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Identification of a 42-Kilodalton Phosphotyrosyl Protein as a Serine(Threonine) Protein Kinase by Renaturation

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We have surveyed fibroblast lysates for protein kinases that might be involved in mitogenesis. The assay we have used exploits the ability of blotted, sodium dodecyl sulfate-denatured proteins to regain enzymatic activity after guanidine treatment. About 20 electrophoretically distinct protein kinases could be detected by this method in lysates from NIH 3T3 cells. One of the kinases, a 42-kilodalton serine(threonine) kinase (PK42), was found to possess two- to fourfold-higher in vitro activity when isolated from serum-stimulated cells than when isolated from serum-starved cells. This kinase comigrated on sodium dodecyl sulfate-gels with a protein (p42) whose phosphotyrosine content increased in response to serum stimulation. The time courses of p42 tyrosine phosphorylation and PK42 activation were similar, reaching maximal levels within 10 min and returning to basal levels within 5 h. Both p42 tyrosine phosphorylation and PK42 activation were stimulated by low concentrations of phorbol esters, and the responses of p42 and PK42 to TPA were abolished by chronic 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment. Chronic TPA treatment had less effect on serum-induced p42 tyrosine phosphorylation and PK42 activation. PK42 and p42 bound to DEAE-cellulose, and both eluted at a salt concentration of 250 mM. Thus, PK42 and p42 comigrate and cochromatograph, and the kinase activity of PK42 correlates with the tyrosine phosphorylation of p42. These findings suggest that PK42 and p42 are related or identical, that PK42 is activated by tyrosine phosphorylation, and that this tyrosine phosphorylation can be regulated by protein kinase C.

Tyrosine-specific protein kinases that span the plasma membrane or associate with the inner face of the plasma membrane can initiate mitogenic signals. To understand how these signals are relayed to the nucleus and other cellular targets, it will be necessary to identify the substrates of the kinases and to determine the consequences of the tyrosine phosphorylation of these substrates.

Recent work has identified two such substrates as cytoplasmic serine(threonine)-specific protein kinases. One is the 74-kilodalton (kDa) Raf-1 protein, the cellular homolog of the v-*raf* oncoprotein. A variety of mitogens, including platelet-derived growth factor and epidermal growth factor, cause the Raf-1 protein to become hyperphosphorylated (34). It is known that with platelet-derived growth factor, this hyperphosphorylation includes tyrosine phosphorylation and is associated with an increase in the in vitro kinase activity of the Raf-1 protein (34). The platelet-derived growth factor receptor can phosphorylate the Raf-1 protein at one or more tyrosine residues when the two kinases are coexpressed in insect cells, and this tyrosine phosphorylation is again associated with an increase in Raf-1 kinase activity in vitro (33). Thus, tyrosine phosphorylation of $p74^{Raf-1}$ may lead to its activation, either directly or by triggering autophosphorylation.

Another tyrosine-phosphorylated serine(threonine) kinase is the *cdc2* gene product. The *cdc2* gene was initially identified in the fission yeast *Schizosaccharomyces pombe*, in which its product appears to play a role in the G_1-to-S and G_2-to-M transitions (36, 44). Homologs are found in the budding yeast *Saccharomyces cerevisiae*, (*CDC28*, a "start" gene [1, 42]) and in a variety of vertebrate and invertebrate cells (10, 13, 27). $p34^{cdc2}$ becomes phosphorylated at tyrosine prior to the M phase in diverse cell types (10, 12, 20, 32). Evidence has been presented implicating the c-*src* protein in this phosphorylation in mammalian cells (12). Phosphorylation of $p34^{cdc2}$ may allow the kinase to associate with a cyclin (10, 11, 15, 38). The cyclin-bound $p34^{cdc2}$ then becomes enzymatically active when the complex is dephosphorylated at the onset of M-phase (10, 12, 27, 32; see also reference 14 for a recent review).

Recently we have undertaken a search for new protein kinases that might be involved in mitogenesis. The method we have used begins by separating cell proteins by denaturing polyacrylamide gel electrophoresis, then transferring the proteins to a blotting membrane and allowing the blotted proteins to renature after treatment with guanidine hydrochloride (3, 17). The renatured, blotted proteins are then overlaid with [γ-^{32}P]ATP, and kinase activities are detected by autoradiography. This method has allowed us to assess the activities of roughly 20 electrophoretically distinct protein kinases in lysates from NIH 3T3 cells and to determine the electrophoretic mobilities of these activities directly and with high precision.

