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Rapid Communication

Nerve Growth Factor Regulates the Subcellular Localization of the Nerve Growth Factor–Inducible Protein PC4 in PC12 Cells

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The immediate early gene (IEG) PC4, which encodes a protein related to γ interferon, is activated at the onset of the neuronal differentiation induced by nerve growth factor (NGF) in PC12 cells. With an antibody raised to a bacterial β gal-PC4 fusion protein, the PC4 protein is detected as an immunoreactive molecular species of 49 kDa, whose synthesis is rapidly induced by NGF in parallel with the induction of its mRNA. Immunofluorescence, electron microscopy and subfractionation studies indicate that the PC4 immunoreactivity is localized in the cytoplasm of PC12 cells, where it is increased transiently by NGF within 3 hr of treatment. In addition, the PC4 immunoreactivity presents an NGF-dependent pattern of intracellular localization. In fact, within 3 hr after addition of NGF, PC4 is also significantly expressed on the inner face of the plasma membrane, to which it is physically associated. After longer NGF treatment, PC4 disappears from the plasma membrane and appears in the nucleus, with reduced cytoplasmic expression. Localization in the nucleus is reversed by removal of NGF and closely parallels changes in the state of differentiation of the cell. The existence within the PC4 protein of a consensus sequence for the addition of myristic acid and of a putative sequence for the nuclear localization suggests possible mechanisms for the NGF-dependent redistribution. For an NGF-inducible IEG product, such growth factor–dependent localization of PC4 is a novel type of regulation in the pathways from the NGF receptor to the adjacent membrane proteins and to the nucleus.

Key words: nucleus, differentiation, proliferation, neurotrophins, immediate early genes

INTRODUCTION

Cell differentiation is a complex process depending on both extracellular and intracellular cues. In vitro and in vivo studies have shown that epigenetic factors, such as transcriptional factors of maternal origin and peptide growth factors, trigger distinct pathways of signal transduction in the target cells (Cross and Dexter, 1991; Brown, 1984). This activation leads to a coordinated induction of distinct subsets of genes, thought to be ultimately responsible for attainment of the differentiated phenotype (Sheng and Greenberg, 1990; Halegoua et al., 1990).

Nerve growth factor (NGF) is required for the development of neural crest–derived nervous tissue, such as sympathetic and some sensory neurons, and also exerts a trophic action on certain populations of cholinergic neurons of the central nervous system (reviewed in Levi-Montalcini, 1987; Barde, 1989). NGF also induces the differentiation into sympathetic neurons of another neural crest–derived tissue, the chromaffin cells of the fetal adrenal medulla (Aloe and Levi-Montalcini, 1979).

PC12 cells, a line derived from a chromaffin cell tumour, constitute a widely studied in vitro model of the NGF-mediated differentiation (Greene and Tischler, 1976). In these cells the NGF receptor consists of two components, $gp140^{trk}$ and $p75^{NGFR}$ (Kaplan et al., 1991a,b; Klein et al., 1991; Radeke et al., 1987). The

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ligand-activated gp140^{trk} is thought to trigger a cascade of events through its intrinsic tyrosine kinase activity (Kaplan et al., 1991a; Klein et al., 1991). Signal transduction presumably involves the sequential activation of molecules associated with the plasma membrane, such as the tyrosine protein-kinase p60$^{c\text{-}src}$ (Koch et al., 1991) and the GTP-binding protein p21$^{c\text{-}ras}$ (Qiu and Green, 1991; Barbacid, 1987). Another plasma membrane protein which appears to be activated by gp140^{trk} is phospholipase C-γ1 (Vetter et al., 1991). Downstream events are the activation of several protein kinases (e.g., MAP kinase, rsk-encoded kinase, protein kinase C; Gotoh et al., 1990; Wood et al., 1992; Hama et al., 1986) and of immediate early genes (IEG) encoding for nuclear proteins, such as c-fos, c-jun, c-myc, and the zinc finger protein NGFI-A (Greenberg et al., 1985; Wu et al., 1988; Milbrandt, 1987). These IEG are all transcriptional modulators (Sheng and Greenberg, 1990). Interestingly, the oncogenic products of src and ras mimic the NGF-induced neurite outgrowth (Bar-Sagi and Feramisco, 1985; Alemà et al., 1985), and induce the transcription of IEG and late expression NGF-dependent genes (Thomas et al., 1991; Rausch et al., 1989; Sassone-Corsi et al., 1989; Guerrero et al., 1988), in a hierarchical order (D'Arcangelo and Halegoua, 1993; Keegan and Halegoua, 1993).

The proteins encoded by the NGF-inducible IEG are not exclusively nuclear. The NGFI-A protein is, in fact, expressed in two molecular forms, one of which is in the cytoplasm (Day et al., 1990), while the c-fos and c-myc proteins translocate from the cytoplasm to the nucleus depending on stimuli such as the density of the cell culture (Vriz et al., 1992) or serum factors (Roux et al., 1990). The src protein p60$^{c\text{-}src}$ also undergoes translocation during platelet activation, from the plasma membrane to the cytoskeleton (Horvath et al., 1992). Moreover, there is one example of PC12 cells of a protein whose intracellular localization is NGF-dependent, the MAP kinase, which translocates from the cytoplasm to the nucleus (Traverse et al., 1992).

We describe here a similar phenomenon for the IEG PC4, which has been recently isolated by us from PC12 cells as an early NGF-inducible gene (Tirone and Shooter, 1989), and by Varnum et al. (1989), in mouse NIH3T3 cells as a tetradecanoyl phorbol acetate–induced gene (called TIS 7). Analysis of the PC4 protein sequence, deduced from the cDNA, indicates a significant correlation with a lymphokine, interferon-γ (Tirone and Shooter, 1989). The interferon-γ protein synergizes with NGF in inducing neuronal differentiation of PC12 cells (Improta et al., 1988). The PC4 protein, however, has no antiviral activity and no functional motifs, such as DNA-binding sequences, thus leaving open the question of its function. Since the PC4 mRNA is expressed at high levels in the embryonic brain in the period related to neuroblast proliferation and differentiation (Tirone and Shooter, 1989), PC4 very likely participates in neurogenesis.

To learn more about the function of PC4 in the process of neuronal differentiation, we studied its expression and sub-cellular localization in the in vitro PC12 cells model. We report that the PC4 protein, besides being expressed in the cytoplasm of PC12 cells, is also localized in the plasma membrane at the onset of NGF-induced differentiation and in the nuclei of differentiated cells. This NGF-dependent intracellular distribution is a novel mechanism for the NGF-inducible IEG products, possibly regulating PC4 function in the process of cell proliferation and/or neuronal differentiation.

MATERIALS AND METHODS

Cell Culture

PC12 cells were obtained from D. Schubert (Salk Institute, 5th passage) and grown in DME with 5% supplemented calf serum (Hy Clone, Logan, UT) and 5% horse serum (Hy Clone) in a humidified atmosphere of 12% CO_2 at 37°C.

Production of the Bacterial Fusion Protein

A fusion protein of β-galactosidase with the NH_2-terminal region 52–144 of the PC4 protein (total length 449 amino acids: see Tirone and Shooter, 1989) was produced by subcloning in the PstI site of the vector pUEX-3 (Amersham, Little Chalfont, England) a *PstI-PstI* fragment 279 base pairs long from the coding region of the PC4 cDNA, and then expressing the construct in *E. coli*. Bacteria were induced to produce the fusion protein by temperature shift (from 30 to 42°C). The pellet from the bacterial lysate was washed sequentially with 0.5% Triton X-100 and 3 M guanidine hydrochloride, until the fusion protein represented about 90% of the total protein as judged by SDS polyacrylamide gel. According to the side-strip procedure described by Harlow and Lane (1988), the fusion protein was then purified by separation on preparative 6% SDS polyacrylamide gel electrophoresis, and by electroelution of the corresponding band excised from the gel.

