A 42-kD Tyrosine Kinase Substrate Linked to Chromaffin Cell Secretion Exhibits an Associated MAP Kinase Activity and Is Highly Related to a 42-kD Mitogen-stimulated Protein in Fibroblasts

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Abstract. The localization of the protein tyrosine kinase pp60$^{c\text{-}src}$ to the plasma membrane and to the membrane of secretory vesicles in neurally derived bovine chromaffin cells has suggested that tyrosine phosphorylations may be associated with the process of secretion. In the present study we have identified two cytosolic proteins of ∼42 and 45 kD that become phosphorylated on tyrosine in response to secretagogue treatment. Phosphorylation of these proteins reached a maximum (3 min after stimulation) before maximum catecholamine release was observed (5–10 min after stimulation). Both secretion and tyrosine phosphorylation of p42 and p45 required extracellular Ca^{2+}. Tyrosine-phosphorylated proteins of similar M_r have previously been identified in 3T3-L1 adipocytes stimulated with insulin (MAP kinase; Ray, L. B., and T. W. Sturgill. 1987. *Proc. Natl. Acad. Sci. USA.* 84:1502–1506) and in avian and rodent fibroblasts stimulated with a variety of mitogenic agents (Cooper, J. A., D. F. Bowen-Pope, E. Raines, R. Ross, and T. Hunter. 1982. *Cell.* 31:263–273; Nakamura, K. D., R. Martinez, and M. J. Weber. 1983. *Mol. Cell. Biol.* 3:380–390). Comparisons of the secretion-associated 42-kD protein of chromaffin cells with the 42-kD protein of Swiss 3T3 fibroblasts and 3T3-L1 adipocytes provide evidence that these three proteins are highly related. This evidence includes comigration during one-dimensional SDS-PAGE, cochromatography using ion exchange and hydrophobic matrices, similar isoelectric points, identical cyanogen–bromide peptide maps, and cochromatography of MAP kinase activity with the tyrosine-phosphorylated form of pp42. This protein(s), which appears to be activated in a variety of cell types, may serve a common function, perhaps in signal transduction involving a cascade of kinases.

Bovine adrenal chromaffin cells, derived from the neural crest during embryogenesis, respond to secretory agonists by releasing the catecholamines epinephrine and norepinephrine in a highly regulated exocytotic process. Through studies using cultured bovine chromaffin cells, evidence has accumulated to suggest that protein phosphorylations provide a link in stimulus-secretion coupling (Amy and Kirshner, 1981; Lee and Holz, 1986; Michener et al., 1986; Creutz et al., 1987; Gutierrez et al., 1988). This finding has been supported by analyses of protein phosphorylation associated with exocytosis in other secretory tissues, including nervous tissue, mast cells, and platelets (Sieghart et al., 1978; DeLorenzo et al., 1979; Nishikawa et al., 1980; Ferrell and Martin, 1988; Golden and Brugge, 1989). The majority of phosphorylations observed thus far in chromaffin cells have been related to activations or inactivations of cAMP-, Ca^{2+}/calmodulin-, or Ca^{2+}/phospholipid-dependent protein kinases (Amy and Kirshner, 1981; Burgoyne and Geisow, 1981; Lee and Holz, 1986; Cote et al., 1986; Giesow and Burgoyne, 1987; Haycock et al., 1988) and have involved phosphorylations primarily on serine or threonine residues of proteins thought to be critical for the onset of or recovery from secretion.

Until recently, alteration in the phosphorylation of proteins on tyrosine residues has been most frequently associated with cell mitogenesis and transformation. These investigations have focused primarily on intracellular tyrosine phosphorylations that are modulated upon infection by oncogenic viruses encoding tyrosine kinases or upon cell surface binding of mitogenic polypeptide growth factors to their cognate receptors, which themselves possess tyrosine kinase activity (Cooper and Hunter, 1981; Cooper et al., 1982; Car-

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penter, 1987; Hunter and Cooper, 1985; Kamps and Sefton, 1988).

Our interest in examining nontransformed, postmitotic chromaffin cells for secretagogue-dependent tyrosine phosphorylations was prompted by several findings: (*a*) localization of pp60^{c-src}, a tyrosine-specific protein kinase, to both the secretory granule membrane and plasma membrane fractions of chromaffin cells (Parsons and Creutz, 1986; Grandori and Hanafusa, 1988); (*b*) modulation of pp60^{c-src} activity after secretagogue stimulation (Oddie et al., 1989); (*c*) identification of two tyrosine kinase substrates, calpactin I and lipocortin I, as members of the chromobindins (Creutz et al., 1987), a group of cytosolic proteins that bind to chromaffin granule membranes in a Ca^{2+}-dependent manner (Creutz et al., 1983); and (*d*) in vitro phosphorylations and functional modulation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase in postsynaptic membranes from the electric organ of *Torpedo californica* (Huganir et al., 1984; Hopfield et al., 1988). Activation of this receptor in chromaffin cells induces exocytosis (for review see Livett, 1984*a*).

Using antibodies specific for phosphotyrosine we describe in this report the identification of proteins in bovine adrenal chromaffin cells with apparent molecular masses of 42 and 45 kD that are phosphorylated on tyrosine in response to several different secretagogues. Experimental evidence supporting the relevance of these endogenous phosphorylations to chromaffin cell secretion includes a dependence of these phosphorylations on extracellular calcium, dose response optima similar to that required for maximum catecholamine release in vitro, and rapid kinetics of phosphorylation after stimulation. The phosphorylated forms of these proteins were localized to the cell cytoplasm.

Proteins in the 42-45-kD mass range have also been identified as targets for tyrosine kinases in transformed cells (Cooper and Hunter, 1981; Kamps and Sefton, 1988), in fibroblasts stimulated with a variety of mitogens, such as epidermal growth factor or platelet-derived growth factor (Cooper et al., 1982; Nakamura et al., 1983; Cooper and Hunter, 1985), in 3T3-L1 adipocytes stimulated by insulin (Ray and Sturgill, 1987, 1988*a*), and in *Xenopus* oocytes undergoing meiosis (Cooper, 1989). Pp42 from mitogen-stimulated fibroblasts is a low abundance cytoplasmic protein that is highly conserved between species (Cooper and Hunter, 1985). Analysis of insulin-stimulated tyrosine phosphorylations in differentiated, nonproliferating 3T3-L1 adipocytes has revealed another tyrosine-phosphorylated 42-kD protein, termed MAP kinase.[1] This protein has been characterized as an insulin-activated serine/threonine kinase capable of phosphorylating the substrates, microtubule-associated protein 2 (MAP-2), and S6 kinase II in vitro (Ray and Sturgill, 1987, 1988*a*; Sturgill et al., 1988). Partial purification of MAP kinase has been achieved by sequential column chromatography using the anion exchange matrix, DEAE, followed by the hydrophobic matrix, phenyl-Superose, and gel filtration on Superose 12 (Ray and Sturgill, 1988*b*). Recent biochemical evidence demonstrates that the mitogenic pp42 protein has an associated MAP kinase activity (Rossomando et al., 1989).

The cytosolic localization, the molecular size, and the rapid and transient tyrosine phosphorylation of pp42 from chromaffin cells, mitogen-stimulated fibroblasts, and insulin-stimulated adipocytes (i.e., MAP kinase) suggested that these three proteins may be highly related. To determine the biochemical and functional relatedness of the secretion-associated pp42 protein to both the mitogen-associated pp42 and insulin-sensitive pp42 (MAP kinase), experiments were conducted comparing chromatographic properties, isoelectric points, phosphoamino acid content, cyanogen bromide peptide maps, and MAP kinase activity. The results demonstrate that the secretion-associated pp42 protein is highly related if not identical to both the insulin-induced and the mitogen-associated pp42 proteins. The tyrosine phosphorylation and activation of the pp42 protein, or highly related proteins in unique cell types, each coupled to distinct cell functions, suggests a central role for this protein, perhaps in intracellular communication from a variety of cell surface receptors.

