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Internalization of Activated Platelet-Derived Growth Factor Receptor–Phosphatidylinositol-3′ Kinase Complexes: Potential Interactions with the Microtubule Cytoskeleton

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Phosphatidylinositol (PI)-3′ kinase catalyzes the formation of PI 3,4-diphosphate and PI 3,4,5-triphosphate in response to stimulation of cells by platelet-derived growth factor (PDGF). Here we report that tyrosine-phosphorylated PDGF receptors, the p85 subunit of PI-3′ kinase (p85), and activated PI-3′ kinase are found in isolated clathrin-coated vesicles within 2 min of exposure of cells to PDGF, indicating that both receptor and activated PI-3′ kinase enter the endocytic pathway. Immunofluorescence analysis of p85 in serum-starved cells revealed a punctate/reticular staining pattern, concentrated in the perinuclear region and displaying high focal concentration at the centrosome. In addition, partial coalignment of p85 with microtubules was observed after optical sectioning microscopy and image reconstruction. The association of p85 with the microtubule network was further evidenced by the microtubule-depolymerizing drug nocodazole, which caused a redistribution of p85 from the perinuclear region to the cell periphery. Interestingly, the most significant effect of PDGF on the distribution of p85 was an increase in the staining intensity of this protein in the perinuclear region, and this effect was eliminated by prior treatment of cells with nocodazole. These results suggest that PDGF receptor-p85 complexes internalize and transit in association with the microtubule cytoskeleton. In addition, the high concentration of p85 in intracellular structures in the absence of PDGF stimulation suggests additional roles for this protein independent of its association with receptor tyrosine kinases.

A unique phosphatidylinositol (PI) kinase has been found to exist in immunoprecipitates of receptor and nonreceptor tyrosine kinases. This PI kinase is distinct in that it phosphorylates PI, PI 4-phosphate (PIns(4)P), and PI 4,5-diphosphate (PIns(4,5)P2) on the D-3 position of the inositol ring (PI-3′ kinase). The products of this phosphorylation reaction, PI 3-phosphate (PIns(3)P), PI 3,4-diphosphate (PIns(3,4)P2), and PI 3,4,5-triphosphate (PIns(3,4,5)P3), are found in intact cells, but the last two appear only after stimulation of cells by polypeptide growth factors such as platelet-derived growth factor (PDGF) (1). The PI-3′ kinase is composed of a 110-kDa catalytic subunit and a noncatalytic 85-kDa subunit which contains two SH2 domains (11, 23, 31). These domains can bind tightly to specific autophosphorylated tyrosines in receptor and nonreceptor tyrosine kinases. As a consequence of these interactions, the lipid kinase comes into close apposition with the plasma membrane, where it can catalyze the phosphorylation of specific phospholipid substrates. The recruitment of SH2 domain-containing proteins to the plasma membrane in response to receptor autophosphorylation and the local formation of specific products are thought to be a paradigm for the general mechanism of growth factor receptor signal transduction (4, 24). The association of PI-3′ kinase with both receptor and nonreceptor tyrosine kinases involved in growth and transformation has suggested that the products of the enzyme could be important intermediates in the cellular signalling pathways that lead to cellular proliferation. However, the biological role of these phospholipids remains unknown.

An important event that is triggered rapidly upon activation of receptors for PDGF and other polypeptide growth factors is the clustering and internalization of the ligand-receptor complex. For PDGF, this process appears to occur through clathrin-coated pits and vesicles and results eventually in the proteolytic degradation of receptor and ligand (22, 27). Whether clustering and internalization lead exclusively to receptor inactivation or are important in triggering or amplifying cellular responses to the growth factor is not known and constitutes an important question. In addition to clustering and internalization, PDGF elicits a complex reorganization of the actin cytoskeleton and a strong chemotactic response (10, 36). The mechanistic relationships between the initial recruitment of cytoplasmic proteins to the plasma membrane, triggered by PDGF receptor autophosphorylation, and the rapid cellular responses of receptor endocytosis and cytoskeletal reorganization are unknown. A potential role for PI-3′ kinase in the regulation of protein trafficking has been suggested by the finding that the catalytic subunit of the enzyme is highly homologous to a gene product responsible for vacuolar sorting in yeast cells (15). This gene product (Vps34p) acts in conjunction with a protein kinase (Vps15p) to deliver soluble hydrolases from the trans-Golgi network (TGN) to the yeast vacuole (13, 14). In this system, soluble hydrolases may act analogously to polypeptide growth factors in triggering the activity of specific receptor-enzyme complexes to mediate efficient targeting of the complex into a specific subcellular compartment (29, 34).

The potential involvement of receptor-binding proteins in

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the endocytosis and intracellular sorting of PDGF receptors has been addressed in this study. We reasoned that the involvement of known receptor-binding proteins in receptor endocytosis or sorting would be manifested by the presence of activated receptor-protein complexes in vesicle intermediates along the endocytic pathway. Because the first intermediate structure in the pathway of receptor endocytosis is the clathrin-coated pit or vesicle, we have looked for the presence and activity of receptors and signalling proteins within clathrin-coated structures. The results presented here indicate that activated PDGF receptors and the p85 subunit of PI-3′ kinase coconcentrate in clathrin-coated vesicles isolated from PDGF-stimulated 3T3-L1 fibroblasts. Moreover, the catalytic activity of PI-3′ kinase was found to be greater in clathrin-coated structures obtained from PDGF-treated cells than in those obtained from nonstimulated cells. We also report that the analysis of the subcellular distribution of PI-3′ kinase by digital imaging immunofluorescence microscopy of the p85 subunit reveals similarities between the distribution of this enzyme and that of various proteins involved in microtubule-based motility. Taken together, our results are consistent with the hypothesis that PI-3′ kinase may play a general role in cellular trafficking processes, a subgroup of which may be the targeting of activated receptor tyrosine kinases to specific intracellular loci.

MATERIALS AND METHODS

Cells. 3T3-L1 cells (American Type Culture Collection) were grown in 150-mm-diameter dishes for experiments involving fractionation or on glass coverslips for immunofluorescence microscopy. Fibroblasts were seeded and fed every 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.75 mg of glutamine per ml, nonessential amino acids (GIBCO), 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 10% fetal calf serum (UBI) and grown under 10% CO_2. At 90% confluence, the medium was removed and cells were serum starved by incubation in DMEM for 18 h. Cells were treated with recombinant AB-PDGF (UBI) at a concentration of 20 ng/ml. In most experiments, PDGF was present for 10 min at 37°C. For the experiment shown in Fig. 1, cells were incubated with PDGF for 60 min at 5°C, after which the medium containing the growth factor was removed and replaced with DMEM at 37°C.