In this report we show that one renaturable kinase, a 42-kDa serine(threonine) protein kinase (PK42), is rapidly but transiently activated in fibroblasts released from serum starvation and we present evidence that the activation of PK42 results from its tyrosine phosphorylation. Recently, Sturgill and co-workers have demonstrated that a serine(threonine) protein kinase, MAP-2 kinase, copurifies with a 42-kDa phosphotyrosyl protein (39–41, 43, 45); PK42 may be related or identical to this kinase.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells (Imperial Cancer Research Fund) were maintained in 5% calf serum or 5% calf serum–5% fetal calf serum. Maintenance cultures were not allowed to become confluent. Cell growth was arrested by incubation

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at an initial density of 3×10^5 cells per 35-mm dish or 2×10^6 cells per 100-mm dish in 0.5% fetal calf serum for 48 h. For mitogen stimulation, the medium was replaced with either 5% calf serum–5% fetal calf serum or 0.5% calf serum containing 0.1% dimethyl sulfoxide and 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Cells were radiolabeled for 6 h in medium containing 4% normal P_i plus 1 mCi of $^{32}P_i$ per ml. Radiolabeled cells were stimulated by adding calf serum and fetal calf serum or TPA in dimethyl sulfoxide directly to the labeling medium.

Lysis. After serum-starved cells were incubated with mitogens for various lengths of time, the cells were washed twice with ice-cold Tris-buffered saline and lysed by one of several methods. For antiphosphotyrosine immunoblotting or kinase renaturation, cells were lysed by addition of gel sample buffer (2.3% sodium dodecyl sulfate [SDS], 62.5 mM Tris, 5 mM EDTA, 10% glycerol, plus 5% 2-mercaptoethanol [pH 6.8]; 250 μl per 35-mm plate or 1 ml per 100-mm plate) which had been preheated to 100°C in a boiling-water bath. Lysates were scraped into microcentrifuge tubes, submerged in boiling water for 3 min, vortexed briskly, and centrifuged for 5 min to pellet insoluble debris. It was found not to be necessary to hydrolyze nucleic acids with DNase and RNase. In some experiments lysates were prepared for immunoblotting or renaturation by lysis with ice-cold immunoprecipitation lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% [vol/vol] aprotinin solution [Sigma Chemical Co.], 100 μg of leupeptin per ml, 200 μg of phenylmethylsulfonyl fluoride per ml, 1 mM sodium orthovanadate [pH 7.4]). Lysates were centrifuged for 5 min to pellet nuclei and insoluble debris. Gel sample buffer was added to the supernatants as a 5× concentrate, and the samples were promptly boiled for 3 min. These two lysis methods yielded similar amounts of PK42 activity and phosphotyrosyl-p42.

Samples for two-dimensional gel electrophoresis were lysed with preheated 0.3% SDS–65 mM dithiothreitol–1 mM EDTA–20 mM Tris (pH 8.0) and treated with DNase and RNase as described previously (18, 23). Nuclease-treated lysates were lyophilized and suspended in isoelectric focusing sample buffer (37).

For in vitro dephosphorylation experiments, cells were scraped into ice-cold hypotonic lysis buffer [25 mM Tris, 25 mM NaCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 200 μM phenylmethylsulfonyl fluoride, 0.5 μg of antipain per ml, 0.5 μg of pepstatin A per ml, 0.5 μg of chymostatin per ml, 0.5 μg of aprotinin per ml, 0.5 μg of leupeptin per ml (pH 7.5 at 4°C)] and homogenized for 25 strokes in a Dounce homogenizer. Debris were pelleted for 10 min in a microcentrifuge. Samples for anion-exchange chromatography on DEAE-cellulose were prepared similarly, except that 1 mM sodium orthovanadate was included in the lysis buffer.

One-dimensional polyacrylamide gel electrophoresis and transfer to PVDF membranes. Samples containing 50 to 100 μg of protein was subjected to discontinuous polyacrylamide gel electrophoresis (8.5% acrylamide; acrylamide/methylene bisacrylamide ratio of 37.5:1 in a separating gel). Prestained molecular weight markers (Sigma) were calibrated against unstained markers (Sigma). Proteins were transferred for 90 min at 40 V to poly(vinylidene difluoride) (PVDF) membranes (Immobilon P; Millipore Corp.) in 192 mM glycine–25 mM Tris transfer buffer without SDS or methanol. Gels were not equilibrated with transfer buffer prior to transfer.

Two-dimensional gel electrophoresis. Isoelectric focusing was carried out in 11-cm tube gels with pH 3 to 10 Pharmalytes as described previously (37). The resulting pH gradient was assessed with a surface electrode. Second-dimension separations were carried out on 8.5 or 10% acrylamide (acrylamide/bisacrylamide ratio of 37.5:1)–SDS gels.