Production of Anti-PC4 Antibodies

A female New Zealand white rabbit (Gennari, Roma) was immunized with 200 μg of the fusion protein. The immunogen was emulsified with an equal volume of complete Freund's adjuvant for the initial injections and incomplete Freund's adjuvant for booster injections, for a total of three injections every 3 weeks, followed thereafter by monthly booster injections. The immune serum was purified by affinity chromatography

through a column of cyanogen bromide–activated Sepharose 4B resin (Pharmacia, Uppsala, Sweden) conjugated to the fusion protein itself, eluted from an SDS polyacrylamide gel onto which the bacterial lysate had been loaded. The affinity-purified antibody was called A451 and used in all the procedures for PC4 detection.

A second antibody was obtained by immunizing a female New Zealand white rabbit (with a protocol similar to that described above) with a peptide having the sequence FKARTKARSKCCRDKRA, which corresponds to residues 428–443 at the carboxy-terminal of the PC4 protein. The peptide, called P1, was synthesized with the branched lysine protocol (by Research Genetics, Huntsville, AL) and therefore did not require coupling to a carrier protein. The antisera obtained was called anti-P1.

In Vitro Transcription and Translation of the PC4 Protein

The PC4 RNA for the in vitro translation was synthesized by the SP6 polymerase, from a BamHI linearized pGEM-3 vector (Promega, Madison, WI) bearing a *BanI-HindIII* fragment from pcD-PC4 (Tirone and Shooter, 1989), subcloned in the sense orientation in the *SalI* site of the polylinker. The reaction was performed at 37°C in 40 mM Tris-HCl buffer, pH 7.5, with 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 20 mM dithiothreitol, 40 units of RNase inhibitor, 4 μg of linearized vector, 40 units of SP6 RNA polymerase, 2.5 mM ribonucleotides, 0.5 mM m7G(5′)ppp(5′), and water to a final volume of 50 μl. After an additional 15 min of incubation with 4 units of RQ1 DNase (Promega), the reaction mixture was extracted with phenol chloroform, precipitated with ethanol and used for translation. Next, 0.5 μg of the synthesized PC4 RNA was added to 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega), 20 μM amino acids (minus Leucine), 5 μCi/ml of [^{14}C]-Leucine (or 1 mCi/ml of [^{35}S] methionine where indicated), 40 units of RNase inhibitor and water to 50 μl, and incubated 60 min at 30°C.

Immunoprecipitation of the Labeled PC4 Protein

The in vitro translated protein was immunoprecipitated, as described by Anderson and Blobel (1979), after addition of 4% v/v (final concentration) SDS followed by 4 min of denaturation in boiling water. The sample was precleared with pre-immune rabbit serum and the supernatant immunoprecipitated with immune serum sequentially after addition of immunoprecipitation buffer (to a final concentration of 150 mM NaCl, 5 mM EDTA, 40 mM Tris HCl pH 7.4, 0.4% SDS, 1% Triton X-100) and of Sepharose-protein A CL-4B (Pharmacia) as immunoadsorbent, at 4°C for 3 hr. The immune complexes were pelleted by centrifugation (5 min at 200*g*, at 4°C), then washed three times in immunoprecipitation buffer with 140 mM NaCl, and once without NaCl; the resin-protein mixture was denatured by heating 5 min at 100°C in 50 mM Tris-HCl, pH 6.8, 8% glycerol, 2% SDS, 0.7 M 2-mercaptoethanol, and separated on 10% SDS-polyacrylamide gel, which was then treated for 45 min with 1 M sodium salicylate, dried and analyzed by autoradiography.

Immunoprecipitation of the labeled PC4 protein in PC12 cells was performed on cultures seeded 24 hr before (about 2×10^6 onto 35 mm plates). PC12 cells were incubated in medium without methionine for 1 hr, and [^{35}S] methionine (100 μCi/ml) was added 90 min before harvesting. Cultures were exposed to NGF (100 ng/ml) prior to harvesting for the time lengths indicated. At the end of the labeling, the cultures were washed in phosphate buffered saline at room temperature, and lysed on the plates by addition of ice-cold 0.5% sodium deoxycholate and 1% NP40, in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, followed by 30 min of incubation at 4°C with occasional rocking. The lysate was collected and boiled 5 min after addition of SDS to a final concentration of 1.6%. Pre-clearing and immunoprecipitation of the sample were then repeated as above. The immunocomplexes were washed three times in 140 mM NaCl, 10 mM Tris-HCl (with the addition of 0.1% Triton X-100, 0.1% gelatin in the initial two washes) and once in 50 mM Tris-HCl, pH 6.8, alone.

Immunofluorescence Microscopy

Cells grown on poly-lysine coated coverslips were washed three times with PBS, fixed for 10 min at room temperature in 4% paraformaldehyde in PBS, then permeabilized with 0.2% Triton, in 0.1 M Tris-HCl pH 7.5. After a PBS wash, the cells were incubated 60 min at room temperature with the primary antibody A451, diluted 1:400 in PBS. Anti-PC4 IgG complexes were detected by incubating the cells 30 min at room temperature with FITC-conjugated (fluorescein isothiocyanate, Myles-Yeda, Rehovot, Israel) goat anti-rabbit. Immunofluorescence was visualized with a Leitz Dialux 22 microscope. Competitive pre-absorption was carried out as a control of the antibody specificity. The purified A-451 antiserum was incubated in PBS for 3 hr at room temperature with increasing concentrations of β gal-PC4 fusion protein (5–180 μg/ml purified as described above), and used for staining. The specificity of the antibody was also verified by staining PC12 cell cultures with pre-immune serum, with identical results.

Cell Fractionation

The procedure followed was similar to that described by Vriz et al. (1992). About 10^7 PC12 cells were harvested in 0.25 M sucrose, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM leupeptin,

and homogenized at 4°C in a Dounce homogenizer (Wheaton, 5 ml, tight-fitting pestle) with 30 strokes. The homogenate was centrifuged at 300*g* for 10 min to pellet nuclei. The supernatant contained the cytoplasmic fraction. The pellet was resuspended and centrifuged through an 0.8 M sucrose cushion at 6,000*g* for 15 min. This pellet was the nuclear fraction, as seen by phase contrast microscopy and from the absence of the cytoplasmic marker tyrosine hydroxylase. The membrane-enriched fraction and the cytosol were separated by centrifugation of the cytoplasmic fraction for 1 hr at 100,000*g*, at 4°C. Plasma membrane separation on a linear sucrose gradient (0.4–2 M) was performed as published (Schweitzer and Kelly, 1985). After homogenization of the cell cultures (10^7 cells) and low-speed centrifugation, as described above, the supernatant (cytoplasmic fraction) was resolved on the gradient at 100,000*g* for 3 hr at 4°C. Ten fractions were collected. The amounts of tyrosine hydroxylase, Na^+-K^+-ATPase, SSR and PC4 were determined by immunoblot.

Immunoblot Analysis

Cell fractions were precipitated overnight in 20% trichloroacetic acid at 4°C, resuspended in 50 mM Tris-HCl, pH 6.8, 2% SDS, 9 M urea, 40 mM DTT, 1 M β-mercaptoethanol, with 1 mM phenylmethylsulfonyl fluoride and 1 mM leupeptin and heated 5 min at 100°C. An aliquot was analyzed by SDS/10% polyacrylamide gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose (4–12 hr at 120 mA in 24 mM Tris-HCl, pH 8.0, 166 mM glycine, 20% methanol). The filters were then incubated for 3 hr with either A451 antiserum or a mouse monoclonal antibody against tyrosine hydroxylase (Boehringer, Mannheim, Germany) or against Na,K-ATPase (from Grazia Pietrini, University of Milano, Italy; see Pietrini et al., 1992) or against the SSR protein (from Giovanni Migliaccio, IRBM, Italy; see Migliaccio et al., 1992). The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Pierce, Rockford, IL), used as per the manufacturer's instructions.