Materials and Methods

Culturing and Stimulation of Cells

Chromaffin cells were prepared from bovine adrenal glands for in vitro culture according to the method of Greenberg and Zinder (1982) as described by Livett (1984*b*) with modifications noted in Creutz et al. (1987). The procedure was further modified by omitting differential plating and maintaining the cells in a serum-free medium lacking 5-fluoro-2'-deoxyuridine (N2 medium) as outlined by Acheson et al. (1984) and Bottenstein and Sato (1979). As determined by uptake of neutral red in different preparations, chromaffin cells were judged to be 85-92% pure. 2-6-d-old cultures were stimulated with 20 μM nicotine, 300 μM carbachol, 100 ng/ml EGF, 100 ng/ml 12-*O*-tetradecanoylphorbol 13-acetate (TPA), or 55 mM K^+. As shown by others (Greenberg and Zinder, 1982; Livett, 1984*b*), concentrations of all stimulants except EGF and TPA were chosen for their ability to induce maximal release of the catecholamine [^{3}H]norepinephrine ([^{3}H]NE; for assay, see below). In a dose-response analysis carried out at 3 min after stimulation, 100 ng/ml EGF, 100 ng/ml TPA, and 10-100 μM nicotine were found to be optimal concentrations for inducing the greatest level of tyrosine phosphorylation of the pp42 and pp45 proteins (data not shown). The first four agents were added either directly to culture media or in a balanced salt solution with glucose (BSSG: 15 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 2 mM $CaCl_2$, 0.5 mM ascorbate, 1.2 mg/ml $NaHCO_3$, and 1 mg/ml BSA), which was applied to the monolayer after a 15-min wash with BSSG at 37°C. TPA was diluted to the appropriate concentration from a 1-mM stock maintained in absolute ethanol. The BSSG buffer was modified to accomodate the increase of KCl to 55 mM by decreasing the molarity of NaCl to 90 mM. Cultures were stimulated at 37°C in a humidified 5% CO_2 atmosphere for the indicated times and then processed for analysis of phosphotyrosine-containing proteins. Where the Ca^{2+} dependency of phosphorylation was tested, stimulations were performed either in BSSG containing 2 mM $CaCl_2$ (as described above), or in BSSG lacking $CaCl_2$ and containing 2 mM EGTA. In those experiments involving both Swiss 3T3 fibroblasts and chromaffin cells, the chromaffin cells were stimulated at 37°C for 3 min in Krebs-Ringer bicarbonate/Hepes buffer (KRB Hepes: 120 mM NaCl, 4.75 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 24 mM $NaHCO_3$, 10 mM Hepes, pH 7.5 [22°C]). Stimulation in KRB Hepes was preceded by a 2-h preincubation at 37°C in this buffer.

PC12 cells (Greene and Tischler, 1976) were maintained on collagen-coated tissue culture dishes in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated horse serum, 5% FCS, and antibiotics. Stimulations were performed in BSSG as described above. Primary cultures of chicken embryo fibroblasts (CEFs) were prepared from 10-d-old gs-negative/chf-negative/Marek-negative embryos (Spafas, Inc., Norwich, CT) and maintained as previously described (Par-

1. *Abbreviations used in this paper*: BSSG, balanced salt solution with glucose; CEF, chicken embryo fibroblast; 1-D, one-dimensional; 2-D, two-dimensional; KRB Hepes, Krebs-Ringer bicarbonate/Hepes buffer; MAP-2, microtubule-associated protein 2; MAP kinase, microtubule-associated protein 2 kinase; NE, norepinephrine; ptyr antibody, phosphotyrosine-specific antibody; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

sons et al., 1979). Confluent monolayers of cells were serum-starved in N2 medium for 24 h before stimulation. The stimulant (EGF or TPA) was added directly to the medium. Swiss 3T3 cells were grown to subconfluence in DME supplemented with 10% FCS and antibiotics, incubated for 2 h at 37°C in KRB Hepes, and stimulated in KRB Hepes at 37°C for 10 min with 100 ng/ml EGF (Collaborative Research, Bedford, MA). 3T3-L1 adipocytes, differentiated according to the method of Rubin et al. (1978), were stimulated in KRB Hepes as described for the 3T3 cells except that 80 nM insulin (Eli Lilly and Co., Indianapolis, IN) was used as the stimulating agent (Ray and Sturgill, 1987).

[^{3}H]Norepinephrine Release

This assay was performed as previously described (Boska and Livett, 1984; Oddie et al., 1989). In those experiments in which the Ca^{2+} dependency of [^{3}H]NE release was tested, stimulations were performed either in BSSG containing 2 mM $CaCl_2$ (as described above), or in BSSG lacking $CaCl_2$ and containing 2 mM EGTA.

One-dimensional Antiphosphotyrosine Western Immunoblots

Stimulated or mock-stimulated cells in 60-mm dishes were lysed directly on the plate without washing by addition of 400 μl (800 μl for 100-mm dishes) ice-cold, modified RIPA (150 mM NaCl, 0.25% Na deoxycholate, 0.1% SDS, 1% NP-40, 1 mM Na orthovanadate, 1 mM PMSF, 50 mg/ml leupeptin, 0.5% aprotinin, 50 mM Tris, pH 7.2). The dishes were scraped with a rubber policeman, and the lysate was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 5 min (~10,000 g) at 4°C. An aliquot was removed from the supernatant for protein determination using a modified Lowry method (Markwell et al., 1978). Laemmli sample buffer (1970) was added to the remainder of the supernatant to attain a 1× concentration; the samples were heated to 100°C for 5 min and then subjected to SDS-PAGE or frozen at −70°C. In some experiments, cell lysates were prepared directly in 95–100°C Laemmli sample buffer, boiled for 5 min, sonicated for 3–4 2-s bursts using a microtip to disrupt the DNA, and processed as described below for anti-ptyr Western immunoblotting. Identical results were obtained using either method of lysate preparation suggesting that the observed phosphorylations did not take place during extract preparation. Samples (each containing 200 μg protein) were electrophoresed through SDS-10.5% polyacrylamide gels, transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH), and processed as previously described (Kamps and Sefton, 1988; Kanner et al., 1989*a*,*b*; Reynolds et al., 1989). The specificity of the antiphosphotyrosine immunoglobulin has been described elsewhere (Kanner et al., 1989*a*,*b*). Briefly, it was shown that only phosphotyrosine (and not phosphoserine or phosphothreonine) completely blocked the binding of antibody to ptyr-containing proteins in a Western immunoblot, and that all in vivo $^{32}P_i$-labeled proteins that were immunoprecipitated with phosphotyrosine antiserum contained phosphotyrosine as determined by two-dimensional (2-D) phosphoamino acid analysis. However, this antisera (like that described by Kazlauskas and Cooper, 1988) was unable to immunoprecipitate either pp42 or pp45 in the native or heat-denatured form (data not shown).

Subcellular Fractionation

Cultured chromaffin cells in 60-mm dishes were stimulated with nicotine and then rinsed twice with cold PBS containing 50 mg/ml leupeptin, 0.5% aprotinin, 1 mM PMSF, and 1 mM Na orthovanadate. All buffers used subsequently in this procedure contained these phosphatase and protease inhibitors. Fractionations were carried out as described by Kanner et al. (1989*a*) and Reynolds et al. (1989).

Mono Q Column Chromatography

Cytosolic fractions obtained from nonstimulated and nicotine-stimulated cells were adjusted to 0.1 M NaCl, applied at a concentration of 2 mg protein/ml to an anion exchange column (FPLC Mono Q; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in buffer A (10 mM Tris, pH 7.4 [22°C], 1 mM $MgCl_2$, 100 mM NaCl, and 4 mM ρNPP) and eluted with a continuous salt gradient from 0.1 to 0.5 M NaCl. All columns were run at 3°C with a constant flow rate of 0.5 ml/min and a back pressure of no more than 1 MPa. Aliquots of 1-ml fractions were analyzed by Western immunoblotting using phosphotyrosine-specific antibody (ptyr antibody) to detect the presence of pp42. Those fractions containing pp42 were pooled, assayed for protein content by the BCA method (Pierce Chemical Co., Rockford, IL) and found to contain ~200 μg total protein/ml eluate.

Phenyl-Superose Column Chromatography

The pooled Mono Q fractions containing pp42 were applied directly to a phenyl-Superose column (FPLC; Pharmacia Fine Chemicals) and fractionated according to the method of Ray and Sturgill (1988*b*), except that the ρNPP concentration was reduced to 4 mM. Aliquots of 1-ml fractions were analyzed for pp42 by the Western immunoblotting technique using ptyr antibody. Pooled fractions were stored at −70°C.

MAP Kinase Assay

The MAP kinase assay was performed as described by Ray and Sturgill (1987). MAP-2 was purified from bovine brain by the method of Kim et al. (1979).

^{32}P Labeling and 2-D Gel Electrophoresis

Monolayer cell cultures were washed two times with KRB Hepes at 37°C and then labeled for 2 h at this temperature in KRB Hepes containing 1 mCi/ml carrier free $^{32}P_i$ (New England Nuclear, Boston, MA) before stimulation, which was achieved by the addition of each inducing agent directly to the labeling media for the indicated times. ^{32}P-labeled pp42 contained in the cytosolic fraction was purified through the phenyl-Superose step described above and prepared for 2-D gel analysis by deoxycholate (0.05%)/TCA (6%) precipitation (Rossomando et al., 1989) of 90 μl of the column fraction containing the peak MAP kinase activity. Pellets were resuspended in 70 μl of nonradiolabeled, EGF-stimulated Swiss 3T3 whole cell lysate (0.5–1 mg protein/ml Garrels' sample buffer) according to the method of Rossomando et al. (1989) and applied to a tube gel containing ampholines of pH 3.5–10, 5–7, and 6–8 (Pharmacia Fine Chemicals) at a ratio of 0.5:1:1, respectively, for electrophoresis. 2-D gel analysis was performed according to the methods of O'Farrell (1975) and Garrells (1979) with modifications according to Rossomando et al. (1989). 10% polyacrylamide gels were used for the second dimension. The pH gradient of the first dimension was determined by measuring the pH of eluates from slices of paired IEF gels eluted in water.