Antiphosphotyrosine immunoblotting. An antiserum against a polymer of phosphotyrosine, glycine, alanine, and keyhole limpet hemocyanin was raised and affinity purified as described originally by Kamps and Sefton (21). Blots were probed with antiphosphotyrosine antiserum followed by ^{125}I-labeled protein A (approximate specific activity, 30 to 40 μCi/μg; ICN Radiochemicals) as described previously (21). There was some variation in the pattern of bands seen in different experiments. Some of this variation might arise from selective depletion of subpopulations of the antiphosphotyrosine antibodies when the serum was reused. Recognition of fibroblast proteins by the antiserum could be blocked with phenylphosphate (40 mM; data not shown).

Kinase renaturation and assay. Kinase renaturation was carried out by a modification (17) of the method of Celenza and Carlson (3). Blotted proteins were treated with 7 M guanidine hydrochloride–50 mM Tris–50 mM dithiothreitol–2 mM EDTA (pH 8.3) for 1 h at 4°C, washed briefly with Tris-buffered saline, and allowed to renature overnight at 4°C in renaturation buffer (140 mM NaCl, 10 mM Tris, 2 mM EDTA, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1% bovine serum albumin [ICN] [pH 7.4]). Blots were blocked with 5% albumin, overlaid with kinase reaction buffer (30 mM Tris [pH 7.4], 10 mM $MgCl_2$, 2 mM $MnCl_2$, [γ-^{32}P]ATP [3,000 to 6,000 Ci/mmol; 50 μCi/ml; 1 ml/10-cm^2 blot area, made with the Promega Gamma Prep kit]) for 30 min, and washed with buffer and with 1 M KOH. Phosphorylation was linear with respect to time through 30 min of reaction (data not shown).

Phosphoamino acid analysis. Regions of interest of the PVDF membranes were excised and hydrolyzed in 6 M HCl for 1 h at 110°C (22). Supernatants were lyophilized, mixed with carrier phosphoamino acids, and analyzed by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5) on thin-layer cellulose plates (100 μm; E. M. Sciences) as described previously (8).

Anion-exchange chromatography. Detergent-free lysates (3 ml; approximately 3 mg of protein per ml) were loaded on a 3-ml DEAE-cellulose column (DE-52; Whatman, Inc.), washed with 12 ml of hypotonic lysis buffer, and eluted with a 12-ml linear salt gradient from 25 to 500 mM NaCl (41). Fractions (1 ml) from the flowthrough, wash, and salt elution were collected and denatured by boiling for 3 min with gel sample buffer, added as a 5× concentrate.

RESULTS

Fibroblasts possess numerous renaturable kinases. Serum-stimulated NIH 3T3 cells were lysed in hot SDS-gel sample buffer. Cell proteins were subjected to denaturing gel electrophoresis and transferred to a PVDF membrane. The blotted proteins were then treated with guanidine and assessed for renaturable kinase activity. About 20 electrophoretically distinct kinases, ranging from about 200 to 30 kDa, could be detected (Fig. 1). Eighteen ^{32}P-labeled kinase bands were excised and subjected to acid hydrolysis and two-dimensional thin-layer electrophoresis. All of the hydrolysates yielded ^{32}P-labeled phosphoamino acids that comigrated with authentic phosphoamino acid standards (data not shown). Of the 18 hydrolysates, 16 yielded [^{32}P]phosphoserine and [^{32}P]phosphothreonine without detectable [^{32}P]phosphotyrosine. Traces of [^{32}P]phosphotyrosine were found with [^{32}P]phosphoserine and [^{32}P]phos-

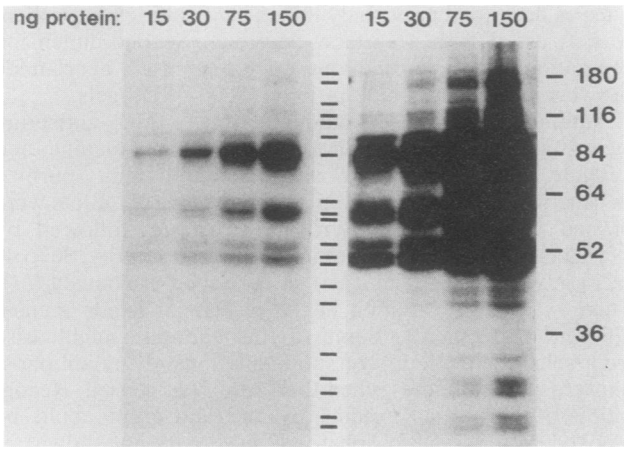

FIG. 1. Renaturation of fibroblast protein kinases. Lysates from serum-stimulated NIH 3T3 cells were prepared, and various amounts of cell protein were subjected to SDS-gel electrophoresis. Proteins were transferred to PVDF membranes, treated with guanidine, allowed to renature, and overlaid with [γ-^{32}P]ATP for 30 min. The autoradiogram on the left is a 3.5-h exposure carried out at room temperature; the one on the right is a 16-h exposure at −75°C. The 20 most prominent kinase activities are denoted by ticks.