Electron Microscopy

PC12 cells, untreated or treated with NGF for 3 or 72 hr, were fixed with glutaraldehyde in PBS for 1 hr at room temperature, partially dehydrated in ethanol and embedded in LR White resin. Thin sections were collected on nickel grids, immunolabeled with the affinity-purified polyclonal antibody A451 and then with protein A-gold (18 nm) prepared by the citrate method (Slot and Geuze, 1981). In control experiments either the antibody from the labeling procedure was omitted or the pre-immune serum was used. All sections were stained with uranyl acetate and lead citrate before EM examination.

Density of labeling was determined as gold particles per μm^2 of cytoplasmic areas or gold particles per μm^2 of plasma membranes. Statistical analysis was done with the Sigma Scan Measurement System (Yandel Scientific, Corte Madera, CA).

RESULTS

Production and Characterization of A451 Anti-PC4 Antiserum

To characterize the PC4 protein, we produced a polyclonal antiserum raised to a bacterial fusion protein that was obtained by linking a fragment of the PC4 cDNA in frame with the β galactosidase gene contained in the pUEX-3 vector (see Materials and Methods). The PC4 protein fused to β galactosidase begins with the cysteine at the nt 299 of the PC4 cDNA (Tirone and Shooter, 1989) and terminates with the isoleucine at the nt 577.

The ability of this antiserum, called A451, to immunoprecipitate the PC4 protein was first tested on the in vitro translated protein, synthesized in a reticulocyte lysate system in the presence of [^{14}C] leucine and of the PC4 RNA. This was previously transcribed with SP6 RNA polymerase from the PC4 cDNA template. The immune complexes were collected after incubation with protein A-Sepharose CL4B, and visualized after SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 1A). The translation product had an apparent molecular mass of approximately 49 kDa, in agreement with the predicted size of the cDNA-deduced protein sequence (49,789 kDa, Fig. 1A, lane 3). This 49 kDa protein form was immunoprecipitated after incubation with the A451 antiserum (Fig. 1A, lane 2), and not with the pre-immune serum (Fig. 1A, lane 1).

To further assess the specificity of the antiserum, we compared the size and the tryptic digestion patterns of immunoprecipitates with A451 antiserum from in vitro synthesized PC4 protein and from whole cell lysates of PC12 cells (Fig. 1B). The cell cultures, labeled with [^{35}S] methionine, were treated with NGF (100 ng/ml) for 2 hr. In vivo, the antiserum immunoprecipitated one molecular species of 49 kDa, identical in size to the PC4 protein translated in vitro and immunoprecipitated (Fig. 1B, respectively lanes 3 and 1). Trypsin digestion of each of these 49 kDa species generated identical patterns of proteolytic fragments, consisting in two peptides with an apparent molecular mass of 8.5 and 4.3 kDa (Fig. 1B, lanes 2 and 4). These peptides corresponded in size to the two major fragments predicted in the PC4 cDNA–deduced protein sequence on the basis of the specificity of recognition of trypsin. The A451 antiserum, therefore,

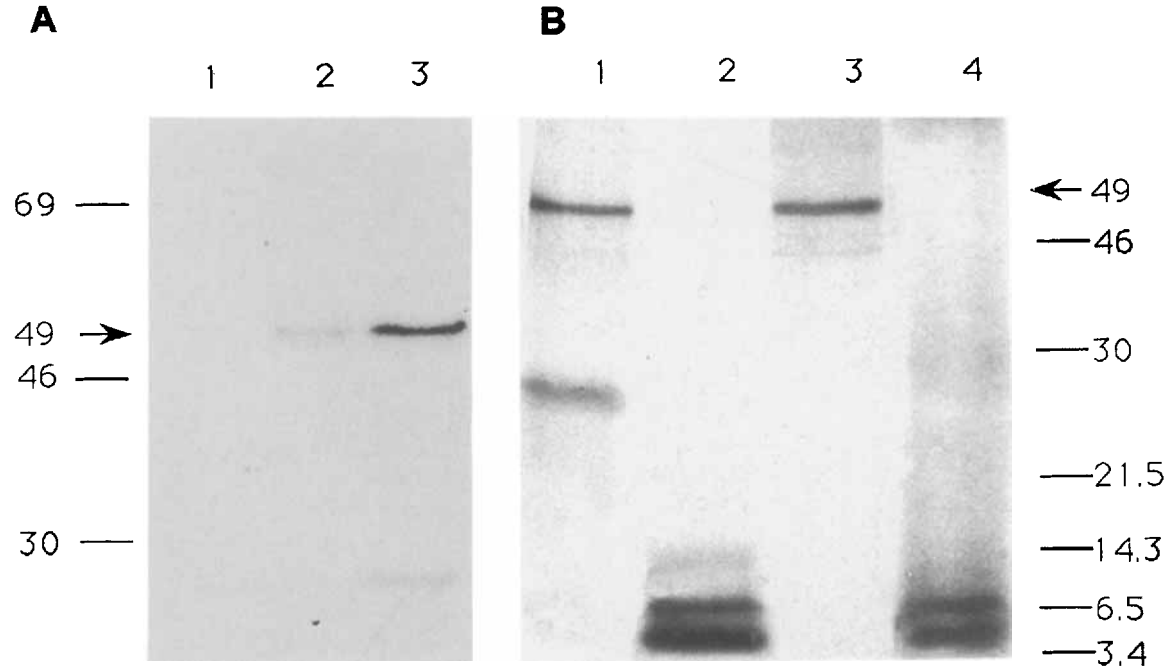


Fig. 1. Specificity of the A451 antiserum. **A:** Immunoprecipitation by A451 antiserum of the PC4 protein [^{14}C] leucine-labeled in vitro. Rabbit reticulocyte lysates were incubated with 0.5 μg of PC4 RNA transcribed with SP6 RNA polymerase. The translation product was immunoprecipitated with pre-immune serum **(lane 1),** with antiserum A451 **(lane 2,** indicated by the arrow), or directly loaded on the 10% sodium dodecyl sulphate-polyacrylamide gel **(lane 3).** The amount of protein loaded in lane 3 was about 2.5 times that used for immunoprecipitation. The gel was analyzed by autoradiography after treatment with 1 M sodium salicylate. The molecular weight standards are indicated. **B:** Trypsin digestion pattern of the 49 kDa species immunoprecipitated with A451, from in vitro translated PC4 and from whole PC12 cell lysates. The A451 antiserum was used to immunoprecipitate the PC4 protein [^{35}S] methionine-labeled in vitro **(lane 1)** and a whole lysate of PC12 cells **(lane 3).** The cell cultures were labeled with [^{35}S] methionine for 90 min; NGF (100 ng/ml) was added 30 min prior to labeling, for a total of 2 hr. The immunoprecipitated products were resuspended in 20 μl of 0.12 M Tris-HCl pH 8.8, 1 mM EDTA, 0.1% SDS, digested for 20 min at 37°C with 5 μg (1.25 units) of trypsin and loaded on a 15% SDS polyacrylamide gel (protein in vitro, **lane 2;** cellular protein, **lane 4**).

specifically recognizes a cellular protein of 49 kDa corresponding to the PC4 protein.