Phosphoamino Acid Analysis

For phosphoamino acid analysis the phenyl-Superose column fraction containing the peak MAP kinase activity was resolved by one-dimensional (1-D) SDS-PAGE, and the ^{32}P-labeled pp42 was localized by autoradiography and excised from the gel. Protein was eluted from the gel slice, TCA precipitated and washed with ethanol according to the method of Beemon and Hunter (1978), and hydrolyzed in 100 μl 5.7 M HCl at 110°C for 1.5 h. Hydrolyzed proteins were mixed with 300 ng each authentic phosphotyrosine, phosphoserine, and phosphothreonine, and resolved by 2-D thin layer electrophoresis (Cooper et al., 1983). Identification of radiolabeled amino acids was made by colocalization with ninhydrin-stained authentic phosphoamino acids.

Peptide Mapping Using CNBr Digestion

Phenyl-Superose-purified, ^{32}P-labeled pp42 was resolved by 1-D SDS-PAGE, eluted, and precipitated by TCA as described for the phosphoamino acid analysis. Each pellet was resuspended in 30 μl of 50 mg/ml CNBr in 70% formic acid and incubated ~18 h at room temperature. Distilled/deionized water was added to the digestion reaction to a final volume of 1 ml and lyophilized. The peptides were then resuspended in 100 μl Laemmli sample buffer, separated by electrophoresis through a 15% acrylamide gel, and detected by autoradiography.

Results

Tyrosine Phosphorylation of 42- and 45-kD Proteins in Chromaffin Cells in Response to Various Secretagogues

To determine tyrosine phosphorylations associated with exocytosis we used antibodies specific for phosphotyrosine. Cul-

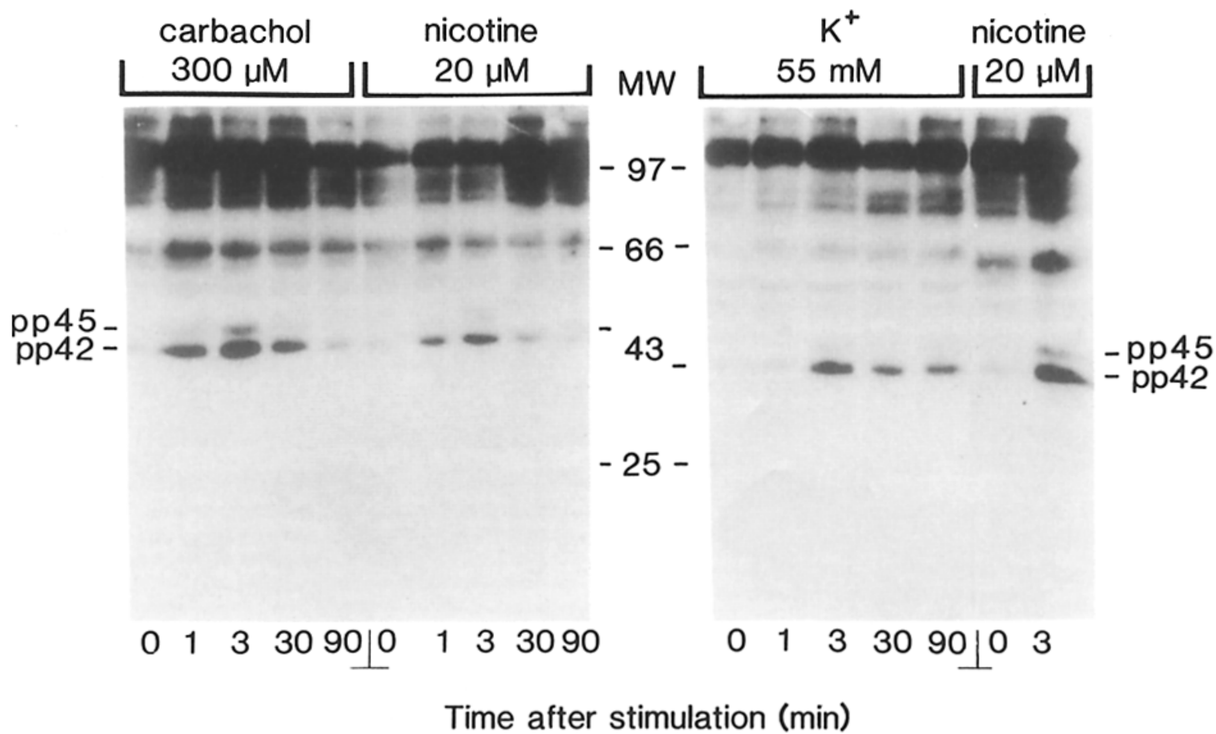

Figure 1. Secretagogue stimulation of cultured bovine chromaffin cells increases the tyrosine phosphorylation of the 42- and 45-kD proteins. Chromaffin cells were cultured from the medulla of fresh bovine adrenal glands and stimulated 5 d later by addition of carbachol, nicotine, or K^+ (at the concentrations indicated in the figure) to the monolayer in BSSG as described in Materials and Methods. At various times afterwards, cultures were prepared for antiphosphotyrosine Western immunoblot analysis by removal of the stimulant and replacement with detergent containing buffer, supplemented with protease and phosphatase inhibitors as outlined in Materials and Methods. Each lane contains 200 μg total cell extract protein obtained from cells stimulated for the time indicated. The migrations of pp42 and pp45 were determined relative to those of prestained protein standards obtained from Bio-Rad, Inc. (Rockville Centre, NY).

tured chromaffin cells were stimulated for various lengths of time with several different secretagogues (nicotine, carbachol, or K^+), and detergent extracts, prepared in the presence of a phosphotyrosine phosphatase inhibitor (sodium vanadate), were subjected to SDS-PAGE and Western immunoblot analysis using rabbit ptyr antibody as described in Materials and Methods. Fig. 1 shows that phosphotyrosine-containing proteins were present in resting, nonstimulated chromaffin cells, and that multiple polypeptides of 120, 80, 65, 45, and 42 kD appeared to become more extensively phosphorylated on tyrosine in response to secretagogues. The most prominent among them were two phosphoproteins with molecular masses of 42 and 45 kD (pp42 and pp45). Enhanced phosphorylation on both proteins could be detected as early as 1 min after stimulation. As determined by densitometric analysis of the immunoblots, tyrosine-specific phosphorylation reached a maximum (∿30-fold over nonstimulated levels for pp42 and 15-fold over nonstimulated levels for pp45) at 3 min after stimulation and returned to nonstimulated levels within 30–180 min.

Dependence of p42 and p45 Tyrosine Phosphorylation on Extracellular Ca^{2+}

Since secretion in chromaffin cells is dependent upon an influx of extracellular Ca^{2+} (for review see Livett, 1984*a*), we wished to determine if tyrosine phosphorylation of the 42- and 45-kD proteins was also dependent upon high concentrations of Ca^{2+}. Cultured cells were stimulated with nicotine in the presence or absence of extracellular Ca^{2+}, and p42 and p45 phosphorylation was monitored as a function of time by Western immunoblot analysis using ptyr antibody. Concomitantly, secretion of catecholamines was assessed by following the release of [^{3}H]NE. As shown in Fig. 2, densitometric analysis of autoradiographs of Western immunoblots revealed that, like the release of [^{3}H]NE, tyrosine phosphorylation of p42 was dependent upon Ca^{2+}. Similar kinetics and dependency on Ca^{2+} of the tyrosine phosphorylation of p45 were also observed (data not shown). The Ca^{2+} dependency of phosphorylation of both p42 and p45 was apparent whether nicotine or K^+ was used as secretagogues. The Ca^{2+} dependency and rapid kinetics of these phosphorylations (which slightly preceded secretion), together with the fact that the optimal dose of nicotine (10–100 μM) that induced these phosphorylations was similar to the optimum for ^{3}H[NE] release in vitro (20 μM) (data not shown), suggested a role for the phosphorylated forms of p42 and p45 in the early stages of exocytosis.