Preparation of clathrin-coated vesicles and clathrin cages. Coated vesicles were isolated by immunoadsorption or by differential centrifugation. For immunoadsorption, cells (one 60-mm-diameter dish) were scraped into 1 ml of an ice-cold buffer (100 mM potassium tartrate, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 1 mM EGTA, 0.5 mM $MgCl_2$ [pH 7.0]) containing 0.1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 10 μg of leupeptin per ml, and 1 mM benzamidine). The lysate was centrifuged for 5 min at 500 × *g*, and 25% of the supernatant was diluted 1:5 with lysis buffer (LB; 20 mM Tris [pH 8.0], 1% Triton X-100, 0.150 M NaCl, 1 mM sodium orthovanadate, protease inhibitors) supplemented with 0.1% sodium dodecyl sulfate (SDS) and used directly for immunoadsorption of the PDGF receptor (see below). The rest of the supernatant was used for immunoisolation of clathrin-coated vesicles. A polyclonal antibody raised against the carboxy-terminal 15 amino acids of the clathrin heavy chain was adsorbed onto 20 μl of protein A-Sepharose (Pharmacia), and the beads were incubated for 1 h at 4°C with the cell lysate. The beads were allowed to settle by gravity and washed once with buffer. The adsorbed vesicles were dissolved in 100 μl of LB containing 1% SDS. After 5 min at room temperature, the SDS concentration was decreased to 0.1% by addition of 900 μl of LB without SDS. The PDGF receptor was then immunoprecipitated with a 2-μl/ml concentration of a polyclonal antiserum against the PDGF receptor cytoplasmic domain (UBI).

For differential centrifugation, three 150-mm-diameter plates of 3T3-L1 fibroblasts were used per condition (3, 9). All procedures were performed at 0 to 4°C in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (100 mM MES, 1 mM EGTA, 0.5 mM magnesium chloride, 0.02% sodium azide, 50 mM sodium fluoride, 500 μM sodium vanadate, 1 mM 1,10-phenanthroline, 10 μg of leupeptin per ml, 1 mM benzamidine, adjusted to pH 6.5). Cell monolayers were scraped into 1 ml of the buffer and broken by the addition of 0.05% Triton X-100 and vortexing. The homogenates were placed in Beckman 1.5-ml microcentrifuge polyallomer tubes and centrifuged for 12 min at 15,600 × *g* in a Beckman TLA 100.3 rotor. The supernatant was removed and centrifuged for 21 min at 36,350 × *g*, yielding a supernatant (cytosol) and a pellet. The pellet was resuspended in 200 μl of buffer, and an equal volume of a solution composed of 12.5% (wt/vol) sucrose and 12.5% (wt/vol) Ficoll (Pharmacia) was added. After vortexing, the suspension was centrifuged for 12 min at 36,350 × *g*. The supernatant was diluted with 5 volumes of ice-cold buffer and centrifuged for 21 min at 72,000 × *g*. The pellet from this centrifugation contained 70 to 80% pure clathrin-coated vesicles, as assessed by negative staining with uranyl acetate. To prepare clathrin cages, coated vesicles were resuspended in 100 μl of buffer, and Triton X-100 was added from a 10% stock to obtain a final concentration of 1%. After 60 min of incubation on ice, the samples were centrifuged for 21 min at 72,000 × *g*. The pellets were resuspended in 250 μl of MES buffer, using a needle and taking care to prevent bubble formation, and centrifuged again. This procedure was repeated twice to remove traces of detergent.

Preparation of total soluble and particulate fractions. Cells (one 150-mm dish) were scraped into 1 ml of MES buffer containing 0.05% Triton X-100 and homogenized by vortexing for 1 min. The suspension was centrifuged for 10 min at 500 × *g* to obtain a postnuclear supernatant, which was centrifuged for 15 min at 150,000 × *g* in a Beckman TLA 100.3 rotor. The pellet was resuspended in 1 ml of MES buffer and used as a total particulate fraction. The supernatant was used as the total soluble fraction.

Protein measurements. An accurate measurement of total protein in coated vesicle, particulate, and soluble fractions was required to calculate the relative amount of p85 present in these fractions. Because the yield of coated vesicles was relatively low, conventional protein assay measurements gave us high variability. To circumvent this problem, we used trace amounts of [^{35}S]methionine (0.5 μCi/ml) to label the cells for 24 h prior to the experiment. The total amount of protein in each fraction could then be measured accurately by scintillation counting of small aliquots.

Agarose gel electrophoresis. Electrophoresis on 0.2% agarose has been shown to effectively separate clathrin-coated vesicles from smooth vesicles, ribosomes, filaments, ribonucleoprotein particles, and other potential particulate contaminants, yielding a virtually 100% pure coated vesicle preparation (19, 28). Approximately 50 μg of protein from the coated vesicle pellet was loaded on a 5- by 1-mm well of a horizontal gel composed of 0.2% agarose in 50 mM MES (pH

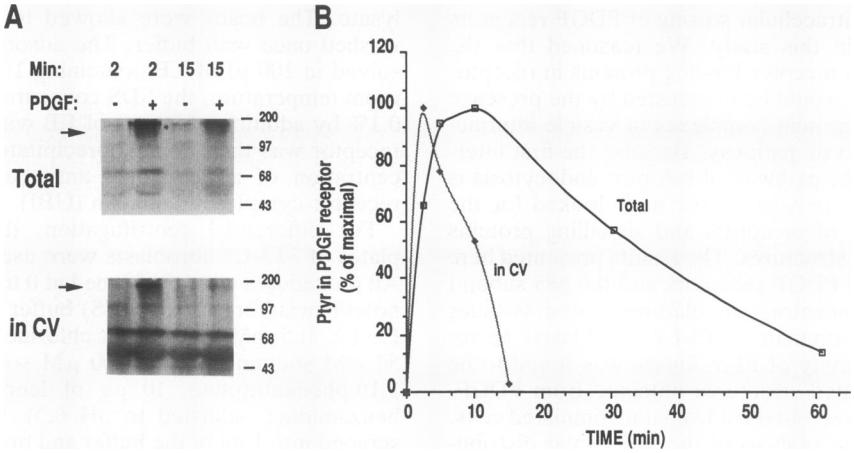


FIG. 1. Transient association of autophosphorylated PDGF receptors with clathrin-coated vesicles. Monolayers of confluent, serum-starved 3T3-L1 fibroblasts were incubated at 5°C without (−) or with (+) recombinant AB-PDGF (20 ng/ml) for 60 min. Cells were warmed by the addition of medium at 37°C. (A) At 2 or 15 min of incubation at 37°C, PDGF receptors were immunoprecipitated from 400 μl of a total lysate (Total) or from clathrin-coated vesicles immunoadsorbed from 1,200 μl of the same lysate (in CV). Receptor immunoprecipitates were analyzed by PAGE and immunoblotting with antiphosphotyrosine antibodies. The position of the receptor is indicated by the arrow, and the positions of prestained molecular mass markers are shown in kilodaltons on the right. (B) At the time points indicated, a total particulate fraction (Total) or clathrin-coated vesicles (in CV) were purified by differential centrifugation. Equal amounts of protein from each fraction were separated by electrophoresis on 7% polyacrylamide gels and analyzed for the presence of activated receptors by immunoblotting with antiphosphotyrosine antibodies. The intensity of the phosphotyrosine signal at the region of the blot corresponding to the PDGF receptor (180 kDa) was measured by densitometric scanning. The results were expressed as a percentage of the highest value obtained in the coated vesicle preparation or in the total membrane preparation. Plotted are the values of one experiment, which was repeated twice with similar results.