In Vivo NGF Induction of PC4 Protein Synthesis

To analyze NGF-mediated PC4 protein induction in PC12 cells, we immunoprecipitated whole cell lysates from cultures labeled with [^{35}S] methionine for 90 min and treated with NGF (100 ng/ml) for increasing times (Fig. 2). The immunoprecipitated product was scarcely detectable at time 0, but after 1 hr accumulation of the 49 kDa protein form previously shown was apparent. Maximal induction was observed after 2 hr, followed by complete decay within 30 hr. These kinetic data resemble those of the PC4 mRNA level (Tirone and Shooter, 1989), which is low in untreated cells but increases rapidly upon addition of NGF. Since the PC4 protein contains 13 cysteine residues, in some experiments the immunoprecipitates were treated with a reducing and denaturing mixture of 9 M urea, 40 mM dithiothreitol and 1 M β-mercaptoethanol, prior to SDS-polyacrylamide gel electrophoresis. The absence of apparent changes of electrophoretic mobility (data not shown) indicated that the observed protein's size was not due to incomplete reduction and/or denaturation.

NGF-Dependent Localization of the PC4 Protein in the Nucleus

At various intervals after NGF stimulation, we visualized the intracellular localization of the PC4 protein in PC12 cells by immunofluorescence microscopy. PC4 staining, as revealed by the A451 antiserum, could not be detected in non-permeabilized cells (data not shown), but was localized homogeneously in the cytoplasm of NGF-treated (4 hr, Fig. 3B) and untreated PC12 cells (Fig. 3A), thus indicating a basal level of expression of the PC4 protein. A slight but definite increase of PC4 staining was observed after NGF treatment for 4 hr. To

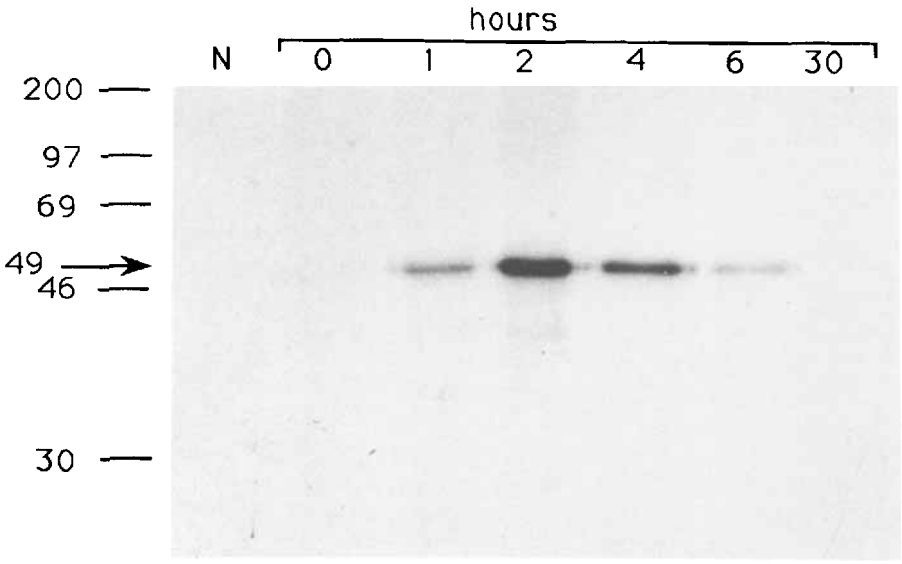


Fig. 2. Time course of the induction of PC12 cells of the PC4 protein synthesis by NGF. PC12 cell cultures (5×10^6 cells/60 mm dish) were incubated in medium without methionine for 1 hr. Immediately after, cells were exposed to [^{35}S] methionine for 90 min and then harvested (= time 0). NGF was added to the cultures prior to time 0 at the time indicated (hours). An aliquot of the whole lysate was immunoprecipitated with A451 antiserum or with pre-immune serum (N). The immune complexes were resolved on a 10% SDS-polyacrylamide gel. Arrow indicates the PC4 molecular species. The molecular weight standards are indicated.

our surprise, longer exposure to NGF (36 hr, 3 and 7 days, Fig. 3C–E) gradually induced nuclear localization of the PC4 staining, in parallel with acquisition of the neuronal phenotype. Nuclear localization of PC4 after prolonged treatment with NGF (2 to 7 days) was also observed using anti-P1 antiserum, directed against the carboxy-terminal region of PC4 (data not shown, see Materials and Methods). However, the immunoreactivity was still detectable in the cytoplasm (Fig. 3C–E). PC4 staining was not observed when A451 was preabsorbed with the fusion protein (see Fig. 3F and Materials and Methods), while no effect was seen after preabsorption with non-recombinant β-galactosidase protein (not shown). Removal of NGF from the culture medium of cells treated for 3 days with NGF induced a nucleus-to-cytoplasm reverse translocation of PC4, in parallel with the return to the undifferentiated phenotype (Fig. 4). To gather more information about this NGF-regulated intracellular distribution of the PC4 protein, we subfractionated a PC12 cell homogenate (see Materials and Methods) into nuclear, membrane-enriched and cytosolic fractions that were analyzed by immunoblot. This revealed one band of apparent molecular mass of 49 kDa (Fig. 5A), in agreement with the previous immunoprecipitation data. In the total cell lysate and cytosol expression of this PC4 immunoreactive species was detected in both control and NGF-treated cells, with a slight and transient increase after 3 hr of treatment. The nuclear fraction showed evident expression of PC4 only in cells treated with NGF for 72 hr (Fig. 5A), in agreement with the results from immunofluorescence microscopy. Surprisingly, the membrane-enriched fraction also showed the presence of the PC4 protein, restricted to the 3-hr NGF treatment. The detection of PC4 in the membrane-enriched fraction did not appear to be due to contaminating cytosol, since the marker tyrosine hydroxylase was in fact almost undetectable (Fig. 5A).

As a further approach to study the nuclear localization of PC4, we measured it by quantitative analysis with electron microscopy. Since by immunofluorescence microscopy the nuclear staining appeared maximal after 7 days of treatment with NGF (Fig. 3 and data not shown), the electron microscopy was performed after this prolonged exposure. The immunoreactivity on the nucleus of untreated cells (labeled with A451) was not significantly higher than in control samples (labeled with pre-immune serum, $P > 0.05$, Table I). In contrast, the nuclear immunolabeling after 7 days of treatment with NGF was significantly higher than in untreated cells ($P < 0.05$, Table I; Fig. 6). The immunolabeling of treated cells appeared homogeneously distributed within the nucleus (Fig. 6).

