration resins, and this kinase appeared to interact with the TSK-400 gel, it seemed likely that a unique S6 kinase was sensitive to mitogenic simulation.

Many of the features of the 3T3 cell FGF-responsive S6 kinase correlated with a 92-kDa kinase that is highly selective for S6 and has recently been purified from frog eggs (18). We have determined that the *Xenopus* oocyte S6 kinase shares identical chromatographic properties with the 3T3 cell S6 kinase on Sephacryl S300 and TSK-400 columns. The RRLSSLRA phosphorylating activity of the amphibian S6 kinase can be stimulated up to eight-fold when oocytes are induced with progesterone to undergo germinal vesicle breakdown (unpublished work; S.L.P., E.G.K., M. Cicirelli).

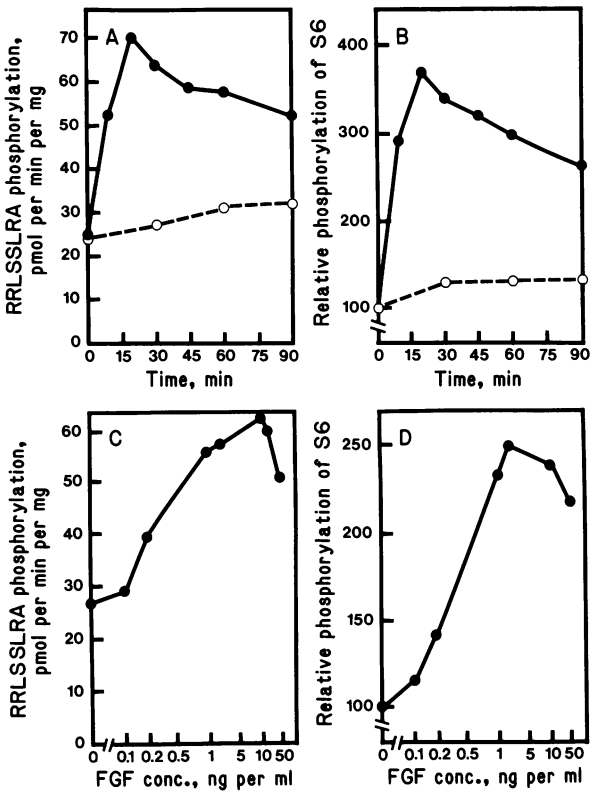

FIG. 4. Time and concentration dependence for acidic FGF activation of the S6 kinase. Cytosolic extracts were prepared from quiescent 3T3-D1 cells incubated for 0–90 min without (○) and with acidic FGF at 10 ng/ml (●) (*A* and *B*) and for 20 min with acidic FGF at 0–50 ng/ml (*C* and *D*). The phosphorylating activity of these extracts was assessed with 15 μM [γ-^{32}P]ATP toward 0.25 mM synthetic peptide RRLSSLRA (*A* and *C*) and 0.4 μM rat liver ribosomal S6 (*B* and *D*).

Studies with PMA-Treated and Protein Kinase C-Deficient Fibroblasts. Blenis and Erickson (10) have recently described an S6 kinase in chicken embryo fibroblasts that was activated by pp60$^{v\text{-}src}$, calf serum, and PMA. Likewise, extracts from PMA-treated 3T3 cells exhibit enhanced RRLSSLRA and S6 phosphorylating activity that comigrates with the FGF-stimulated S6 kinase on Sephacryl S300 and TSK-400 columns (unpublished data; S.L.P., E.G.K.). Therefore, protein kinase C could potentially mediate the action of those mitogens that accelerate phosphatidyl-phosphoinositol turnover and induce S6 phosphorylation.

Basic FGF has been shown to elicit rapid diacylglycerol formation, protein kinase C activation, and Ca^{2+} mobilization when added to quiescent cultures of Swiss 3T3 cells (28), although acidic FGF has not been tested. There is as yet no direct evidence that either FGF alters tyrosine protein phosphorylation in responsive cells (29). Consequently, it was feasible that protein kinase C activation initiated by FGF-induced phosphatidyl-phosphoinositol breakdown led to stimulation of the S6 kinase, possibly via direct phosphorylation by protein kinase C.

Blackshear *et al.* (6) have reported that protein kinase C in 3T3 cells can be almost completely down-regulated by prolonged exposure to phorbol esters. Such protein kinase C-deficient cells should be incapable of enhanced S6 kinase activity in response to FGF if protein kinase C served as the sole link.