6.5) and electrophoresed at a constant voltage of 0.75 V/cm at 5°C in the same buffer. After 24 h, the agarose gel was carefully sliced into 20 pieces, which were lyophilized, boiled in 200 μl of SDS sample buffer, and analyzed by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Immunoblotting. Aliquots containing equal amounts of protein from each fraction were electrophoresed on 7.5% polyacrylamide gels and transferred onto nitrocellulose paper. The blots were probed with an anti-clathrin heavy-chain monoclonal antibody (Chc5.9; ICN), antiphosphotyrosine monoclonal antibody (UBI), or anti-p85 polyclonal antiserum (UBI). The primary antibodies were detected with polyclonal goat anti-mouse or anti-rabbit immunoglobulins coupled to horseradish peroxidase, using enhanced chemiluminescence (Amersham). The intensity of the bands was quantified with an LKB Ultroscan XL laser densitometer. To ensure that the signals obtained in all fractions compared were within the same linear range, four serial dilutions of the particulate, soluble, and coated vesicle fractions were run simultaneously in each gel, and several exposure times of the enhanced chemiluminescence immunoblots were analyzed.

PI-3 kinase assays. Coated vesicles or cages were washed once and resuspended in a buffer composed of 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM sodium orthovanadate (TNE). Sonicated PI (Avanti) and [γ-^{32}P]ATP (Amersham) were added to final concentrations of 0.2 mg/ml and 50 μM (5 Ci/mmol), respectively. After 10 min at room temperature, lipids were extracted, deacylated, and separated by high-pressure liquid chromatography (HPLC) as described previously (1).

Immunofluorescence. Cells were grown to confluence on glass coverslips, washed rapidly twice with 2 ml of phosphate-buffered saline (PBS) at room temperature, and fixed either by incubation for 10 min in 4% formaldehyde or by immersion in −20°C methanol for 6 min. The latter fixation

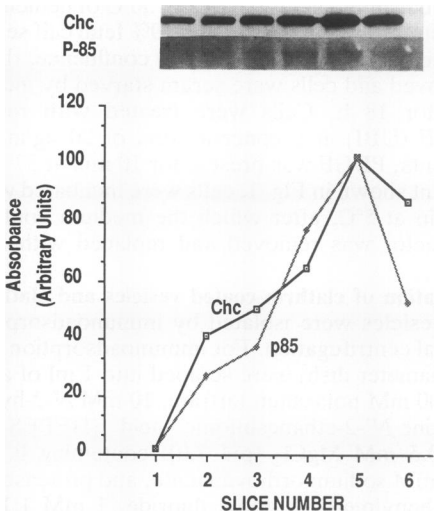


FIG. 2. Comigration of clathrin heavy chain and p85 on nonsieving agarose gels. Clathrin-coated vesicles were prepared from 3- by 150-mm dishes of confluent, serum-starved fibroblasts that were incubated for 10 min in the continual presence of AB-PDGF (20 ng/ml). Vesicles were electrophoresed for 24 h on a 0.2% agarose gel. The gel was sliced, and fractions were lyophilized, boiled in SDS sample buffer, and analyzed by immunoblotting with anti-clathrin heavy-chain (CHc) or anti-p85 (p85) antibodies. The intensity of the clathrin and p85 bands in each fraction was quantified by densitometric scanning. Plotted values represent the percentage of the maximal value for each protein, where the maximal value is 100% and the lowest is 0%. Gels show the signals obtained in the 170- to 190-kDa (Chc) and 80- to 90-kDa (P-85) regions of the blot.

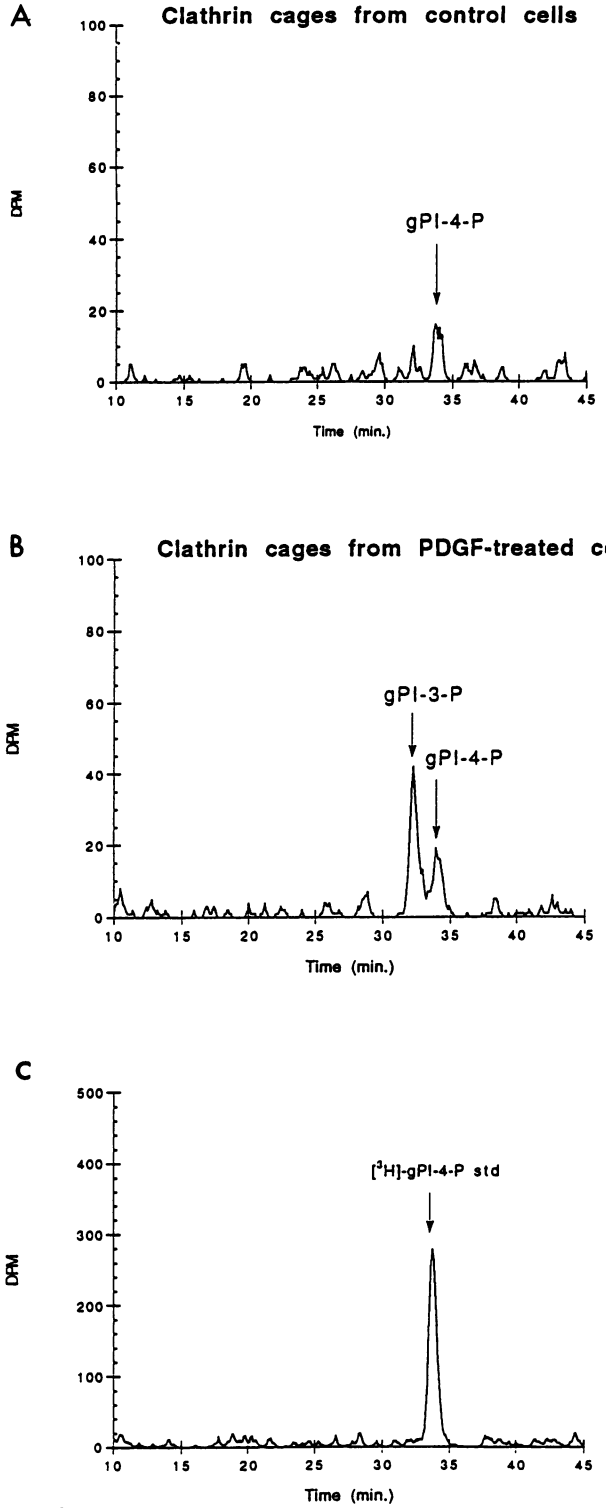
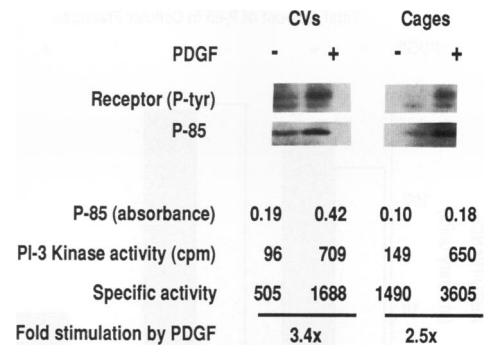


FIG. 3. PI-3 kinase activity in clathrin cages from PDGF-treated cells. Clathrin cages were obtained from three 150-mm dishes of nonstimulated (A) or PDGF-stimulated (B) serum-starved cells as described in Materials and Methods. Lipid kinase activity was measured after a 10-min incubation with exogenous PI and [γ-^{32}P]ATP. Lipid products were extracted, deacylated, and separated by HPLC. Values on the abscissa represent elution times in minutes. The migration of ^{3}H-labeled glycero- (g)PIns(4)P ([^{3}H]-gPI-4-P std) standards is indicated in panel C. The expected position of gPIns(3)P (gPI-3-P) is indicated by the left arrow in panel B.