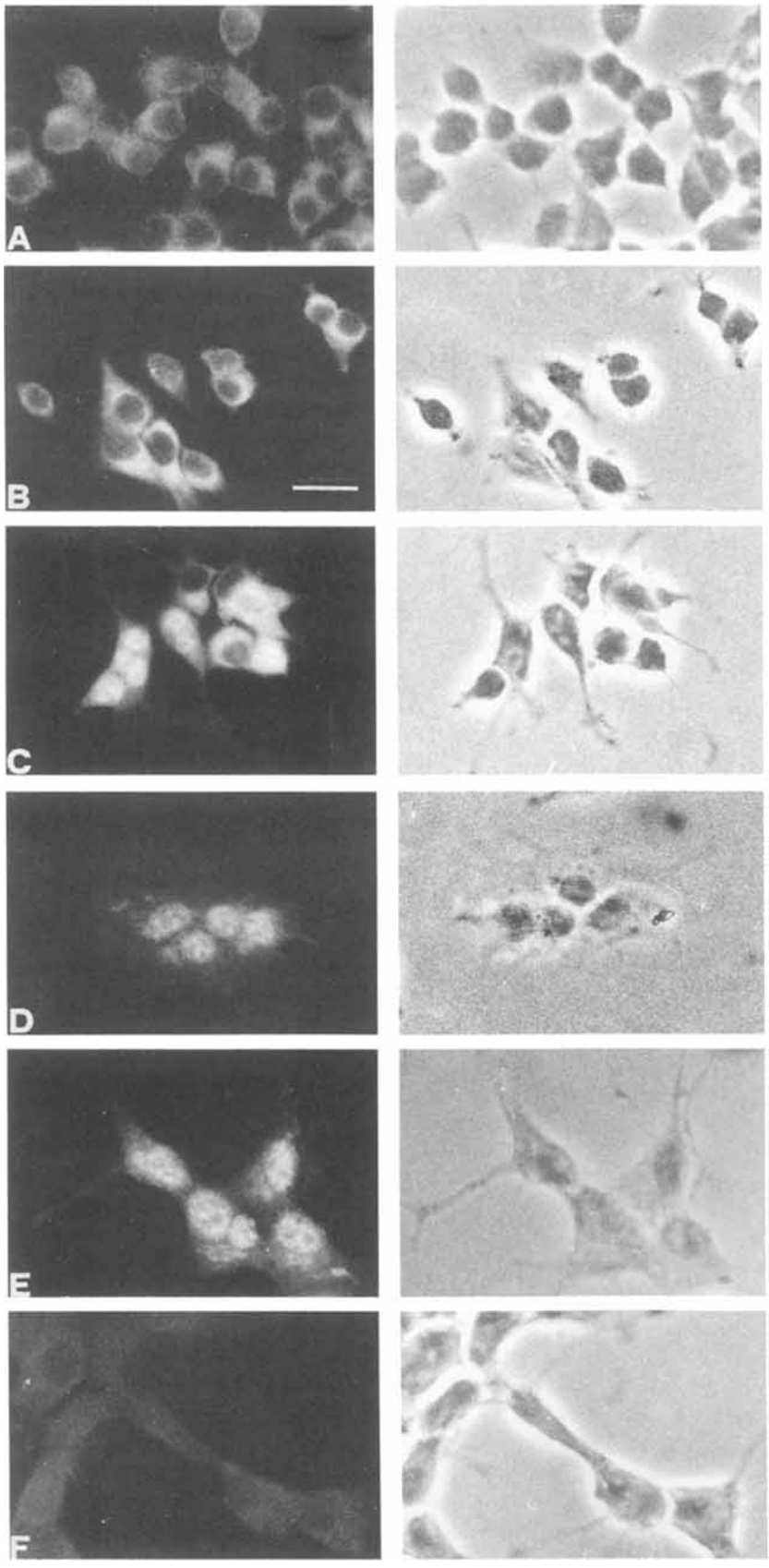


Fig. 3. Localization of PC4 by immunofluorescence in control and differentiated cultures of PC12 cells. Exponential-phase cultures, either untreated **(A)** or treated with NGF for 4 hr **(B)**, 36 hr **(C)**, 3 days **(D)** or 7 days **(E)** were prepared for immunofluorescence **(left panels)** as described in Materials and Methods; **right panels** show the corresponding phase contrast fields. The antigen was revealed by FITC staining after incubation with A451 purified serum. As a control, the A451 purified serum was preabsorbed with β gal-PC4 fusion protein (15 μg/ml) and used to stain cultures treated 7 days with NGF **(F)** or untreated (not shown). The bar indicates 40 μm (A–D, F) or 30 μm (E).

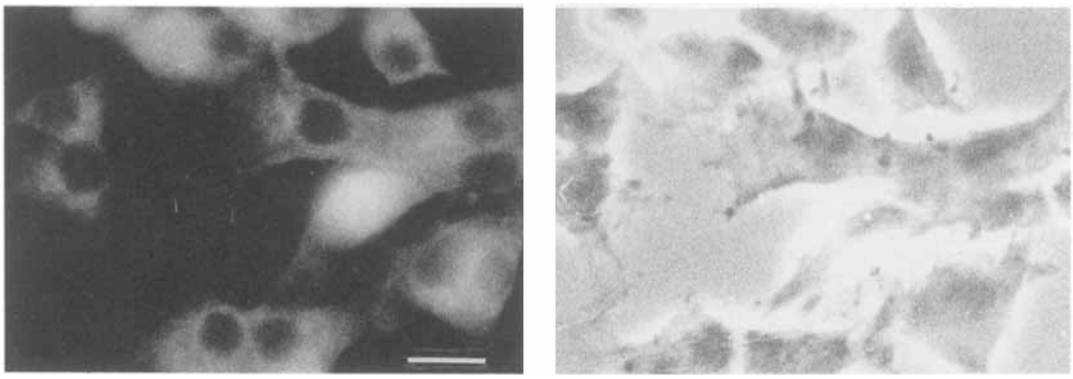


Fig. 4. Localization by immunofluorescence of PC4 in differentiated and NGF-deprived cultures of PC12 cells. Cultures treated for 3 days with NGF were washed and refed medium without NGF for 5 days, in the presence of a polyclonal anti-NGF antiserum. Detection of the antigen and competitive preabsorption (not shown) was performed as in Figure 3. The bar indicates 30 μm.

NGF-Dependent Expression of the PC4 Protein in the Plasma Membrane

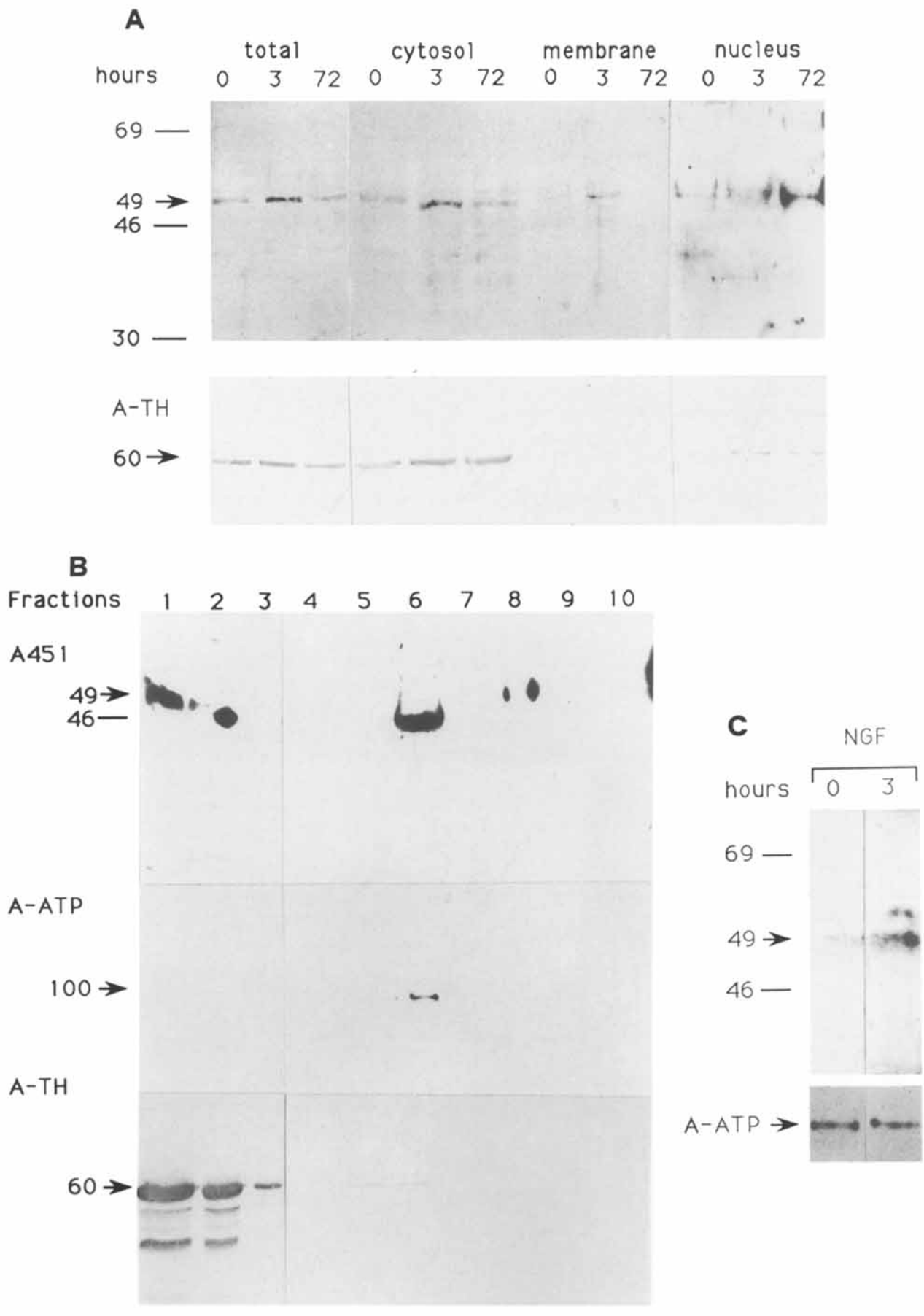
Our data indicated that there were sub-cellular changes in the PC4 protein levels in the presence of NGF, with differing patterns of increase: slow and permanent in the nucleus, rapid and transient in the cytosol and in the membrane fraction.