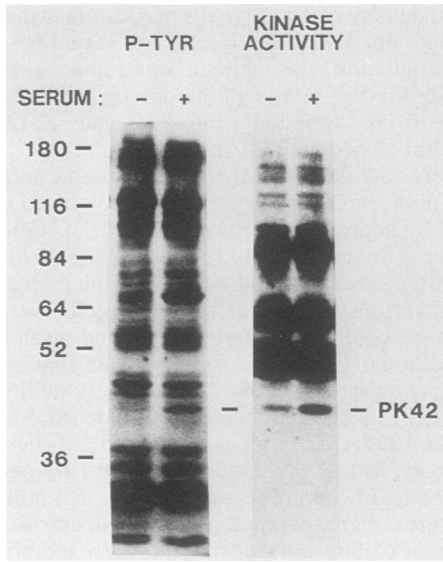

FIG. 2. Comigration of PK42 and p42. Lysates from serum-starved (lanes −) and serum-stimulated (lanes +) NIH 3T3 cells were subjected to SDS-gel electrophoresis, and proteins were transferred to PVDF membranes. The membranes were cut in half. The left half (P-TYR) was analyzed by antiphosphotyrosine immunoblotting; the right half (KINASE ACTIVITY) was treated with guanidine, allowed to renature, and overlaid with [γ-^{32}P]ATP for 30 min.

phothreonine in the reaction products of the 86- and 56-kDa kinases (data not shown).

Serum stimulates PK42 activity. NIH 3T3 cells were serum starved for 48 h and then treated with serum for 10 min or left untreated. The cells were lysed, and the denatured lysates were subjected to gel electrophoresis. Duplicate samples were loaded on the left and right sides of the gel. Proteins were transferred to a PVDF membrane, and the membrane was then cut in half. One half was reserved for antiphosphotyrosine immunoblotting (described below). The other half was treated with guanidine, allowed to renature, and assessed for kinase activity. One relatively minor kinase, a 42-kDa protein designated PK42, was found to possess markedly higher activity when isolated from serum-stimulated cells than when isolated from serum-starved cells (Fig. 2). The magnitude of this activation ranged from two- to fourfold in several experiments. Similar results were obtained with NIH 3T3 cells that were serum starved for only 2 h prior to serum stimulation (data not shown).

The amino acid specificity of PK42 was determined by acid hydrolysis of the ^{32}P-labeled reaction products followed by two-dimensional thin-layer electrophoresis. PK42 phosphorylated serine and threonine residues exclusively (Fig. 3). The identities of the proteins phosphorylated by PK42 on the blotting membrane were investigated by eluting the reaction products as described previously (17) and subjecting them to gel electrophoresis. Reaction products were found to include a 42-kDa protein that could be either PK42 itself or a comigrating species, a 68-kDa protein that is probably albumin, and a number of other bands of uncertain identity (data not shown). The 42-kDa phosphoprotein was a minor component of the phosphorylated reaction products.

PK42 comigrates with a phosphotyrosyl protein, p42. The other half of the blot shown in Fig. 2 was probed with a polyclonal antiphosphotyrosine antiserum. A number of bands were recognized, ranging in molecular mass from 180 kDa to less than 30 kDa (Fig. 2). One phosphotyrosyl protein, hereafter called p42, was found to be identical in electrophoretic mobility to PK42. The tyrosine phosphorylation of p42 increased markedly in response to serum (Fig. 2).

Changes in p42 antiphosphotyrosine immunoreactivity in vivo and in vitro are accompanied by changes in PK42 activity. The time courses of PK42 activation and p42 tyrosine phosphorylation are shown in Fig. 4. PK42 was maximally activated 5 min after serum stimulation, began decreasing in activity within 3 h, and returned on its basal level of activity within 5 h. Similarly, p42 antiphosphotyrosine immunoreactivity peaked within 5 to 30 min of serum stimulation and returned to its initial low level within 5 h. The decrease in p42 immunoreactivity could result from dephosphorylation, proteolysis, or secretion of p42.