	CVs		Cages	
P-85 (absorbance)	0.19	0.42	0.10	0.18
PI-3 Kinase activity (cpm)	96	709	149	650
Specific activity	505	1688	1490	3605
Fold stimulation by PDGF		3.4x		2.5x

FIG. 4. Increased concentration and specific activity of PI-3 kinase in clathrin-coated structures in response to PDGF. Clathrin-coated vesicles (CVs) or clathrin cages (Cages) were obtained from 3- by 150-mm dishes of nonstimulated (−) or PDGF-stimulated (+) serum-starved cells as described in Materials and Methods. Phosphorylated PDGF receptor [Receptor (P-tyr)] and p85 (P-85) were measured by immunoblotting with antiphosphotyrosine and anti-p85 antibodies, and the intensity of the p85 bands was quantified by densitometric scanning [P-85 (absorbance)]. Lipid kinase activity was measured after a 10-min incubation with exogenous PI and [γ-^{32}P]ATP. The radioactivity in PIns(3)P (PI-3 kinase activity) was measured by Cerenkov counting of the deacylated products separated by HPLC as described in Materials and Methods. Representative examples of the immunoblots are shown at the top. Values represent averages of three independent experiments.

procedure resulted, in our hands, in a significantly better preservation of the microtubular network. The methanol was removed, and coverslips were briefly air dried before immersion in PBS containing 1% fetal calf serum (FBS). After 30 min, coverslips were washed once with PBS–1% FBS and transferred into PBS–1% FBS containing primary antibodies at the following concentrations: monoclonal anti-α-tubulin (Sigma), 5 μg/ml; and monoclonal anti-clathrin heavy chain (Chc5.9; ICN), 5 μg/ml. Two independent rabbit polyclonal anti-p85 antibodies raised against glutathione *S*-transferase fusion proteins containing either the full-length 85-kDa subunit or the N-terminal SH2 domain of p85 were used at a final dilution of 1:600. Both polyclonal antibodies gave indistinguishable images. In addition, the intensity of the immunofluorescence signal was decreased by >90% when the anti-full-length p85 antiserum was preincubated with immobilized glutathione *S*-transferase fusion protein of the full-length p85. Primary antibodies were detected by using a rhodamine-coupled goat anti-mouse immunoglobulin M (IgM) to detect anti-clathrin Chc5.9, a fluorescein isothiocyanate (FITC)-coupled anti-mouse IgG for antitubulin, and either rhodamine- or FITC-coupled anti-rabbit Igs for anti-p85. All secondary antibodies were supplied by TAGO. Samples were visualized on a Zeiss IM-35 microscope, using a Nikon Apo 60/1.4 oil immersion lens and an 8× or 16× eyepiece. To generate more precise images of the localization of p85, we used digital imaging microscopy and a powerful deconvolution algorithm that reverses the blurring introduced by the microscope optics (5). For these experiments, 26 serial two-dimensional images were recorded at 0.2-μm intervals, using a thermoelectrically cooled charged-coupled device camera (Photometrics Ltd.). Each image was corrected for lamp intensity variations and photobleaching. Blurring of fluorescence from regions above and below the plane of focus was reversed by using an iterative constrained deconvolution algorithm based on the theory of ill-posed problems (5).

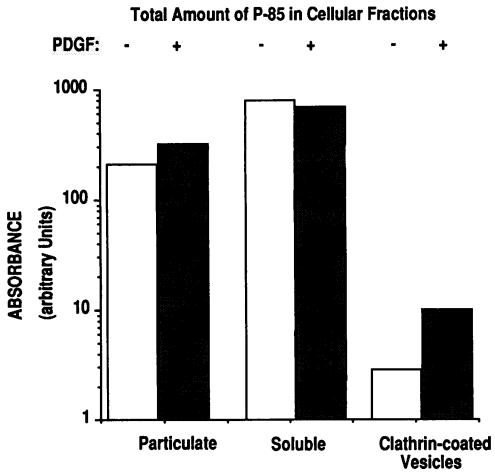


FIG. 5. Subcellular distribution of p85. Total particulate and soluble fractions and clathrin-coated vesicles were obtained from serum-starved cells which were not stimulated or were stimulated with PDGF (20 ng/ml) for 10 min, as indicated. The amount of p85 in each fraction was measured by immunoblotting, and the total yield of protein was used to calculate the relative amount of p85 present in each fraction. Values represent averages of three independent experiments.

RESULTS

Localization of activated PDGF receptors in clathrin-coated vesicles. Morphological studies using labeled PDGF have shown that its initial internalization occurs through coated pits and that the ligand is targeted to lysosomes for degradation (22, 27). Biochemical and morphological studies of the PDGF receptor have also indicated that the receptor is internalized and degraded. Interestingly, the kinase activity, and thus autophosphorylation of the receptor, appears to be important but not absolutely necessary for receptor degradation (33). Thus, the possibility exists that the receptor enters the endocytic pathway in a dephosphorylated form unbound to signalling proteins. To determine whether the PDGF receptor enters the endocytic pathway in an activated form, we examined the tyrosine phosphorylation state of the receptor in the first intermediate of the endocytic pathway, the clathrin-coated vesicle.

Serum-starved 3T3-L1 fibroblasts were incubated at 5°C with AB-PDGF for 60 min, after which the nonbound ligand was removed. Monolayers were warmed by the addition of medium at 37°C and at different times after warming processed to obtain either purified coated vesicles or a total particulate fraction. In Fig. 1A, coated vesicles were isolated by immunoadsorption to an anti-clathrin heavy-chain polyclonal antiserum, and the PDGF receptor was then immunoprecipitated from the total lysate or from the immunoisolated vesicles. The phosphotyrosine content of the receptors in each fraction was then analyzed by immunoblotting with antiphosphotyrosine antibody. Alternatively, clathrin-coated vesicles or a total particulate fraction were prepared by differential centrifugation and directly analyzed by immunoblotting with antiphosphotyrosine antibody. Densitometric scanning of the main phosphorylated band, which corresponded to the molecular weight of the PDGF receptor, is shown. By both procedures, tyrosine-phosphorylated receptors could be readily detected in coated vesicles, where they reached a maximal concentration in the first 2.5 min of rewarming and decreased rapidly thereafter, becoming almost undetectable after 15 min (Fig. 1). Assuming a complete recovery of endocytic coated vesicles by immunoadsorption, the results obtained suggest that 15 to 20% of activated receptors are associated with coated vesicles after 2 min of incubation at 37°C. These kinetics contrast with those observed in the total lysate or total particulate fraction, where maximal levels of phosphorylation were observed between 5 and 10 min after rewarming, and were more sustained, decreasing only by 30 to 50% in the first 15 min. The rapid, transient association of tyrosine-phosphorylated receptors with coated vesicles suggests that the receptor concentrates and internalizes through clathrin-coated pits and vesicles as an active autophosphorylated kinase and subsequently progresses through the endocytic pathway.

p85 internalizes with the activated receptor. To determine whether the PI-3′ kinase internalizes with or dissociates from the activated receptor prior to internalization, we assayed clathrin-coated vesicles for the presence of the p85 subunit of the kinase, using immunoblotting with a polyclonal antibody against this protein. Clathrin-coated vesicles from PDGF-stimulated cells were further purified by electrophoresis on nonsieving concentrations of agarose. This procedure removes noncoated vesicles, as well as filaments, ribosomes, and loosely bound proteins which contaminate the preparation to a small extent (19, 28). The p85 subunit completely comigrated with clathrin heavy chain in these gels (Fig. 2).