Therefore, we initially sought to verify this latter point and to define the membrane component to which PC4 might have been associated. By subfractionation on a continuous sucrose gradient of the cytoplasmic fraction (see Materials and Methods) from PC12 cells treated for 3 hr with NGF, we found that the PC4 immunoreactivity was associated with the fractions at the top of the gradient (fractions 1–2, Fig. 5B), containing the cytosolic marker tyrosine hydroxylase (fractions 1–3, Fig. 5B), and with fraction 6 containing the plasma membrane marker Na,K-ATPase (Fig. 5B). Fraction 7, and to a lesser extent fraction 6, contained membranes from the endoplasmic reticulum (respectively about 90% and 10% of the total amount, data not shown) as judged by the presence of the signal sequence receptor protein (SSR), considered as a marker for these membranes (see Migliaccio et al., 1992). Immunoblot analysis of fraction 6 from lysates of control cells and cells treated for 3 hr with NGF showed that the PC4 levels were induced by NGF (Fig. 5C), as previously observed in the membrane-enriched fraction (Fig. 5A).

Thus, considering that fraction 6 contained only trace amounts of cytosol and that the membranes from the endoplasmic reticulum were localized mainly in fraction 7, subfractionation by continuous gradient indicated that PC4 was associated with a membranous component of fraction 6, plausibly the plasma membrane; an association of PC4 with the endoplasmic reticulum membrane appeared unlikely. However we could not exclude the association of PC4 with some other unidentified membranous organelle having the density of fraction 6 (1.12–1.13 g/cm^3), e.g. the Golgi apparatus (see Harder and Bonisch, 1984; Evans, 1987).

Therefore, to unambiguously identify the membranous cellular component to which PC4 was associated, further investigation was carried out by transmission electron microscopy.

The ultrastructural appearance of untreated PC12 cells was similar to that previously reported (Luckenbill-Edds et al., 1979; Roda et al., 1980), with round cells with smooth plasma membranes and a cytoplasm with homogeneously distributed chromaffin granules. Immunolabeling of untreated cells with the anti-PC4 A451 antibody and protein A-colloidal gold was scattered all over the cytoplasm, without any clear localization on the plasma membrane or on intracellular organelles. Golgi apparatus (Fig. 7B), secretory chromaffin granules and endoplasmic reticulum (Fig. 7A,B) were unlabeled. Quantitative analysis in control cells showed weak immunolabeling of the cytoplasm, which was significantly greater than that of control cells in experiments with pre-immune serum ($P < 0.05$, Table II). When cells were treated with NGF for 3 hr before fixation, morphological observation indicated an increase in the number of microvilli at the cell surface (not shown). The density of the immunolabeling was clearly increased in the cytoplasm (Fig. 7C,D; $P < 0.001$, Table II). We were most interested to see that the pattern of distribution had changed, the gold particles being clearly associated with the inner side of the plasma membrane, either along its smooth regions or on the microvilli (Fig. 7C; $P < 0.001$, Table II); no labeling was detected in the endoplasmic reticulum or in Golgi cisternae or in chromaffin granules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A-ATP

Fig. 5.

TABLE I. Quantitative Analysis of the Immunolabeling of the PC4 Protein in Nuclei of Untreated and NGF-Treated PC12 Cells

	Density of labeling ± S.E.M. (gold/μm^2)
Control (pre-immune)	5.90 ± 0.448 (n = 10)
Untreated	6.43 ± 0.553 (n = 13)
7 days NGF-treated cells	21.80 ± 1.258 (n = 15)*

n = nuclei counted.
*$P < 0.001$ vs. the corresponding value in untreated cells (Student's t-test).

TABLE II. Quantitative Analysis of the Immunolabeling of the PC4 Protein in Untreated and NGF-Treated PC12 Cells

	Density of labeling ± S.E.M. (gold/μm^2)	
	Plasma membrane	Cytoplasm
Control (pre-immune)	0.150 ± 0.03	0.766 ± 0.183
Untreated	0.369 ± 0.51	3.907 ± 0.449
3 hr NGF-treated cells	2.035 ± 0.221*·***	12.244 ± 1.217*
72 hr NGF-treated cells	0.526 ± 0.48	3.912 ± 0.057**

Plasma membranes and cytoplasmic areas analyzed were taken randomly from photographed cells from three different experiments. Control cells were incubated with pre-immune serum.
*$P < 0.001$ vs. the corresponding value in untreated cells (Student's t-test).
**$P > 0.05$ vs. the corresponding value in untreated cells (Student's t-test).
***Significant difference between the ratio [3 hr NGF-treated cells/untreated cells] observed in the plasma membranes (= 5.5) and the corresponding ratio in the cytoplasm (= 3.13): $P < 0.05$, computated with ANOVA in conjunction with Tukey's test (df = 3, 55). Each cytoplasm value had been previously normalized by division for the coefficient [cytoplasm untreated/plasma membrane untreated] = 3.907/0.369 = 10.58.

(Fig. 7C,D). Furthermore, after 3 hr of treatment, the increase of PC4 along the plasma membrane was significantly greater than the increase in the cytoplasm ($P < 0.05$, Table II). After 72 hr of treatment with NGF, the PC12 cells had acquired the typical neuronal features of differentiated cells (Luckenbill-Edds et al., 1979), i.e., the appearance of neurites with dense packages of chromaffin granules. At this time, the density of immunolabeling in the cytoplasm had markedly declined from that observed after 3 hr, returning to the level of control cells ($P > 0.05$, Table II). Similarly, the density of the plasma membrane immunolabeling after 72 hr of NGF treatment was lower than that of cells treated with NGF for 3 hr ($P < 0.001$, Table II) although still higher than control cells ($P < 0.05$, Table II). At this same time, and therefore throughout the entire time course, the Golgi complex, the endoplasmic reticulum and the chromaffin granules remained virtually unlabeled (Fig. 7D,F and data not shown).