Subcellular Localization of pp42 and pp45

To determine the subcellular localization of pp42 and pp45, nicotine- and mock-stimulated chromaffin cells were lysed in hypotonic buffer, and the cytosolic and crude membrane fractions were obtained as described in Materials and Methods. Cell equivalents of the total starting lysate, cytosolic, and membrane fractions were analyzed for phosphotyrosine-containing proteins. As shown in Fig. 3 *A*, the majority of

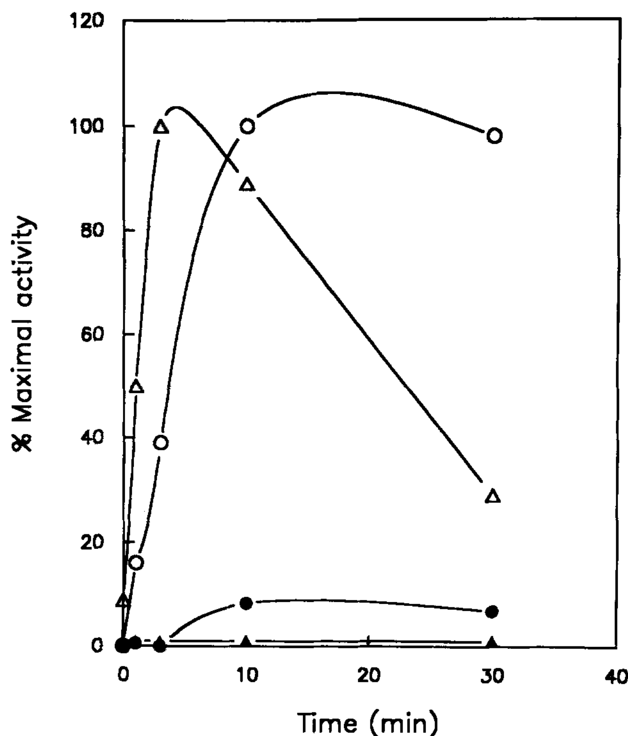

Figure 2. Comparison of the kinetics and Ca^{2+} dependency of secretion and p42 tyosine phosphorylation in nicotine-stimulated chromaffin cells. Freshly prepared chromaffin cells were seeded at a density of $10^5/cm^2$ in collagen-coated 24-well cluster dishes or 60-mm tissue culture dishes. 5 d later, cells in cluster dishes were assayed for secretory activity in response to 20 μM nicotine in BSSG containing either 2 mM Ca^{2+} (○) or 2 mM EGTA (●) by the [^{3}H]NE release assay as described in Materials and Methods. Amounts of [^{3}H]NE released as a function of time after stimulation are plotted as a percent of maximal release (22.4% of total cellular content, corrected for spontaneous release), which occurred at 10 min. Cells in 60-mm dishes were stimulated in the presence (△) or absence (▲) of Ca^{2+} as described above, and cell extracts were analyzed by the antiphosphotyrosine Western immunoblotting procedure as described in the legend to Fig. 1. Relative levels of pp42 tyrosine phosphorylation were determined from densitometry tracings of the autoradiograms.

the phosphotyrosine-containing forms of p42 and p45 partitioned with the cytosolic fraction. Addition of 2 mM $CaCl_2$ (Fig. 3 *B*) or 2 mM EGTA (Fig. 3 *C*) to the hypotonic buffer in which the cells were lysed did not alter the subcellular localization. These results indicate that neither the presence of high concentrations of Ca^{2+} nor the depletion of Ca^{2+} (by EGTA addition) in the lysis buffer induced the association of phosphotyrosine-containing pp42 and pp45 to membranes, where the majority of $pp60^{c\text{-}src}$ has been shown to reside (Parsons and Creutz, 1986; Grandori and Hanafusa, 1988).

Identical Migration of the Secretion-associated, the Mitogen-associated, and the Insulin-sensitive pp42 and pp45 Proteins on 1-D Gels

The rapid tyrosine phosphorylation in response to receptor activation, the molecular size, and the cytosolic location of pp42 and pp45 are properties shared with a family of proteins previously described by Cooper et al. (1982), Bishop et al. (1983), Nakamura et al. (1983), Gilmore and Martin (1983), Cooper et al. (1984), Kohno (1985), Cooper and Hunter (1985), Isacke et al. (1986), Kohno and Pouyssegur (1986), Contor et al. (1988), Vila and Weber (1988), Ray and Sturgill (1987, 1988*a*), and Rossomando et al. (1989). In murine fibroblasts and CEFs and in murine adipocytes these proteins become transiently phosphorylated on tyrosine residues in response to various agents, such as platelet-derived growth factor, EGF, TPA, thrombin, and insulin. To investigate the possibility that pp42 and pp45 of chromaffin cells may be related to proteins of similar size in different cell types stimulated with a variety of agents, we examined extracts of the following cells for phosphotyrosine-containing proteins by the Western immunoblot technique: (*a*) neurally derived PC12 cells stimulated with the secretory agent, 55 mM K^+; (*b*) 3T3-L1 adipocytes stimulated with insulin: (*c*) Swiss 3T3 fibroblasts stimulated with EGF; and (*d*) CEFs and chromaffin cells stimulated with EGF and TPA. Fig. 4 *A* shows that two proteins with electrophoretic migrations identical to those observed in the chromaffin cells became phosphorylated on tyrosine when PC12 cells (a cell line de-

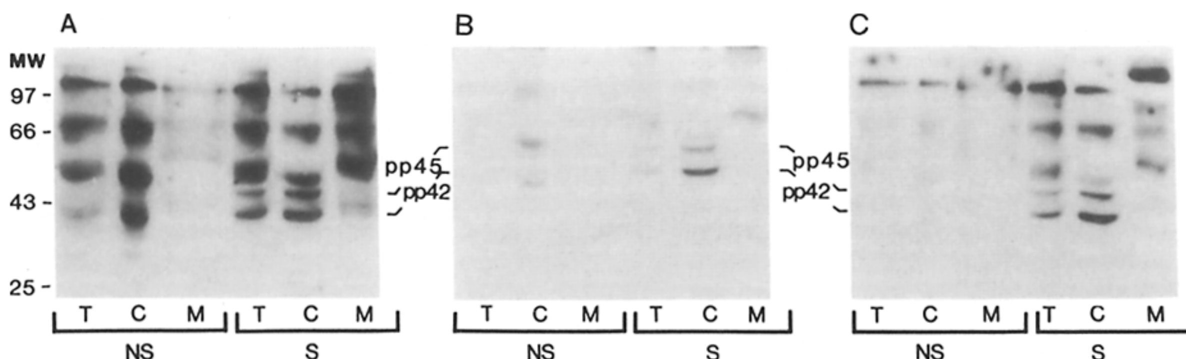

Figure 3. Subcellular localization of pp42 and pp45 in bovine chromaffin cells. Paired cultures of cells were either nonstimulated (*NS*) or stimulated (*S*) for 3 min with 20 μM nicotine in BSSG, and crude membrane (*M*) and cytosolic (*C*) fractions were prepared in hypotonic lysis buffer containing (*A*) no extra additives, (*B*) 2 mM $CaCl_2$, or (*C*) 2 mM EGTA as described in Materials and Methods. All fractions were brought to equal volume in Laemmli sample buffer, and 50 μl of each of the appropriate samples was loaded into the *C* and *M* lanes, while lanes marked *T* (total extract) contained 50 μl each of the cytosolic and membrane fractions. Phosphotyrosine-containing proteins were detected by the Western immunoblotting technique as described in the legend to Fig. 1. The cytosolic protein in extracts from nonstimulated cells which exhibited a M_r of ~40 kD was not consistently observed in repeat experiments.