The presence of the p85 subunit of PI-3 kinase in clathrin-coated vesicles suggested that the enzyme and its lipid products might be concentrated in these structures. To test this hypothesis, we sought to assess both the lipid composition and the lipid kinase activities associated with isolated coated vesicles derived from nonstimulated or stimulated cells. We were unable to determine the concentration of 3′-phosphoinositides in purified coated vesicles from [γ-^{32}P]P_i-labeled cells, because 3′-phosphorylated lipids degraded almost immediately after homogenization of the cells under conditions suitable for coated vesicle purification. However, we were able to assess the lipid kinase activity present in isolated coated vesicles by incubating these structures with exogenous PI and [γ-^{32}P]ATP. In initial assays, endogenous lipids were removed from clathrin-coated vesicles by using 1% Triton X-100. The resulting clathrin cages were then incubated in the presence of exogenous PI and [γ-^{32}P]ATP. The lipid products of the reaction were extracted, deacylated, and analyzed by HPLC. Clathrin cages from control cells were found to phosphorylate PI, and HPLC analysis revealed that this phosphorylation was mainly catalyzed by a PI-4′ kinase activity (Fig. 3A). In contrast, the phosphorylation of PI by clathrin cages from PDGF-treated cells was catalyzed by both PI-4′ and PI-3′ kinase activities (Fig. 3B). The activity of PI-4′ kinase was not changed in response to PDGF stimulation. The appearance of PI-3′ kinase activity in clathrin cages from PDGF-stimulated cells suggests that a catalytically active PI-3′ kinase is internalized with the PDGF receptor through the clathrin-coated pit/vesicle pathway. The concentration of activated PDGF receptors and PI-3′ kinase in coated vesicles was specific in that it was not observed after incubation of serum-starved cells with ligands such as transferrin or epidermal growth factor (not shown).

The specific activity of PI-3′ kinase is stimulated in response to PDGF. Despite the low levels of PI-3 kinase activity present in clathrin-cages obtained from nonstimulated cells, immunoblotting analysis of non-Triton X-100-extracted clathrin-coated vesicles consistently revealed the presence

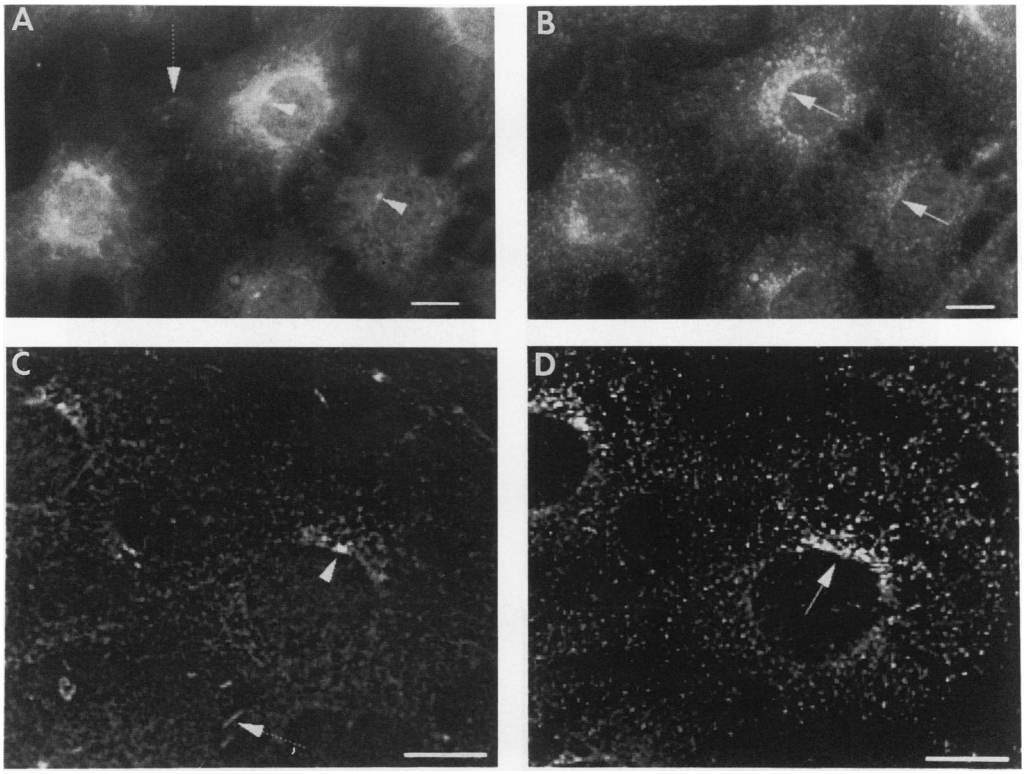


FIG. 6. Immunofluorescence analysis of clathrin heavy chain and p85 distribution reveals distinctly different staining patterns. Cells were fixed as described in Materials and Methods and stained with anti-clathrin heavy-chain monoclonal antibody Chc5.9 (B and D) and rabbit anti-p85 polyclonal antibody (A and C). Primary antibodies were detected by using anti-mouse and anti-rabbit polyclonal antibodies coupled to rhodamine and FITC, respectively. (A and B) Two-dimensional images before restoration; (C and D) optical sections from the middle of the cell after restoration. Indicated are sites of focal concentration of p85, at regions of cell-cell contact (broken arrows, A and C) or in the center of the TGN (arrowheads, A and C). The region of juxtanuclear concentration of clathrin which corresponds to the TGN is indicated by the arrows in panels B and D. Bars, 10 μm.

of a significant amount of p85. These results suggested that p85 in clathrin-coated vesicles obtained from nonstimulated cells might be more soluble in detergent than that present in coated vesicles from PDGF-treated cells. Alternatively, the specific activity of the kinase present in clathrin-coated structures might be increased in response to PDGF. To distinguish between these possibilities, we performed experiments to compare within the same preparation the levels of tyrosine-phosphorylated PDGF receptors, of p85 immunoreactivity, and of PI-3′ kinase activity in clathrin-coated vesicles and clathrin cages obtained from nonstimulated and PDGF-stimulated fibroblasts.