Decreases in p42 antiphosphotyrosine immunoreactivity in vitro were also accompanied by deactivation of PK42. Serum-starved NIH 3T3 cells were incubated with or without serum for 10 min. The cells were washed, scraped into hypotonic lysis buffer containing a variety of protease inhibitors, and homogenized. The homogenates were centrifuged, and supernatants were collected. The supernatants were incubated at 37°C for various lengths of time and were

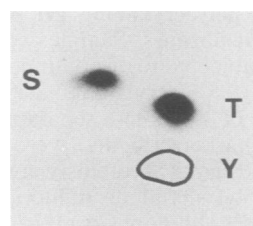

FIG. 3. Phosphoamino acid analysis of PK42 reaction products. The sample was analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 (horizontal) and pH 3.5 (vertical). Abbreviations: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

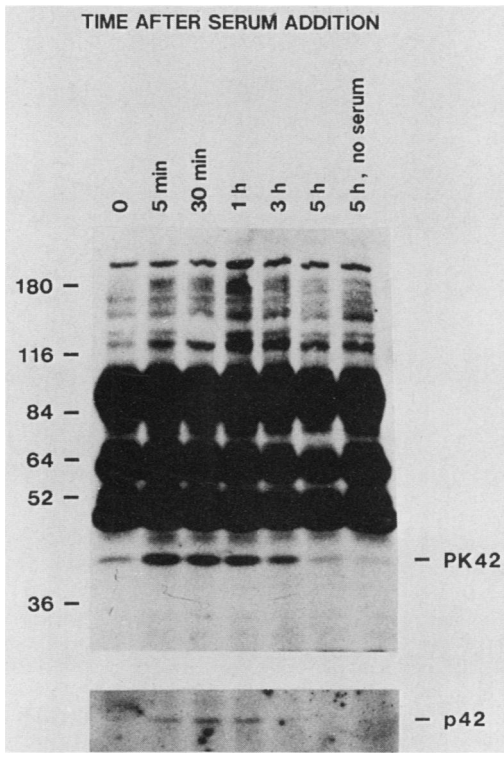

FIG. 4. Time course of PK42 activation and p42 tyrosine phosphorylation. Serum-starved NIH 3T3 cells were treated with serum for various lengths of time as indicated. The top autoradiogram is a kinase assay performed on renatured, blotted proteins. The bottom autoradiogram is an antiphosphotyrosine immunoblot.

subjected to antiphosphotyrosine immunoblotting and kinase renaturation. The phosphotyrosine immunoreactivity of p42 rapidly fell to the undetectably low level seen in unstimulated cells, presumably through the agency of endogenous phosphatases or noninhibited proteases (Fig. 5). The decrease in p42 immunoreactivity was accompanied by a decrease in PK42 activity to roughly the levels seen in unstimulated cells (Fig. 5). This result suggests that p42 and PK42 are related and that PK42 can be inactivated by dephosphorylation, although other processes could be responsible.

The role of protein kinase C in PK42 activation and p42 tyrosine phosphorylation. We examined the effects of the phorbol ester TPA on PK42 activity and p42 activation in TPA-naïve NIH 3T3 cells and in cells that were chronically treated with high doses of TPA to down regulate protein kinase C (Fig. 6a). In addition, parallel dishes of TPA-naïve and chronically TPA-treated cells were radiolabeled with $^{32}P_i$, and the phosphorylation of p80, an endogenous substrate of protein kinase C, was assessed after two-dimensional gel electrophoresis (Fig. 6b).

In TPA-naïve cells, TPA increased the tyrosine phosphorylation of p42 and increased the activity of PK42 to about the same level seen in serum-stimulated cells (Fig. 6a, first three lanes for each panel). The concentration of TPA required for half-maximal stimulation of both p42 tyrosine phosphorylation and PK42 kinase activity was 5 to 10 ng/ml (data not shown). TPA caused about a 12-fold increase in p80 phosphorylation in TPA-naïve cells (Fig. 6b). Serum stimulation caused a more modest rise in p80 labeling, about fourfold in the same experiment (Fig. 6b).

Chronic TPA treatment led to about a twofold elevation in the basal level of p80 phosphorylation (Fig. 6b), as previously reported (23). Chronic TPA treatment abolished the responses of p80, p42, and PK42 to a subsequent dose of TPA (Fig. 6). These findings suggest that the TPA-stimulated increase in p42 tyrosine phosphorylation and PK42 activity is dependent upon protein kinase C. Chronic TPA treatment also abolished the response of p80 to serum (Fig. 6b), but only partially inhibited the serum-induced increase in p42 tyrosine phosphorylation and PK42 activity (Fig. 6a).