Fig. 5. Expression of the PC4 protein in PC12 cell subfractions. **A:** Immunoblot analysis with A451 and with anti-tyrosine hydroxylase antibody (A-TH) in cell fractions from cultures treated with NGF for the time indicated; total lysate (total), enriched-membrane fraction (membrane), cytosol, nucleus. The quantities of proteins analyzed in each fraction were obtained from equal amounts of starting material (the lysate from 10^7 cells); 15–20% of each fraction was loaded. The protein contents of samples from different treatments within each fraction were equalized. **B:** Immunoblot analysis with A451 antiserum, with anti-Na,K-ATPase antibody (A-ATP), or with anti-tyrosine hydroxylase antibody (A-TH), of protein fractions separated on a continuous sucrose gradient from cultures treated for 3 hr with NGF, as described in Materials and Methods; 30% of each fraction from the same experiment was analyzed in the blots. **C:** Immunoblot analysis with A451 antiserum of equal amounts of proteins from cultures untreated and treated for 3 hr with NGF, separated on continuous sucrose gradients (sixth fraction, as in B; the identity of the fractions and the presence in each one of equal amounts of proteins was checked by Na,K-ATPase immunoreactivity (A-ATP). Signal detection was obtained by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (the latter for A-ATP and A-TH), followed either by colorimetric development or by chemiluminescent assay (the latter for nuclear fraction in A, and A451 in B, C). The protein molecular weights are indicated.

DISCUSSION

The PC4 protein can be detected in PC12 cells by the A451 antiserum as a 49 kDa species, whose synthesis is greatly but transiently induced by NGF (as shown by immunoprecipitation of in vivo labeled cells, Fig. 2), in parallel with the temporal pattern of induction of the PC4 mRNA levels (Tirone and Shooter, 1989). The common size of the in vitro synthesized PC4 protein and of the in vivo PC4 suggests the absence of post-translational modifications involving the addition of large groups (e.g., glycosylation).

As shown by the electron microscopy, immunofluorescence and cellular fractionation findings (Figs. 3, 5, 7), PC4 protein is present at a basal level in the cytoplasm of undifferentiated cells, and its expression is increased by treatment with NGF. The NGF effect can be best appreciated by electron microscopy (Table II), presumably because this technique is less affected by the dilution of the newly synthesized protein in the existing cytoplasmic pool.

A similar NGF-mediated induction of the PC4 protein was also detected by subcellular fractionation in a membrane compartment, identified by electron micros-

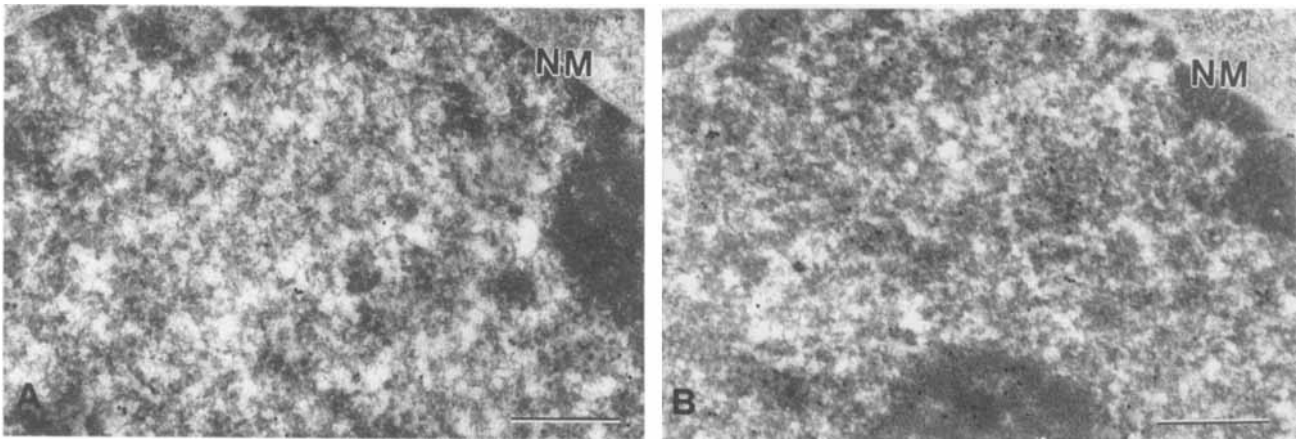

Fig. 6. Immunoelectron microscopic localization of the PC4 protein in the nucleus of PC12 cells. Sections from cell samples representative of those analyzed in Table I are shown: untreated PC12 cells (**A**) and PC12 cells treated for 7 days with NGF (**B**). Gold immunolabeling is increased in density over the nuclei of NGF-treated cells. NM, nuclear membrane. The bars indicate 0.5 μm.

copy as the plasma membrane. However, unlike in the cytosol, electron microscopy and immunoblots showed that the presence of PC4 in the plasma membrane was evident only after a few hours of treatment with NGF, as it was low or undetectable after prolonged exposure to NGF and in control cells. In fact, cells with NGF-differentiated morphology have scarcely detectable PC4 immunoreactivity in the neuritic protrusion of the plasma membrane. The presence of PC4 in purified fractions of membranes strongly suggests a physical association of the protein with the lipid bilayer. If this is the case, the observation that PC4 is a soluble protein raises the question of the molecular mechanism of this NGF-induced association. A search for active sites in the PC4 cDNA–deduced protein with the program Prosite (Bairoch, 1990) indicated a consensus sequence (aa. 13 to 26, see Tirone and Shooter, 1989) for the addition of myristic acid. The existence of such a motif for myristoylation, which would lead to membrane localization of the protein (for review see Grand, 1989), offers an attractive hypothesis. Recent evidence has indicated that myristoylated proteins bind to specific receptors at the inner leaf of the plasma membrane, rather than associating by mere insertion of the fatty acid moiety into the lipid bilayer (Resh, 1989; Resh and Ling, 1990). The association of myristoylated proteins with the plasma membrane has been shown to be regulated by phosphorylation, as demonstrated by the "myristoylated alanine-rich C kinase substrate" protein (Thelen et al., 1991). A similar mechanism might account for the NGF-dependent targeting of PC4 to the cytoplasmic side of the plasma membrane, as indicated by immunogold labeling. Intracellular membranes completely fail to show PC4 immunoreactivity (Figs. 7 and 5B). We do not yet know whether the subsequent disappearance of PC4 from the plasma membrane depends on NGF-induced events (e.g., neo-synthesis of proteins and/or post-translational modifications). For instance, detachment of PC4 might follow an interaction with membrane-associated proteins involved in the NGF pathways, such as the NGF receptor trk (Kaplan et al., 1991a,b; Klein et al., 1991), $P21^{ras}$ (Barbacid, 1987) or $p60^{src}$ (Resh, 1989).

Furthermore, we could not detect any secretion of PC4 protein, as its partial sequence homology with the interferon-γ (Tirone and Shooter, 1989) would have suggested. In fact, PC4 was found neither in vesicular structures nor in the Golgi apparatus nor in the culture medium of PC12 cells treated with secretagogues (data not shown). However we cannot rule out the possibility that a truncated portion of the molecule, not recognized by the A-451 antibody, is secreted.

Fig. 7. Immunoelectronmicroscopic localization of the PC4 protein in control PC12 cells or PC12 cells treated with NGF. **A,B:** In untreated cells, the gold immunolabeling is scattered all over the cytoplasm. Plasma membranes (A), chromaffin granules (A,B) and Golgi apparatus (B) are unlabeled. **C,D:** In cells treated with NGF for 3 hr, the density of the labeling has increased over the cytoplasm and numerous gold particles appear to be associated with the inner sides of the plasma membranes (C, arrows). Chromaffin granules (C,D) and Golgi apparatus (D) are unlabeled. **E,F:** In cells treated with NGF for 72 hr, the density of the labeling in the cytoplasm and on the plasma membranes has decreased. The densely packaged chromaffin granules (E,F) and Golgi apparatus (F) are unlabeled. M, mitochondria; er, endoplasmic reticulum; PM, plasma membranes; cg, chromaffin granules; G, Golgi apparatus; ly, lysosome. The bars indicate 0.5 μm.