Immunoblotting with antiphosphotyrosine antibodies confirmed the previously observed appearance of tyrosine-phosphorylated receptor in clathrin-coated vesicles from PDGF-stimulated cells (Fig. 4, top lanes). Occasionally, a tyrosine-phosphorylated band which migrated slightly faster than the receptor on polyacrylamide gels was detected in coated vesicles from nonstimulated cells, but this was not observed reproducibly. The phosphorylated receptor associated with clathrin-coated vesicles was relatively resistant to solubilization by Triton X-100 and remained associated with clathrin cages. The low solubility of the receptors present in clathrin-coated structures suggests that the receptor participates in multiple protein-protein interactions, which potentially involve both signalling proteins and structural components of the clathrin coat.

p85 was present in clathrin-coated vesicle preparations from both nonstimulated and PDGF-stimulated cells and in both cases remained associated with clathrin cages after Triton X-100 solubilization (Fig. 4, P-85). This resistance to detergent was not exhibited by all components of the isolated coated vesicle preparation, as the activity of PI-4 kinase was decreased by 80% upon extraction with the detergent (not shown). The concentration of p85 in clathrin-coated vesicles and cages increased in response to PDGF by a factor of 2 to 3 (Fig. 4, P-85). The simultaneous measurement of p85 immunoreactivity by densitometric scanning of the immunoblots (Fig. 4, P-85) and of PI-3′ kinase activity by incubation with exogenous PI and [γ-^{32}P]ATP (Fig. 4, PI-3 Kinase activity) reveals that the specific activity of PI-3′ kinase associated with coated structures (Fig. 4, Specific activity) increased by a factor of 2.5- to 3.5-fold in response to PDGF (Fig. 4, Fold stimulation). These results suggest that at least two regulatory mechanisms can affect the function of PI-3′ kinase: changes in subcellular distribution and changes in catalytic activity.

p85 is distributed between soluble and particulate fractions in nonstimulated cells. The presence of PI-3′ kinase in clathrin-coated structures derived from both nonstimulated and PDGF-stimulated cells suggested the possibility that this enzyme or its lipid products are involved in the process of clathrin coat assembly, a process which occurs both at the TGN and at the plasma membrane and which is stimulated by growth factors (7, 8). To further explore the interaction between PI-3′ kinase and clathrin lattices, we sought to

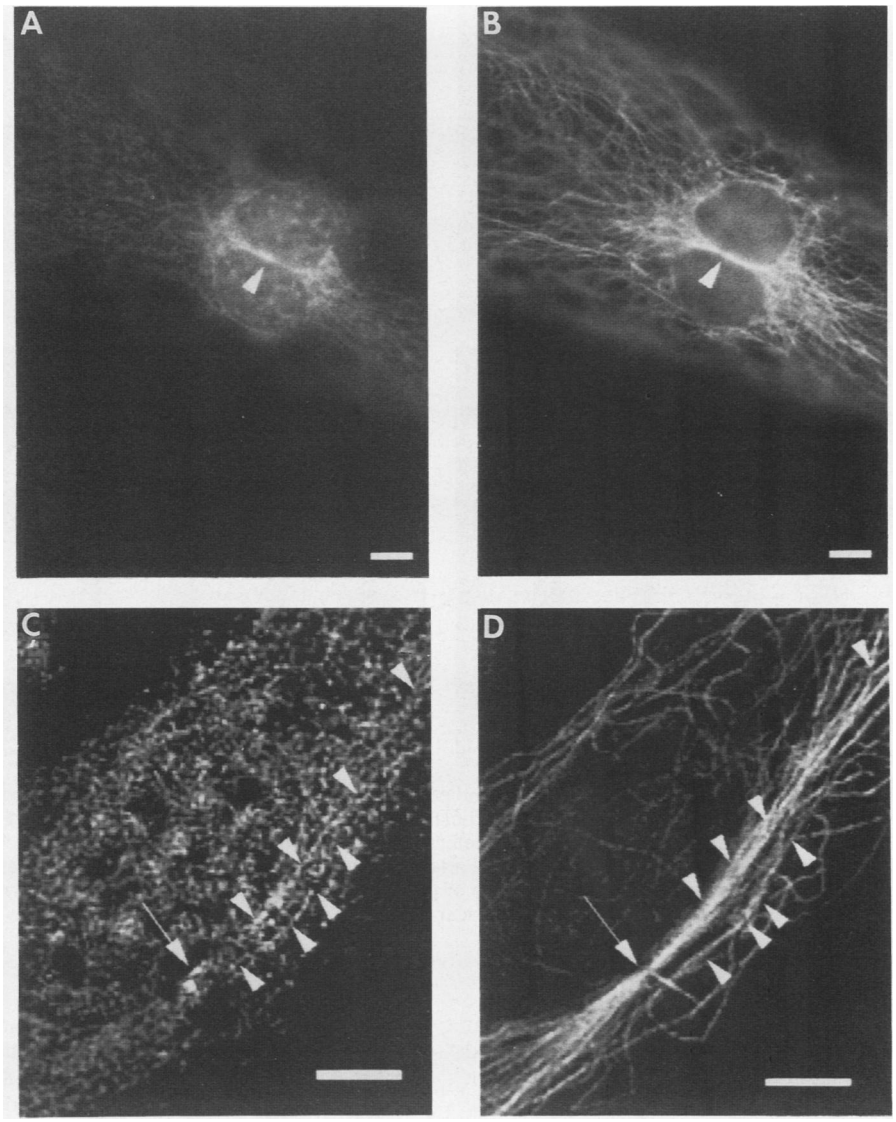


FIG. 7. Immunofluorescence analysis of tubulin and p85 reveals a high concentration of p85 at the MTOC and coalignment with microtubules. Cells were fixed in methanol as described in Materials and Methods and stained with antitubulin mouse monoclonal antibody (B and D) or anti-p85 rabbit polyclonal antibody (A and C). Primary antibodies were detected by using anti-mouse and anti-rabbit antibodies coupled to FITC and rhodamine, respectively. (A and B) Single two-dimensional images focused at the MTOC, which is indicated by the arrowheads. (C and D) Sum of the information contained in 10 two-dimensional images taken at 0.25-μm intervals after removal of out-of-focus blur. The MTOC is indicated by the arrow, and arrowheads point to tubular arrays of p85 which coalign with microtubules. Bars, 5 μm.

determine the extent to which p85 copurified with clathrin-coated vesicles.

The total amount of p85 in the particulate, soluble, and clathrin-coated vesicle fractions obtained from a determined number of cells was quantified by immunoblotting. An unexpectedly high proportion of cellular p85 (20%) was found to be in the particulate fraction in nonstimulated cells, and this amount was increased by only a factor of 1.5 in response to PDGF stimulation (Fig. 5). However, whereas over 30% of the clathrin heavy chain detected in the total particulate fraction was recovered in the clathrin-coated vesicle preparation (not shown), less than 2% of the total particulate p85 copurified with clathrin-coated structures (Fig. 5). In response to PDGF, the amount of p85 in clathrin-coated vesicles increased by a factor of 3. These results are consistent with the hypothesis that PDGF receptor–PI-3 kinase complexes concentrate transiently in clathrin-coated vesicles. However, a significant amount of p85 is particulate even in the absence of PDGF, and it does not appear to purify with clathrin coats.