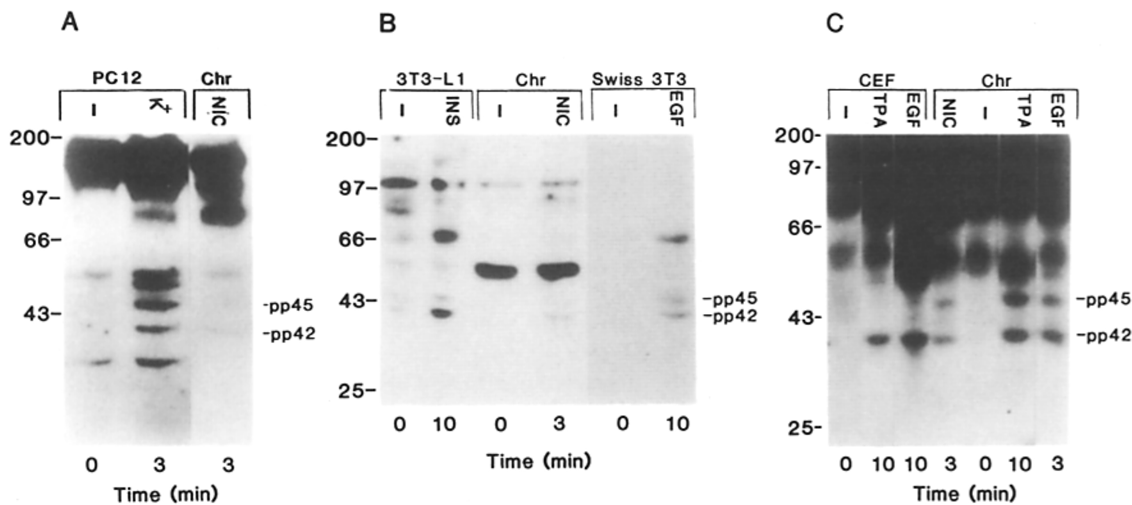

Figure 4. 1-D PAGE comigration of pp42 and pp45 from PC12 pheochromocytoma cells, 3T3-L1 adipocytes, Swiss 3T3 fibroblasts, CEFs, and chromaffin cells. In *A–C*, samples were subjected to antiphosphotyrosine Western immunoblot analysis as described in the legend to Fig. 1. (*A*) Logarithmically growing cultures of PC12 cells were stimulated with 55 mM K^+ for 3 min in BSSG as outlined in Materials and Methods, and the migrations of tyrosine-containing proteins were compared to those from chromaffin cells stimulated with 20 μM nicotine for 3 min. 200 μg extract protein was analyzed per lane. [^{3}H]NE release in this experiment reached a maximum of 15.7% of total cellular content (corrected for spontaneous release) 60 min after addition of K^+. (*B*) Cytosols were obtained as described in Materials and Methods from 3T3-L1 adipocytes stimulated with 80 nM insulin, Swiss 3T3 fibroblasts stimulated with 100 ng/ml EGF, and chromaffin cells stimulated with 20 μM nicotine. Stimulations took place for times indicated in the figure. For each cell type equal amounts of protein from nonstimulated (0 time) and stimulated samples were analyzed; 600 μg from 3T3-L1 adipocytes, 250 μg from Swiss 3T3 fibroblasts, and 200 μg from chromaffin cells. (*C*) Cultured chromaffin cells were stimulated with 100 ng/ml EGF, 20 μM nicotine, or 100 ng/ml TPA for the indicated time. Confluent monolayers of secondary cultures of CEFs were starved of serum in N2 medium for 18 h before the addition of 400 ng/ml EGF or 50 ng/ml TPA in fresh N2 medium for 10 min at 37°C before analysis of 200 μg/lane total cellular protein.

rived from a rat pheochromocytoma, which releases NE in response to secretory stimulation [Greene and Rein, 1977; see also legend to Fig. 4 *A*]) were treated with 55 mM K^+. The kinetics of phosphorylation and dephosphorylation of these two proteins in PC12 cells were also similar to those in the chromaffin cells. Furthermore, three additional proteins (M_r = ~50, 48, and 35), not seen in the chromaffin cells, were tyrosine phosphorylated in a stimulation-dependent fashion. These results indicate that tyrosine phosphorylation of p42 and p45 was enhanced in at least two different cell populations which responded to secretory stimuli.

Similarly, cytosolic fractions obtained from 3T3-L1 adipocytes treated with insulin (Fig. 4 *B*), from Swiss 3T3 fibroblasts treated with EGF (Fig. 4 *B*) and from CEFs treated with EGF or TPA (Fig. 4 *C*), contained two proteins which exhibited enhanced tyrosine phosphorylation in response to stimulation and identical electrophoretic migration in 1-D gels as did pp42 and pp45 from nicotine-, EGF-, or TPA-stimulated chromaffin cells. Phosphorylation in all cell types took place within comparable times after stimulation. Cellular fractionation experiments (performed as described in Materials and Methods) confirmed the cytosolic localization of the ptyr-containing proteins in EGF- and TPA-stimulated chromaffin cells (data not shown). These experiments show that two proteins of 42 and 45 kD became phosphorylated on tyrosine in chromaffin cells not only in response to secretagogues, but also in response to the fibroblast growth-promoting agents EGF and TPA. Furthermore, these two proteins exhibited electrophoretic mobilities in 1-D PAGE analyses identical to proteins whose phosphorylation on tyrosine could be induced in a variety of other systems.

Although the contribution of the nonchromaffin cells (fibroblasts) in chromaffin cell cultures to the tyrosine phosphorylation of p42 and p45 in response to EGF and TPA cannot be entirely eliminated, several lines of evidence indicate that the majority of the tyrosine phosphorylation of these proteins observed in response to either secretagogues or mitogens was induced in the chromaffin cells. First, we have detected no tyrosine phosphorylations of either p42 or p45 in pure murine fibroblast cultures (Swiss 3T3 and C3H10T1/2) treated with nicotine (data not shown). Second, the intensity of p42/p45 labeling observed in Western immunoblots of chromaffin cells treated with either EGF or TPA was comparable to, or slightly greater than, the intensity seen in preparations of cells stimulated with secretagogues. Third, the level of p42 and p45 tyrosine phosphorylations induced by EGF or TPA treatment of a pure culture of fibroblasts was nearly equal to the level of p42 and p45 tyrosine phosphorylation induced by nicotine, EGF, or TPA treatment of an equal number of chromaffin cells (data not shown). And lastly, repeated visual examinations and staining of preparations with neutral red have confirmed the low fibroblast content (<15%) of the cell cultures, which were maintained in serum-free medium to minimize fibroblast growth. Thus, it is unlikely that the contaminating fibroblasts or other nonchromaffin cells (which amount to 8–15% of the population) were solely responsible for the EGF and TPA responses.

Lack of Requirement for Extracellular Ca^{2+} for the EGF- and TPA-induced Tyrosine Phosphorylations of p42 and p45 from Chromaffin Cells

To compare the Ca^{2+} dependency of the mitogen- and secre-

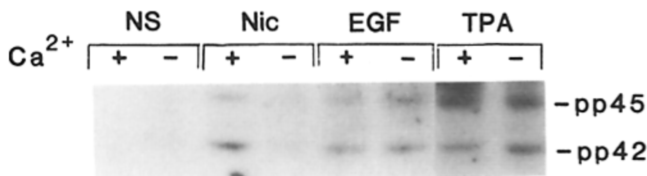

Figure 5. Calcium-independent tyrosine phosphorylation of pp42 and pp45 induced by EGF and TPA in chromaffin cells. Cultured chromaffin cells were either nonstimulated (*NS*, fluid changed) or stimulated for 3 min at 37°C with 20 μM nicotine, 100 ng/ml EGF, or 100 ng/ml TPA in the presence (+2 mM $CaCl_2$) or absence (+2 mM EGTA) of calcium as described in Materials and Methods. 200 μg extract protein was electrophoresed through an SDS–8.5% polyacrylamide gel, transferred to nitrocellulose, and probed with ptyr antibody.

tagogue-induced tyrosine phosphorylations on p42 and p45, cultured chromaffin cells were stimulated with EGF, TPA, or nicotine in the presence or absence of extracellular Ca^{2+}, as described in Materials and Methods. Fig. 5 depicts a 1-D anti-ptyr Western immunoblot analysis of the cell lysates. While the enhanced tyrosine phosphorylation of p42 and p45 in response to nicotine stimulation required extracellular Ca^{2+} (lanes *3* and *4*), the phosphorylations of p42 and p45 in response to EGF and TPA did not exhibit such a strict dependence (lanes *5–8*), suggesting that at least two different signaling pathways could mediate the tyrosine phosphorylation of cytosolic proteins with similar molecular mass, kinetics of phosphorylation, and subcellular localization.