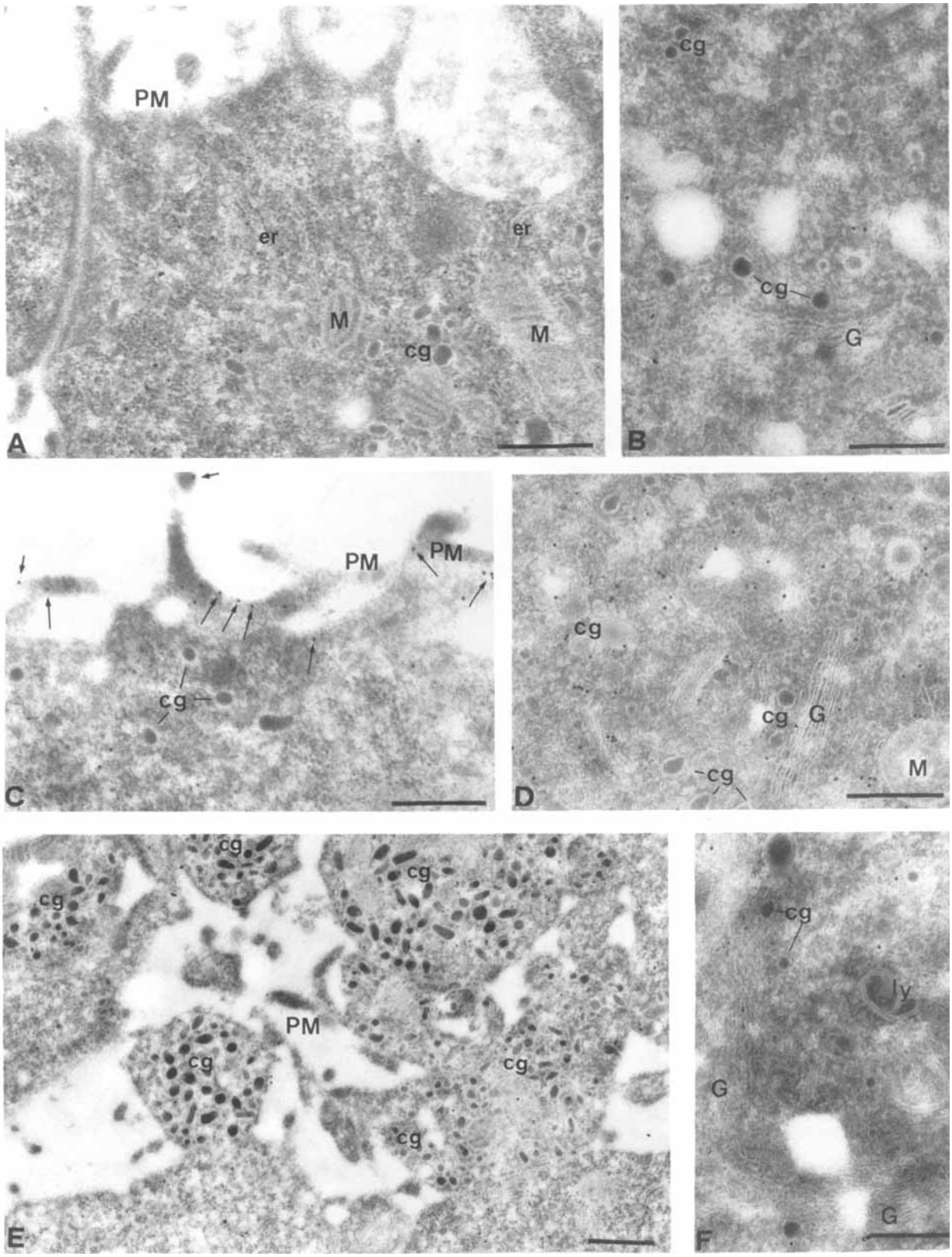
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Fig. 7.

In addition to a rapid redistribution into the plasma membrane, after a prolonged treatment with NGF the PC4 protein can also be localized in the nucleus, as shown by immunofluorescence and immunoblot analysis of nuclear fractions. This is confirmed by quantitative analysis with electron microscopy, indicating a significant increase of the immunogold labeling (Table I), and by immunofluorescence staining with a different antisera—anti-P1—against the carboxy-terminal of PC4. The latter result would also indicate that the PC4 protein localizes in the nucleus without processing at the carboxy-terminal region. Nuclear PC4 may derive either from newly synthesized PC4 protein or from a pre-existing pool. The latter possibility is in agreement with the reduction of PC4 protein levels in the cytoplasm and plasma membrane (Table II) and also with the decrease of PC4 protein synthesis (Fig. 2), which are observed at the time of nuclear localization. Translocation into the nucleus is known to be determined by active nuclear localization sequences (NLS) within the protein. By binding to these specific sequences, cytoplasmic carriers—together with other proteins of the nuclear envelope—direct the movement of NLS-containing proteins through the nuclear pores (reviewed in Schmitz et al., 1991; Gerace, 1992; Garcia-Bustos et al., 1991). There are some other examples of translocation to the nucleus regulated, for instance, by developmental cues (as in the case of the dorsal protein; Steward, 1989) or by hormones (as shown for the glucocorticoid receptor by glucocorticoids and for the MAP protein kinase by NGF; Picard and Yamamoto, 1987; Traverse et al., 1992). In some cases the regulation occurs via the unmasking of the NLS region from other proteins, which can be brought about by phosphorylation of amino acid residues near the NLS region. At present, we can only speculate about the mechanism underlying the regulated translocation of PC4 to the nucleus; it is certainly worth noting that its cDNA-deduced protein sequence (aa. 2–6; 130–136; 323–334; 429–442) has several putative NLS (Gomez-Marquez and Segade, 1988; Garcia-Bustos et al., 1991), one potential phosphorylation site for tyrosine kinase (aa. 355) and many for protein kinase C (as indicated by the program Prosite, Bairoch, 1990).

Whatever the nature of the mechanism involved, nuclear localization of PC4 follows a temporal pattern matching that of the NGF-induced arrest of cell proliferation and differentiation (Rudkin et al., 1989), and, in fact, it can be clearly detected only in cells with differentiated morphology. Accordingly, the nuclear localization is gradually reversed during the loss of the differentiated state that follows removal of NGF from the medium, and is almost undetectable in fully de-differentiated PC12 cells (Fig. 4 and data not shown). Thus, the nuclear translocation of PC4 is associated not only with the initial phase of morphological differentiation but also with the permanence of the neuronal phenotype.

Certainly, to give a clear functional meaning to these observations, it will be helpful to investigate the effects on PC4 intracellular localization in PC12 cells of other factors, affecting proliferation and differentiation separately. Interestingly, in the mouse skeletal myoblast cell line C2C12 where PC4 expression is also regulated during differentiation, the protein remains localized in the cytoplasm (A.M., M.T.C., F.T., in preparation). This suggests that nuclear translocation is a cell type-specific or hormone-specific process. That NGF does not affect myoblast differentiation it is in line with the latter possibility.

What might be the function of a molecule involved in such a complex pattern of sub-cellular expression? The NGF-dependent induction of PC4 levels in the plasma membrane, followed by translocation to the nucleus, suggests a role in the transduction of signals across the plasma membrane to the nucleus. The absence in the PC4 sequence of any known DNA binding motif stands against transcriptional regulation activity. However, by altering the expression of PC4 in C2C12 myoblasts, we have found that the induction of myogenin, a key factor in myogenesis (Edmondson and Olson, 1989), is affected (A.M., M.T.C., F.T., in preparation). Therefore, we cannot rule out an effect of PC4 on gene transcription in PC12 cells. Presently, on the basis of the control exerted by NGF on the sub-cellular expression and localization of an IEG product, shown here, we suggest the existence of an additional level of regulation in the NGF-dependent pathways, in which PC4 might play a part.

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