Immunofluorescence staining indicates that p85 is concentrated near the TGN. Immunofluorescence analysis was performed to gain insight into the nature of the particulate structures with which p85 associates. Cells were double stained with a monoclonal antibody against the clathrin heavy chain and a rabbit polyclonal antiserum against p85. Shown in Fig. 6 are images obtained before (Fig. 6A and B) and after (Fig. 6C and D) optical sectioning and image restoration. Staining with anticlathrin resulted in a characteristic discrete punctate pattern (Fig. 6B and D), with an

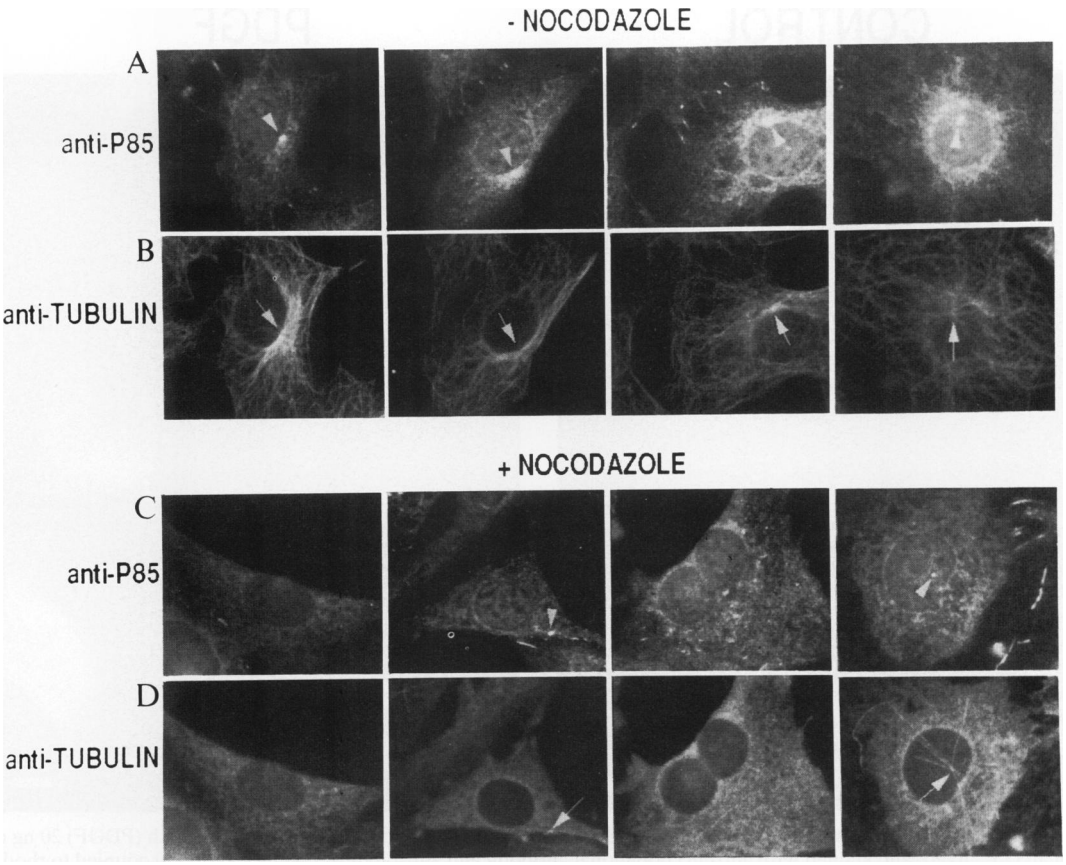


FIG. 8. Effects of microtubule depolymerization on p85 distribution. Cells were treated without (top) or with (bottom) 40 μg of nocodazole per ml for 60 min, fixed, and double stained with anti-p85 rabbit polyclonal antibody (anti-P85) or antitubulin mouse monoclonal antibody (anti-TUBULIN), as indicated. Primary antibodies were detected by using anti-mouse and anti-rabbit antibodies coupled to rhodamine and FITC, respectively. The sites of high focal concentration of p85 (arrowheads) coincide with the MTOC (arrows). The presence of high focal concentration of p85 at the remnants of the MTOC in some nocodazole-treated cells is illustrated in two of the images in panels C and D.

area of juxtanuclear concentration at the TGN (Fig. 6B and D, arrows), where clathrin cages participate in the traffic of lysosomal enzymes to a prelysosomal compartment (12). The pattern observed with anti-p85 was distinctly different (Fig. 6A and C); staining was not as punctate but rather had a tubular appearance; it was weaker in the cell periphery but tended to concentrate focally at sites of cell-cell contact (Fig. 6A and C, broken arrows) and in the perinuclear region, predominantly in an area which corresponded with the TGN, as evidenced by its colocalization with juxtanuclear clathrin (compare Fig. 6A and C with Fig. 6B and D). High focal concentration of p85 in a bright spot localized at the center of the TGN was also detected (Fig. 6A and C, arrowheads). The localization of this bright, juxtanuclear focal staining to the center of the TGN suggested that p85 might be concentrated near the centrosome/microtubule organization center (MTOC).

Double staining with anti-p85 and antitubulin reveals that p85 localizes to the MTOC. To localize the centrosome/MTOC, we double stained cells with antitubulin (Fig. 7B and D) and anti-p85 (Fig. 7A and C). The bright spot of juxtanuclear p85 was found to coincide with the region from which microtubules originate (Fig. 7A and B, arrowheads). Furthermore, p85 appeared to concentrate in the regions where microtubules were bundled. Figures 7C and D represent the summed information contained in 10 two-dimensional images of the perinuclear region of a cell taken at 0.25-μm intervals. The bright spot of p85 coincides with the MTOC (arrow). In addition, the restoration process reveals more clearly the vesicular/tubular staining pattern of p85 and a low level of staining within the nucleus. A colocalization of p85-labeled structures with microtubules extending from the MTOC can be appreciated in these images (arrowheads).

Effects of nocodazole on the localization of p85. The colocalization of p85 with microtubules and its high focal concentration at the centrosome suggest that soluble p85 or endosomes containing p85 might associate with the microtubule cytoskeleton. In an effort to distinguish between these possibilities, the effect of microtubule depolymerization on p85 distribution was explored. Figure 8 illustrates the results of experiments in which cells were treated for 60 min with nocodazole (40 μg/ml) prior to fixation and double staining for p85 and tubulin. In nontreated cells (Fig. 8A), the characteristic perinuclear distribution of p85 and the high centrosomal concentration can be observed. Treatment of cells with nocodazole resulted in a pronounced change in the distribution of p85. Most of the signal was now present in a granular pattern, distributed homogeneously throughout the cell, and the focal centrosomal staining disappeared (Fig. 8C). The staining pattern of p85 in nocodazole-treated cells was distinct from that of depolymerized tubulin (Fig. 8D), which was less granular and more diffusely distributed