The RRLSSLRA phosphorylating activity with [γ-^{32}P]ATP was examined in soluble extracts from quiescent cells (*A*), cells exposed to 100 nM PMA for 10 min (*B*), and cells pretreated with 5 μM PMA for 15 hr without (*C*) and with (*D*) acidic FGF at 10 ng/ml during the last 20 min prior to harvest. The specific kinase activities (pmol·min^{-1}·mg^{-1}; ± SEM of three sets of pooled cells) were (*A*) 28.5 ± 1.5, (*B*) 55.5 ± 2.1, (*C*) 22.5 ± 0.5, and (*D*) 46.5 ± 0.9, respectively. Similar trends were obtained with S6 in 40S ribosomes (data not shown). Moreover, in protein kinase C-depleted 3T3-L1 fibroblasts, impure basic FGF but not PMA stimulated phosphorylation of a 31-kDa protein, tentatively identified as S6 (6). Other responses to FGF in 3T3 cells such as enhanced phosphorylation of a 22-kDa protein (6), Ca^{2+} mobilization, and induction of DNA synthesis (28, 30) are also preserved in protein kinase C-deficient cells. Consequently, FGF responses can be mediated via protein kinase C-independent pathways.

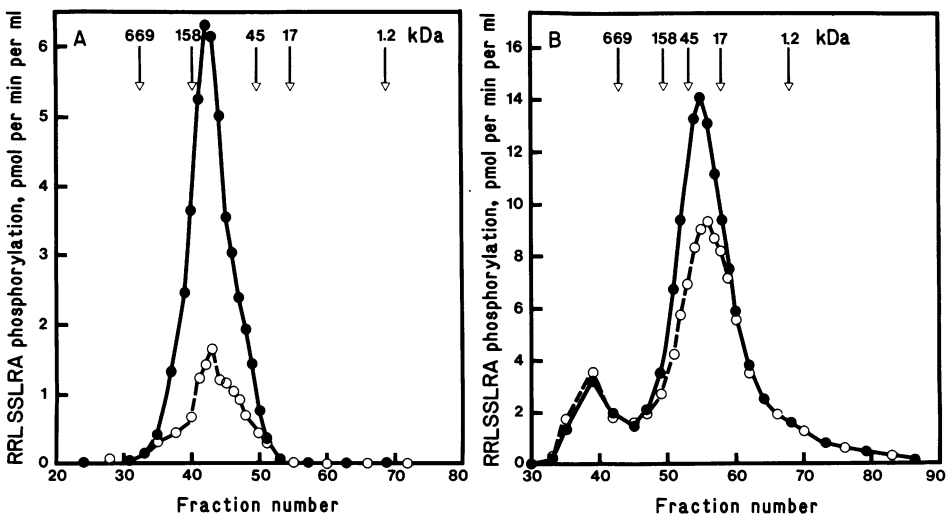

FIG. 5. Gel filtration chromatography of the FGF-stimulated S6 kinase. Cytosolic protein from untreated (○) and acidic FGF (10 ng/ml)-treated (●) 3T3-D1 cells was subjected to Sephacryl S300 (*A*) and TSK-400 (*B*) chromatographies. Both columns were equilibrated and eluted with buffer A plus 100 mM NaCl and 0.05% Brij 35. Four hundred micrograms of cytosolic protein was applied to a 1 × 55-cm Sephacryl S300 column (Pharmacia) and eluted at a flow rate of 0.15 ml/min into 0.5-ml fractions. Three hundred micrograms of cytosolic protein was chromatographed on a 0.75 × 30 cm TSK-400 GSWP HPLC column (Bio-Rad) at a flow rate of 0.5 ml/min and collected into 0.25-ml fractions. Fractionated extracts were assayed for RRLSSLRA peptide (0.25 mM) phosphorylating activity with 15 μM [γ-^{32}P]ATP in the presence of 400 nM protein kinase inhibitor-derived peptide. Each fractionation was performed at least three times with identical results. Elution positions of the marker proteins, thyroglobulin, IgG, ovalbumin, myoglobin, and vitamin B_{12} are indicated (left to right).

Although the acidic form of FGF was more thoroughly examined, the effect of the 19-kDa bovine brain basic FGF on S6 kinase activity was also investigated in this study in addition to the experiments detailed above. Both FGFs produced comparable activation of the S6 kinase with respect to mitogen dose, time, and fold stimulation (not shown). These data are consistent with the finding that both factors compete for a common 165-kDa receptor on 3T3-D1 fibroblasts (21). The binding constants and ED_{50} values for stimulation of [^{3}H]thymidine incorporation into DNA corresponded to approximately 0.7 ng/ml for both acidic and basic FGF (21) which is comparable with the ED_{50} value derived here for half-maximal stimulation of the S6 kinase (0.4 ng/ml, Fig. 4 *C* and *D*).

The early events in the cascade that are initiated upon FGF binding to its receptor remain obscure. Apparently, FGF-induced phosphatidyl-phosphoinositol turnover is not mandatory for activation of the S6 kinase. Since platelet-derived growth factor, insulin, insulin-like growth factor-1, and epidermal growth factor stimulated the protein-tyrosine kinase activities of their respective receptors and similarly activate an S6 kinase, it may be that the FGF receptor is closely linked with a protein-tyrosine kinase activity.

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