PK42 and p42 copurify on DEAE-cellulose. Serum-starved

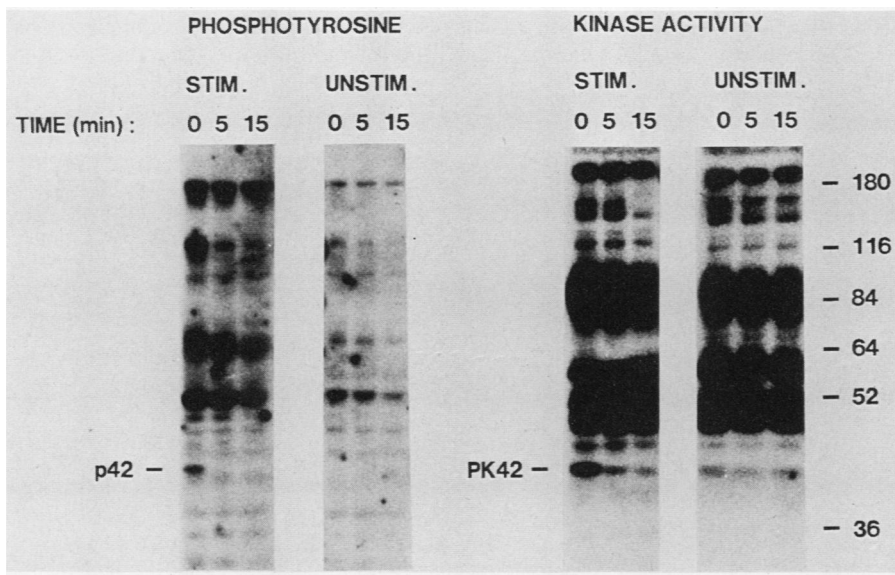

FIG. 5. p42 dephosphorylation and PK42 deactivation in vitro. Lysates from serum-stimulated (STIM.) and serum-starved (UNSTIM.) NIH 3T3 cells were allowed to undergo dephosphorylation in vitro in the presence of protease inhibitors for various lengths of time as indicated. Samples were taken and analyzed by antiphosphotyrosine immunoblotting (left) or by kinase assay performed on renatured, blotted proteins (right).

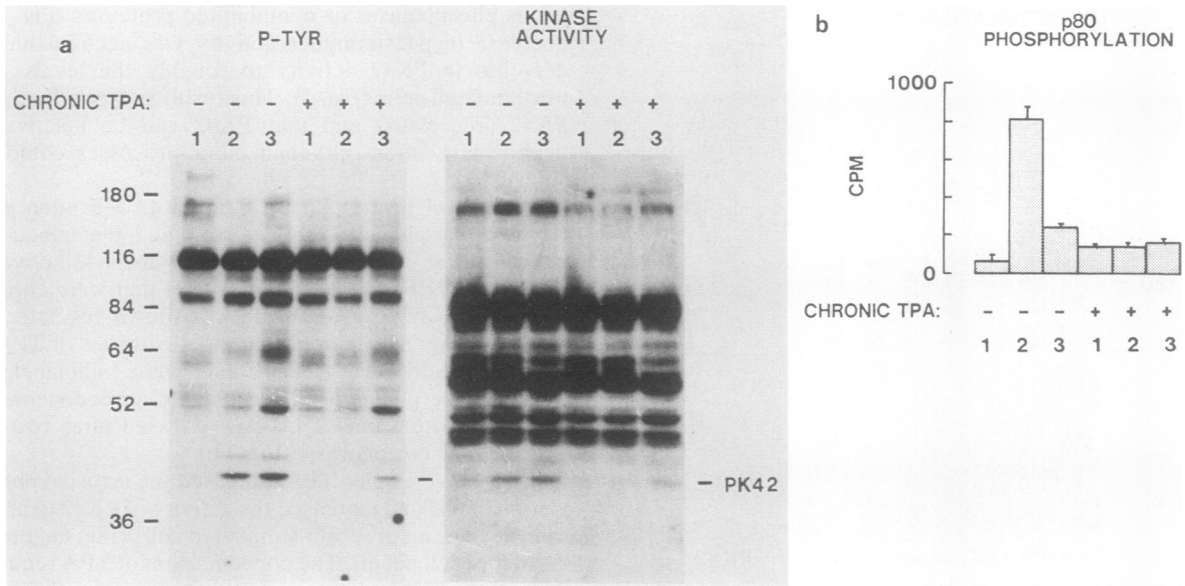

FIG. 6. Effect of chronic TPA treatment on TPA- and serum-stimulated p42 tyrosine phosphorylation and PK42 kinase activity. (a) NIH 3T3 cells were serum starved in the absence (lanes −) or presence (lanes +) of TPA (1 μg/ml). Cells were then left untreated (lanes 1) or treated with 50 ng of TPA per ml (lanes 2) or 10% fetal calf serum (lanes 3). (b) Parallel experiment performed on ^{32}P-labeled cells. TPA-treated (lanes +) and TPA-naïve (lanes −) cells were incubated with no agent (lanes 1), TPA (lanes 2), or calf serum (lanes 3) as in panel a, and p80 phosphorylation was assessed after two-dimensional gel electrophoresis. A sample containing 5×10^4 trichloroacetic acid-precipitable cpm was loaded for the first-dimension gels. Data shown are mean ± standard error of the mean for gels run in duplicate.