Chromatographic Characteristics of the Chromaffin Cell pp42

Previous investigations by Ray and Sturgill (1988*b*) showed that the MAP kinase protein binds to the anion exchange matrix DEAE and elutes at ∼0.2–0.3 M NaCl. In addition, this protein was shown to have a high affinity for the hydrophobic matrix, phenyl-Superose, eluting as the only alkali-stable phosphoprotein at ∼37% ethylene glycol. More recently, it was observed that the mitogen-associated pp42 in Swiss 3T3 fibroblasts shared identical chromatographic characteristics with MAP kinase (Rossomando et al., 1989). To determine the chromatographic behavior of the secretion-associated pp42 and pp45 proteins, two FPLC columns were tested for the binding and elution of these proteins, the anion exchange

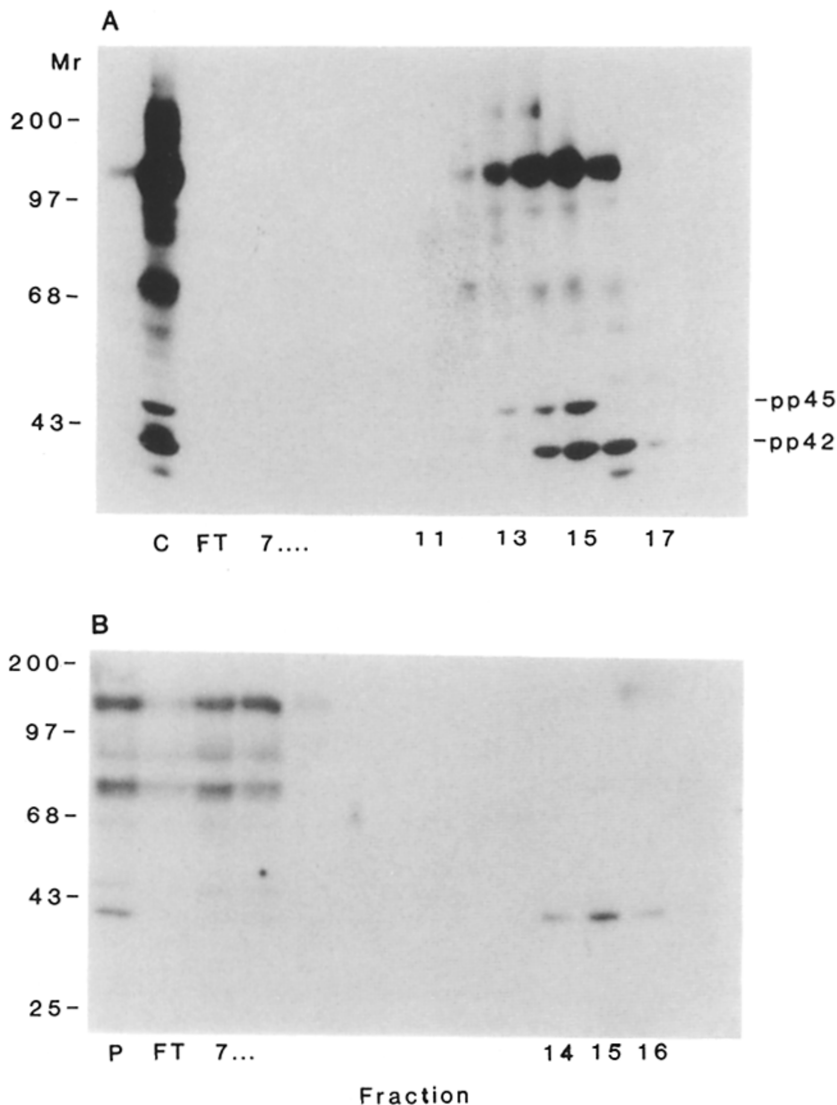

Figure 6. Antiphosphotyrosine Western immunoblot analysis of chromotographic fractions of extracts from nicotine-stimulated chromaffin cells. (*A*) Mono Q chromatography. 2 × 10^8 cells were stimulated for 3 min in culture medium containing 20 μM nicotine. Cells were lysed in hypotonic buffer, and the cytosolic (*C*) fraction was obtained by differential centrifugation. The cytosol was further fractionated on an FPLC Mono Q column as described in Materials and Methods. 100 μl of each 1-ml fraction was analyzed for pp45 and pp42 by the antiphosphotyrosine Western immunoblotting technique as in Fig. 1. Fractions 1–6 contained the flow through (*FT*). (*B*) Phenyl-Superose chromatography. Mono Q column fractions 13–17 were pooled (*P*) and applied directly to the phenyl-Superose column. 200 μl of each 1-ml fraction was analyzed for pp42 and pp45 as in *A*. Fractions 1–6, flow through (*FT*).

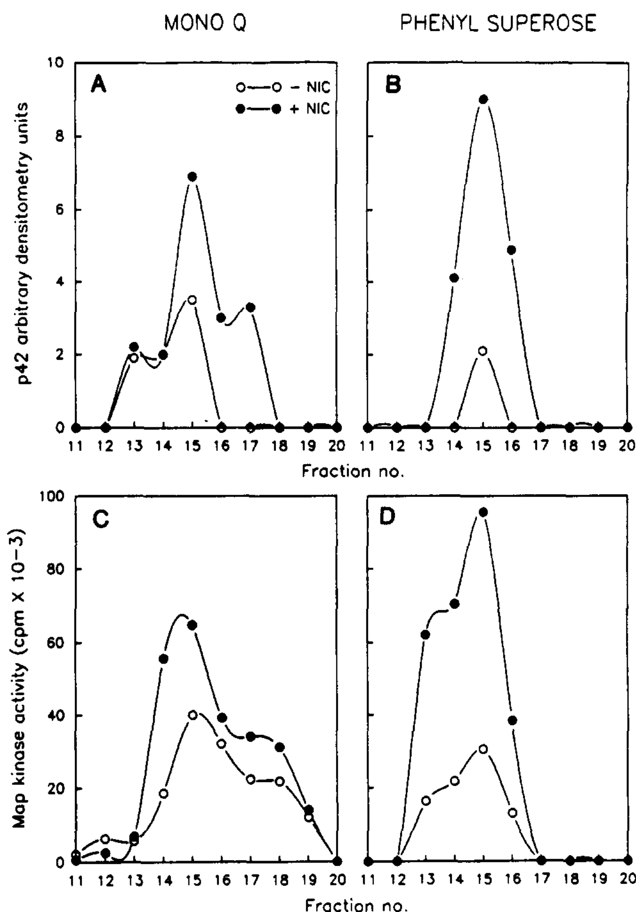

Figure 7. Cochromatography of pp42 and MAP kinase from nicotine-stimulated chromaffin cells. Autoradiograms of Western immunoblots prepared from Mono Q (*A*) and phenyl-Superose (*B*) column fractions were analyzed by densitometry for the amount of phosphotyrosine contained in the 42-kD band. The data are expressed as arbitrary densitometry units. MAP kinase activity contained in the Mono Q (*C*) and phenyl-Superose (*D*) column fractions was determined as described in Materials and Methods.

column Mono Q, and the phenyl-Superose column mentioned above. All fractions eluted were tested by the Western immunoblotting technique with ptyr antibody. The results in Fig. 6 *A* show that both pp42 and pp45 bound to the FPLC Mono Q column at low ionic strength and eluted in a continuous salt gradient at ~0.2–0.3 M NaCl. This result is consistent with the step elution of MAP kinase and Swiss 3T3 mitogen-associated pp42 from a DEAE matrix using 0.35 M NaCl. The pp42/pp45-containing fractions from chromaffin cells that had been eluted from the Mono Q column were pooled, applied directly to an FPLC phenyl-Superose column, and eluted under conditions used for the MAP kinase protein (Ray and Sturgill, 1988*b*), except that the *p*NPP concentration was reduced to 4 mM. The results in Fig. 6 *B* show that the secretion-associated pp42 bound with high affinity to this column and eluted at 37% ethylene glycol as the major protein in these fractions detected by the ptyr antibody, whereas the pp45 protein was only slightly retained on the column and eluted at 5–10% ethylene glycol along with several other phosphotyrosine-containing proteins. These results demonstrate that pp42 (but not pp45) from chromaffin cells exhibited identical chromatographic properties on phenyl-Superose as the MAP kinase protein and pp42 from EGF-stimulated Swiss 3T3 cells. Therefore, subsequent efforts were focused on the further characterization of pp42 from chromaffin cells and its relationship to MAP kinase and the mitogen-sensitive pp42.

MAP Kinase Activity of Partially Purified Chromaffin Cell pp42

To examine chromaffin cell preparations for MAP kinase activity, column fractions containing peak pp42 tyrosine phosphorylation were tested for their ability to phosphorylate MAP-2, isolated from bovine brain (Kim et al., 1979). Column fractions from nonstimulated samples were tested in parallel. Fig. 7 shows that MAP kinase activity eluted in a pattern similar to the tyrosine-phosphorylated pp42 protein from both anion exchange (*A* and *C*) and hydrophobic matrices (*B* and *D*). Furthermore, MAP kinase activity was elevated in nicotine-stimulated cells when compared to nonstimulated cells, particularly in the more purified phenyl-Superose fractions.