NIH 3T3 cells were treated with serum for 10 min and then lysed by homogenization in hypotonic buffer. Supernatants were prepared, loaded on a DE-52 DEAE-cellulose column, and washed. Bound proteins were then eluted with an increasing salt gradient. Samples of each fraction from the flowthrough, wash, and salt elution were dissolved in gel sample buffer and analyzed on two parallel gels. Proteins were then blotted and subjected to either renaturation or antiphosphotyrosine immunoblotting. Both p42 and PK42 bound to DEAE-cellulose, and both eluted as single peaks at a salt concentration of 250 mM (Fig. 7). The coelution of p42 and PK42 again suggests that they are related or identical, although further purification would be required to establish this identity definitively.

DISCUSSION

By overlaying renatured, blotted proteins from fibroblast lysates with [γ-^{32}P]ATP, we have identified a large number of electrophoretically distinct protein kinase activities, all of which catalyze the transfer of phosphate from the γ-position of ATP to protein(s). Each kinase band comprises at least one serine(threonine) protein kinase; in addition, traces of tyrosine kinase activity were found at 86 and 56 kDa. It is formally possible that denaturation and renaturation allow proteins to acquire protein kinase activity artifactually, but we consider this to be unlikely; at least four enzymes that normally phosphorylate nonprotein substrates (creatine kinase, fructose-6-phosphate kinase, myokinase, and pyruvate

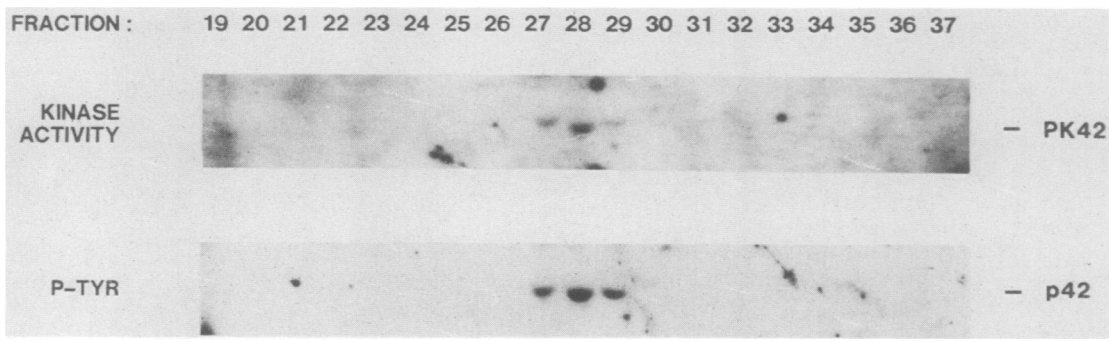

FIG. 7. Coelution in p42 and PK42 from DE-52 cellulose. Serum-starved NIH 3T3 cells were treated with 5% calf serum–5% fetal calf serum for 15 min. Cells were lysed, and approximately 9 mg of cell protein was loaded onto a 3-ml DE-52 anion-exchange column, washed, and eluted with a linear salt gradient (25 to 500 mM NaCl from fractions 23 to 34). Two samples (0.1 ml) of each 1-ml fraction were run on parallel gels (two gels per sandwich in an SE-400 electrophoresis apparatus [Hoefer Scientific Instruments]). Each gel was blotted. One blot was treated with guanidine followed by renaturation buffer and [γ-^{32}P]ATP (top autoradiogram). The other blot was probed with antiphosphotyrosine antiserum and ^{125}I-labeled protein A (bottom autoradiogram). Only the regions around 42 kDa are shown. Fraction 28, which constitutes the peak of PK42 activity and phosphotyrosyl-p42, corresponds to an NaCl concentration of 250 mM.

kinase) fail to acquire artifactual protein kinase activity after renaturation (17). The overall magnitude of renaturable kinase activity found in fibroblast lysates is roughly twofold lower than the activity found in equal amounts of protein from human platelets (17), which are unusually rich in a variety of proteins involved in signal transduction.

We have characterized one of the renaturable fibroblast kinases, PK42, in detail. The kinase activity of PK42 increases two- to fourfold following mitogen treatment. Since this increase is manifest in an in vitro assay that presumably would separate PK42 from noncovalently associated regulatory cofactors, PK42 activation must arise by covalent modification. The increased activity of PK42 could be due to either increased availability of phosphoacceptor sites (e.g., dephosphorylation of an autophosphorylation site) or increased intrinsic activity of PK42 itself. If PK42 is identical to MAP-2 kinase (see below), it would imply that the intrinsic activity of PK42 is increased after mitogen treatment.

The antiphosphotyrosine immunoreactivity of a 42-kDa protein, p42, correlates with the kinase activity of PK42 in a variety of experiments. First, tyrosine phosphorylation of p42 in vivo is accompanied by activation of PK42 (Fig. 2, 4, and 6). Decreases in p42 antiphosphotyrosine immunoreactivity in vivo (Fig. 4) and in vitro (Fig. 5) are accompanied by deactivation of PK42. TPA stimulates tyrosine phosphorylation of p42 and activates PK42, and both the tyrosine phosphorylation and kinase activation are abolished by chronic TPA treatment (Fig. 6). Finally, chronic TPA treatment only partly abolishes serum-stimulated tyrosine phosphorylation of p42 and activation of PK42 (Fig. 6). PK42 and p42 also comigrate on polyacrylamide gels, and the two proteins cochromatograph on anion-exchange medium. These findings suggest that the two proteins are related or identical.

These findings also suggest that the activity of PK42 is regulated by tyrosine phosphorylation and that there is a simple relationship between the overall level of tyrosine phosphorylation of PK42 and its kinase activity. It is also possible that the changes in PK42 tyrosine phosphorylation are accompanied by other covalent modifications, such as changes in serine or threonine phosphorylation, that we have not measured and that those changes are responsible for the changes in PK42 activity. A more detailed understanding of this regulation of PK42 awaits purification of the protein.

The tyrosine phosphorylation of PK42 can arise as a result of protein kinase C activation. PK42 tyrosine phosphorylation is stimulated by TPA, and this stimulation is lost in cells chronically treated with TPA. It is possible that PK42 is tyrosine phosphorylated in a protein kinase C-independent fashion as well, since chronic phorbol ester treatment appears to decrease but not eliminate the tyrosine phosphorylation and activation of PK42 in response to serum. Alternatively, it is possible that the amount of protein kinase C remaining after chronic TPA treatment is sufficient to bring about the tyrosine phosphorylation and activation of PK42 in response to serum, but not in response to TPA. This alternative possibility would be difficult to reconcile with the observation that serum did not stimulate the protein kinase C-mediated phosphorylation of p80 in chronically TPA-treated cells.

The relationship of PK42 to other known 42-kDa phosphotyrosyl proteins. One of the first phosphotyrosyl proteins to be identified in nontransformed cells was a 42-kDa protein, pp42 (6, 9, 19, 35). pp42 is scarce (7) and evolutionarily conserved (5) and becomes tyrosine phosphorylated to high stoichiometry in response to diverse mitogens (2, 5–7, 9, 19, 24, 25, 35, 46). pp42 can be detected by some (43), but not all (23), antiphosphotyrosine antibodies. The electrophoretic mobility, reactivity with antiphosphotyrosine antibodies, and mitogen dependence of PK42 suggest that it may be related or identical to pp42. In addition, staphylococcal V8 protease cleaves PK42 to yield a major 32-kDa phosphotyrosyl peptide and a minor 16-kDa phosphotyrosyl peptide (data not shown), consistent with the patterns previously reported for pp42 (5, 45; T. Gilmore, Ph.D. thesis, University of California, Berkeley, 1984). It has been previously hypothesized that pp42 is MAP-2 kinase, because MAP-2 kinase activity and pp42 copurify over several column steps (43). At the stage of purification shown in Fig. 7, the peak fractions for PK42 still contain several renaturable protein kinase activities, precluding a simple assay for MAP-2 kinase. A definitive test of the relationship between PK42, pp42, and MAP-2 kinase awaits further purification of the protein(s).

Tyrosine kinases as regulators of regulatory proteins. PK42 is one of several substrates of cellular tyrosine kinases to have been identified recently. The other substrates include the γ form of phosphatidylinositol-specific phospholipase C (29, 30, 47); GTPase-activating protein, a protein that can stimulate the GTPase activity of *ras* proteins (16, 31); and two serine(threonine) protein kinases, the Raf-1 oncoprotein (33, 34) and the *cdc2* protein (10, 12, 20, 29). It is becoming increasingly clear that one of the principal functions of tyrosine kinases is to regulate other regulatory proteins and to influence and perhaps coordinate diverse signal transduction pathways.

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