Identical Migration of the Secretion-associated and Mitogen-associated pp42 Proteins in 2-D Gels

To verify that the electrophoretic migrations of partially purified pp42 from both mitogen- and secretagogue-stimu-

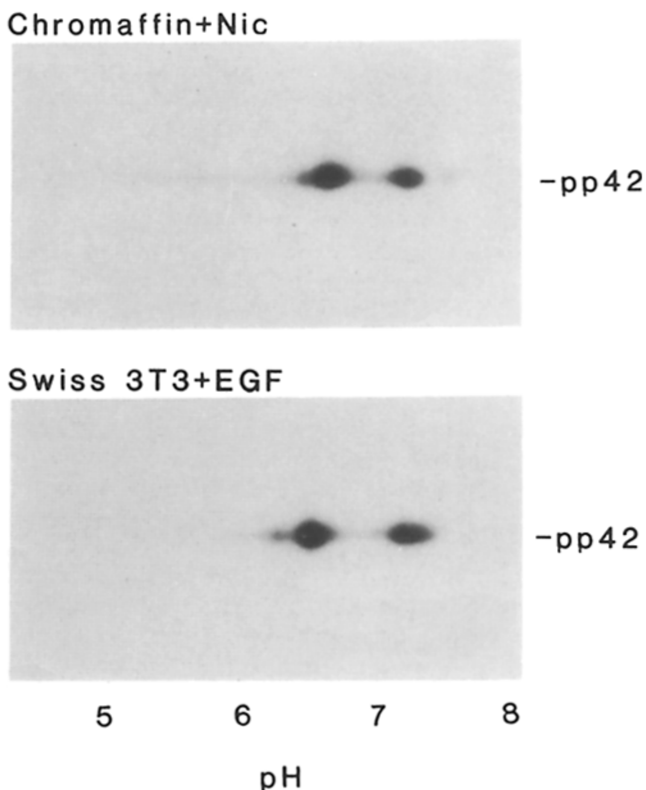

Figure 8. 2-D gel analysis of pp42 from nicotine-stimulated chromaffin cells and EGF-stimulated Swiss 3T3 fibroblasts. ^{32}P-labeled pp42 from each cell type was purified through the phenyl-Superose step and subjected to 2-D gel analysis as described in Materials and Methods. (*Top*) Nicotine-stimulated chromaffin cell proteins (1,543 cpm applied); (*bottom*) EGF-stimulated Swiss 3T3 cell proteins (926 cpm applied). Exposure was for 6 h.

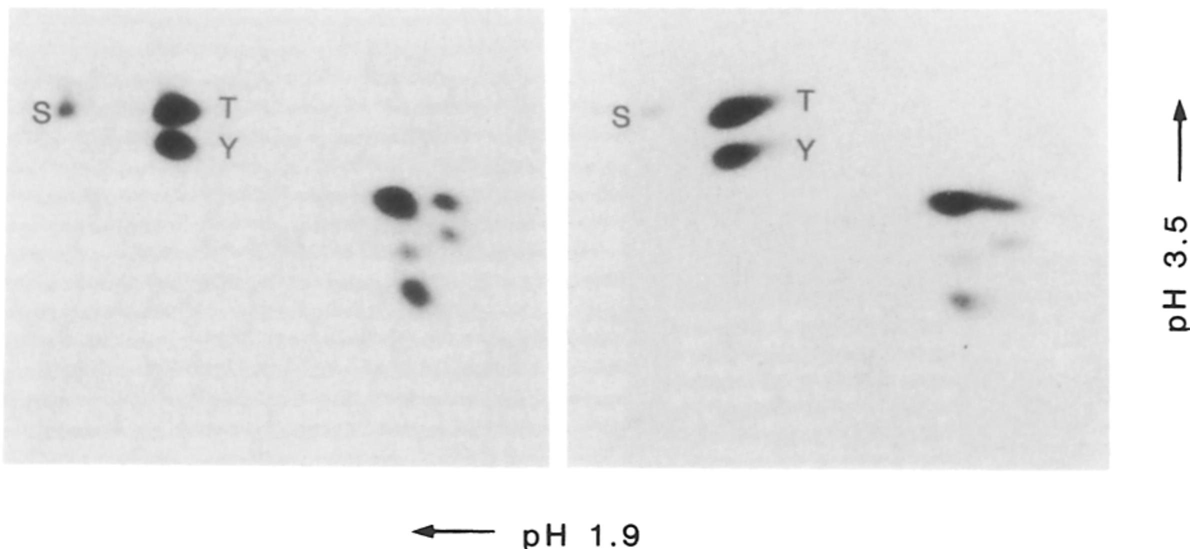

Figure 9. Phosphoamino acid analysis of pp42 from nicotine-stimulated chromaffin cells and EGF-stimulated Swiss 3T3 fibroblasts. ^{32}P-labeled pp42 from each cell type was purified through the phenyl-Superose step and resolved by 1-D SDS-PAGE. pp42 bands were excised from gels, eluted, and resolved by 2-D electrophoresis as described in Materials and Methods. 1,000 cpm of chromaffin cell pp42 and 1,500 cpm of 3T3 cell pp42 were applied to thin layer plates. Exposure was for 3 d. *Y*, phosphotyrosine; *T*, phosphothreonine; *S*, phosphoserine.

lated cells were the same, a comparative analysis of their mobility in 2-D gels was undertaken. Swiss 3T3 and chromaffin cells were labeled in vivo with $^{32}P_i$ and stimulated with either EGF or nicotine, respectively, and the phosphorylated 42-kD proteins were partially purified through the phenyl-Superose step in preparation for 2-D electrophoretic analysis as described in Materials and Methods. The migrations of these proteins in two dimensions are shown in Fig. 8. Acidic and basic forms of pp42 (Cooper et al., 1984) with nearly identical migrations were observed in both cell types, providing another criterion for their relatedness.

Phosphoamino Acid Analysis of EGF-stimulated Swiss 3T3 pp42 and Nicotine-stimulated Chromaffin Cell pp42

The phosphoamino acid analysis of the in vivo ^{32}P-labeled, phenyl-Superose-purified pp42 protein excised from 1-D SDS-polyacrylamide gels showed that both the EGF-stimulated Swiss 3T3 pp42 and the nicotine-stimulated chromaffin cell pp42 proteins were phosphorylated predominantly on tyrosine and threonine with minor phosphorylation on serine (Fig. 9). These results are consistent with the previously reported phosphoamino acid content of MAP kinase (Ray and Sturgill, 1988*a*). In addition, peptides resulting from partial acid hydrolysis during this procedure showed an identical pattern of fragments (Fig. 9).

Peptide Mapping of pp42 by Cyanogen Bromide Digestion

Cyanogen bromide digestion of pp42 proteins from stimulated chromaffin cells or Swiss 3T3 fibroblasts, which had been labeled in vivo with $^{32}P_i$, purified through the phenyl-Superose step, and excised from 1-D SDS-polyacrylamide gels, generated peptides of virtually identical size as determined by comigration during 1-D SDS-PAGE. These results provide further evidence for the relatedness of the pp42 proteins from chromaffin cells and Swiss 3T3 fibroblasts (Fig. 10).

Discussion

Analysis of phosphotyrosine-containing proteins in chromaffin cells has revealed that the most striking changes in phosphotyrosine content in response to secretagogues occurred in two proteins of 42 and 45 kD. These proteins underwent a simultaneous increase in phosphotyrosine content, which reached maximum levels (30- and 15-fold above control, respectively) 3 min after stimulation with three different classes of secretagogue: carbachol, which activates both muscarinic and nicotinic acetylcholine receptors; nicotine, which stimulates the nicotinic acetylcholine receptor alone; and K^+, which functions through membrane depolarization and activation of Ca^{2+} channels (reviewed in Livett, 1984*b*). A common effect of all three secretagogue treatments is an increase in the intracellular Ca^{2+} concentration, derived in large part from extracellular sources. Indeed, efficacious release of catecholamines is dependent upon such an influx (Livett, 1984*a*,*b*; Creutz, 1984; Fig. 2). The dependency of p42 and p45 tyrosine phosphorylations upon extracellular Ca^{2+} (Fig. 2) suggests that these proteins may be considered candidates for mediating some event(s) associated with secretion. Although characteristics of chromaffin cell pp42 and pp45 are shared with one another (kinetics and Ca^{2+} dependency of tyrosine phosphorylation and subcellular localization), whether they are unique proteins with similar properties or identical proteins with different posttranslational modifications, or products of differentially spliced mRNAs, remains to be determined.

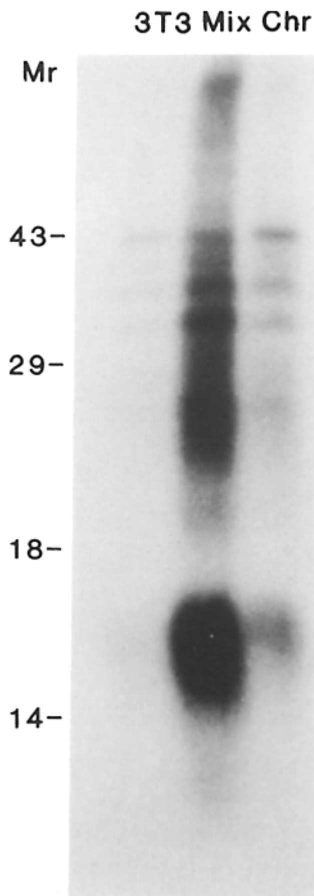

Figure 10. Cyanogen bromide peptide maps of pp42 from nicotine-stimulated chromaffin cells and EGF-stimulated Swiss 3T3 fibroblasts. ^{32}P-labeled and phenyl-Superose-purified pp42 from each cell type was resolved by 1-D SDS-PAGE, eluted from the gel slice, and digested with cyanogen bromide as described in Materials and Methods. The resulting peptides were separated by 1-D SDS-PAGE. 828 cpm of 3T3 pp42 and 1,380 cpm of chromaffin cell pp42 were loaded into the appropriate wells. The mix contained 828 cpm of 3T3 pp42 and 1,380 cpm of chromaffin cell pp42. Exposure was for 4 d.

Studies supporting a possible role for protein phosphorylation in chromaffin cell exocytosis include analysis by 1- and 2-D PAGE of total cell phosphoproteins (Gutierrez et al., 1988) or of the chromobindin fraction of cytosolic proteins which have been labeled in vivo with $^{32}P_i$ before stimulation (Michener et al., 1986), or in vitro with γ-[^{32}P]-ATP in permeabilized cells (Lee and Holz, 1986). Further in vitro studies have followed the endogenous phosphorylations of cytosolic (Wise and Costa, 1985) and membrane fractions (Wise and Costa, 1985; Burgoyne and Geisow, 1981; Geisow and Burgoyne, 1987) in the presence or absence of Ca^{2+}. While virtually all reports have specified a Ca^{2+} dependency for the phosphorylation of proteins in the 40–45-kD range, including those that partitioned to both soluble and particulate fractions, few of these studies have specified a role for tyrosine phosphorylations. The majority of the investigations have emphasized phosphorylations mediated by protein kinase C, cAMP-dependent protein kinases, and Ca^{2+}/calmodulin-dependent kinases, all of which are specific for the phosphorylation of serine or threonine residues. However, Grandori and Hanafusa (1988) have described a chromaffin cell membrane-associated protein of 38 kD, which can be found in stable complex with pp60$^{c\text{-}src}$, and which becomes tyrosine phosphorylated in vitro in a pp60$^{c\text{-}src}$-specific immune complex. It is not yet known whether this protein is associated with the process of secretion in these cells.

From an independent study (data not shown) we have concluded that pp42 and pp45 are unlikely to be the chromobindins calpactin I (36) and lipocortin I. This conclusion is based on (*a*) the inability of rabbit antibovine calpactin, which recognizes both calpactin and lipocortin (Drust, D., and C. Creutz, manuscript in preparation) to immunoblot either pp42 or pp45; (*b*) the distinct migration of pp42 and pp45 in 1-D gels when compared to purified bovine lung calpactin; (*c*) different chromatographic properties of the four proteins on DEAE; and (*d*) the inability of pp42 and pp45 to associate with cell membranes in the presence of calcium as has been demonstrated for calpactin, lipocortin, and other chromobindins (Creutz et al., 1983; Drust and Creutz, 1988). However, the possibility still remains that unphosphorylated p42 or p45 may exhibit many of the characteristics of calpactin and/or lipocortin. The availability of immunological reagents to pp42 and pp45 will allow for their further characterization as well as aid in the identification of the kinase(s) responsible for their tyrosine phosphorylations.

The results of the present studies do suggest, however, that secretagogue-treated chromaffin cells contain a 42-kD tyrosine kinase substrate(s) that is highly related to proteins found in mitogen-stimulated Swiss 3T3 fibroblasts and in insulin-stimulated 3T3-L1 adipocytes. The principal lines of evidence supporting this conclusion can be summarized as follows: (*a*) pp42 proteins from each of the three cell lines exhibited nearly identical chromatographic properties on anion exchange and hydrophobic matrices (Figs. 6 and 7; Ray and Sturgill, 1988*b*; Rossomando et al., 1989); (*b*) all three proteins exhibited an agonist-depdendent activation of a MAP kinase activity and an enhanced tyrosine phosphorylation of a 42-kD protein (Figs. 1, 4, and 7; Ray and Sturgill, 1987, 1988*b*; Rossomando et al., 1989); (*c*) MAP kinase activity from each stimulated cell type cochromatographed with a tyrosine-phosphorylated pp42; and (*d*) pp42 proteins from the three cell types contained predominantly phosphothreonine and phosphotyrosine and possessed nearly identical isoelectric points (Figs. 8 and 9; Ray and Sturgill, 1988*a*; Rossomando et al., 1989). Furthermore, V-8 partial proteolytic peptide analysis revealed that the major peptides generated from partially purified MAP kinase were present as a subset of peptides in digests of pp42 from EGF-stimulated Swiss 3T3 cells (Rossomando et al., 1989). In addition we show that partial acid hydrolysis and CNBr digestion of purified pp42 proteins from stimulated chromaffin cells and Swiss 3T3 fibroblasts generated indistinguishable peptide profiles (Figs. 9 and 10). Based on these criteria, it appears that pp42 is common to several different species and cell types and can be found in cells programmed to carry out specific functions in response to different stimuli, e.g., secretagogue-stimulated exocytosis, EGF-induced proliferation, and insulin-stimulated glucose uptake.

In this paper we have referred to the pp42 bands that copurify with MAP kinase as MAP kinase. This is a working hypothesis that is supported by evidence outlined in detail by Ray and Sturgill (1987, 1988*a*,*b*) and Rossomando et al. (1989) and briefly summarized above. The data presented here also favor this hypothesis. However, definitive proof of the identity of these proteins awaits the availability of specific antibody reagents and isolation of the gene.

Nicotine stimulated the phosphorylation on tyrosine of a 45-kD protein as well as a 42-kD protein in chromaffin cells. Fig. 6 shows that pp45 and pp42 exhibited nearly identical chromatographic properties on Mono Q, but significantly different properties on phenyl-Superose. pp45 isolated from

insulin-stimulated 3T3-L1 adipocytes and EGF-stimulated Swiss 3T3 cells also exhibited a chromatographic profile that was virtually identical to that of pp45 from chromaffin cells (data not shown). While these data, together with the identical comigration during 1-D SDS-PAGE (Fig. 4), suggest that the pp45 proteins from chromaffin cells, 3T3-L1 adipocytes, and Swiss 3T3 fibroblasts may be related, the extent of the similarity between the three proteins awaits further investigation.

Although the three pp42 proteins from chromaffin cells, 3T3-L1 adipocytes, and Swiss 3T3 fibroblasts appear to possess MAP kinase activity in vitro, the relevance of this activity in vivo, if any, is unknown. If MAPs are physiologically relevant substrates of MAP kinase, perhaps a common cytoskeletal alteration accompanies the onset of secretion, glucose uptake, and mitogenesis. This would be consistent with existing data which have indicated that the cytoskeleton plays a significant role in secretory granule mobilization (Kondo et al., 1982; Trifaro et al., 1985; Burgoyne et al., 1986; Perrin et al., 1987; Matter et al., 1989), in vesicular translocation of glucose transporters (Blok et al., 1988), as well as in endocytic vesicle movement accompanying mitogen receptor activation and down modulation (Herschman, 1985; Schroer and Kelly, 1985; Brodsky, 1988; Kasaian and Neet, 1988). The work of Sturgill et al. (1988) has suggested that S6 kinase II may also be an in vivo substrate for MAP kinase. Such a finding would associate this protein with the process of protein synthesis, which is known to be required for cell division (Todaro et al., 1965; Coffino and Groppi, 1981) and thought to be involved in the regeneration of secretory peptides after exocytosis (Livett, 1984*a*,*b*). Regardless of the precise substrate for this protein, it appears that pp42 acts as one member of a series of kinases which may serve to transduce membrane-induced signals throughout the cell and/or to regulate intracellular processes, such as organelle movement or protein synthesis.

We have found that both EGF and the tumor promoter, TPA, induced the tyrosine phosphorylation of a 42-kD protein in chromaffin cells that comigrated with the secretagogue-sensitive protein, yet neither of these agents was capable of inducing proliferation or secretion in chromaffin cells (data not shown). In contrast to secretagogue treatment, neither EGF- nor TPA-induced tyrosine phosphorylation of p42 or p45 appeared to be dependent on extracellular Ca^{2+} (Fig. 5). Furthermore, it is unclear what biological effect EGF and TPA might have on adult chromaffin cells. The phosphorylations induced by these agents may represent vestigial or ablated signals for the transmission of proliferative or differentiative messages during early embryogenesis or neurological differentiation. Identity of the secretagogue-stimulated pp42 with the mitogen-stimulated pp42 (as indicated by the work presented in this communication) implies that the tyrosine phosphorylation of this protein is not a sufficient signal for either the process of secretion or cell proliferation. Perhaps cellular context and specific substrates of MAP kinase are the important factors in determing the cell function ultimately triggered by different cell surface receptor-activated pathways that converge on this protein.

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