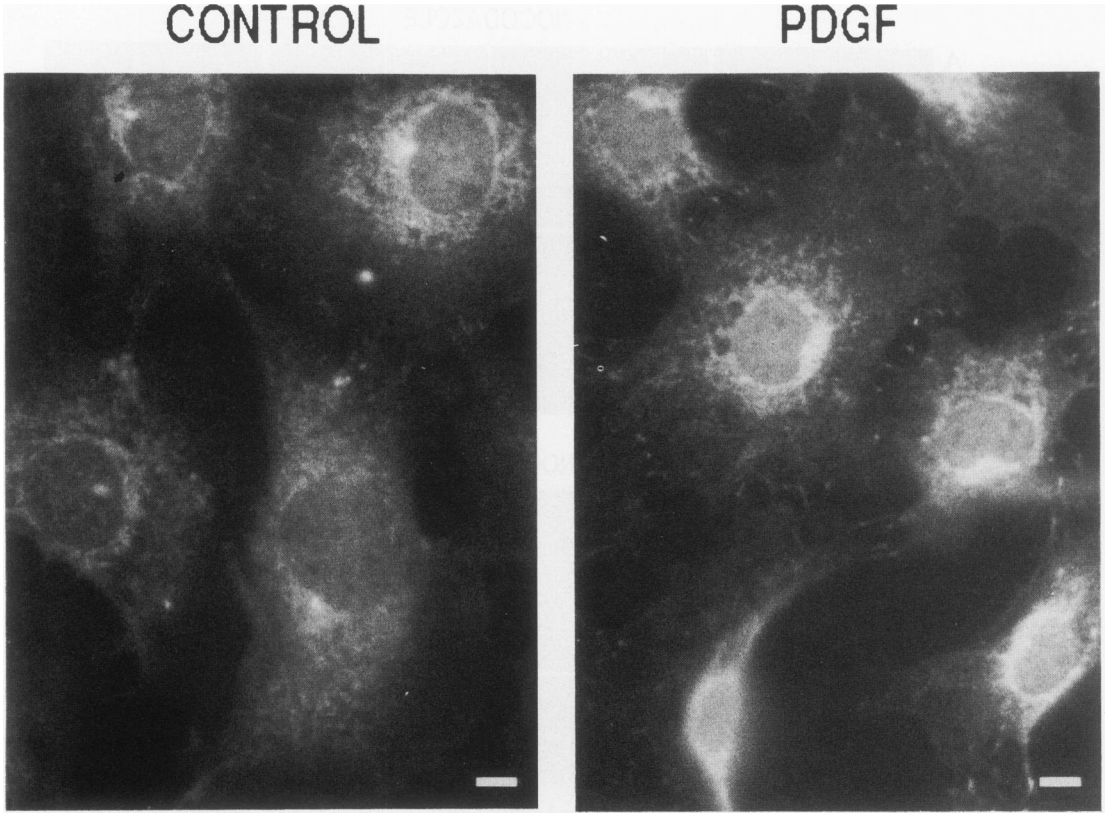


FIG. 9. Effect of PDGF on the localization of p85. Serum-starved cells were treated without (Control) or with (PDGF) 20 ng of AB-PDGF per ml for 10 min at 37°C, fixed, and stained with anti-p85 polyclonal antibody and anti-rabbit secondary antibody coupled to rhodamine. Bars, 5 μm.

throughout the cytoplasm. These images suggest that p85 remains associated with particulate structures, such as endosomes, after microtubule depolymerization and that the cellular localization of structures containing p85 is dependent on microtubule integrity.

Depolymerization of the microtubule network by nocodazole was not complete in all cells; we could frequently find cells in which a few stubs of microtubules could be seen stemming from the MTOC (Fig. 8C and D, arrows). In these cells, most of the p85 was dispersed, but a spot remained which coincided with the MTOC. Thus, the degree of microtubule depolymerization correlates well with the dispersion of the p85 signal, suggesting that the localization of the protein is dependent on its direct or indirect association with microtubules.

Stimulation with PDGF increases the concentration of p85 in the perinuclear region. The comparison between the staining pattern of p85 in nonstimulated and PDGF-stimulated cells did not reveal a dramatic change in response to PDGF stimulation (Fig. 9). This result is consistent with the subcellular distribution of p85 determined by immunoblotting after subcellular fractionation, which indicated only a 1.5-fold increase in the amount of p85 present in the particulate fraction after treatment with PDGF (Fig. 5). In general, a more clear demarcation of the cell periphery was apparent in images of cells that were exposed to PDGF continuously for 10 min, consistent with an increase in p85 at the plasma membrane. More noticeable was an increased staining of the perinuclear region around the TGN after exposure to PDGF. The intensity of the centrosomal staining appeared to decrease in PDGF-stimulated cells. However, images obtained with shorter photographic exposure times (not shown) revealed this to be the result of reduced contrast; the brightness of the areas surrounding the MTOC obscure its presence. The magnitude of the effect of PDGF was estimated by measuring the mean fluorescence intensity in concentric regions of the cell centered on the nucleus (Fig. 10A). The intensity of fluorescence in the central regions of the cell, which encompass the nucleus, perinuclear region, and TGN, was increased by a factor of 2 after 10 min of continual exposure of the cell to PDGF (Fig. 10B).

Nocodazole inhibits the effect of PDGF on p85 distribution. We next examined whether the effects of PDGF on the distribution of p85 were dependent on the apparent interactions of p85 with the microtubule cytoskeleton. Figure 11 illustrates the results of experiment in which cells were treated without or with nocodazole prior to exposure to PDGF. The localization of p85 and tubulin was then analyzed by immunofluorescence. The perinuclear concentration of p85 was enhanced after 10 min following exposure of cells to PDGF (compare extreme left top and bottom images). Treatment of cells with nocodazole resulted in the dispersion of p85 to the cell periphery, although a small spot which coincided with the remnants of the MTOC could be observed (compare center left and extreme right top images). Exposure of nocodazole-treated cells to PDGF did not result in a detectable change in the distribution of p85 (compare center left top and bottom images). These results suggest that the effects of PDGF on the distribution of p85 are

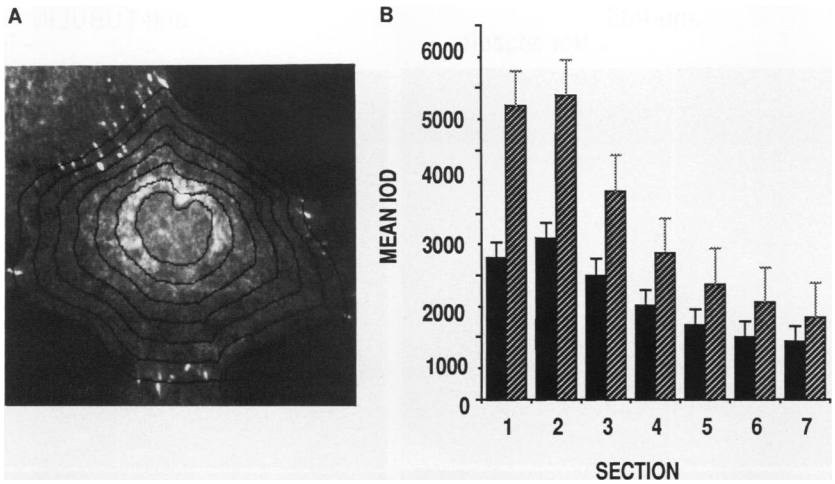


FIG. 10. Quantitative analysis of p85 immunofluorescence. Serum-starved cells were treated without or with 20 ng of AB-PDGF per ml for 10 min at 37°C, fixed, and stained with anti-p85 polyclonal antibody and anti-rabbit secondary antibody coupled to rhodamine. Images from control and PDGF-treated cells were taken with identical exposure times, and care was taken to minimize photobleaching. Digitized images were analyzed by using a program that sections the cell into concentric rings centered on the nucleus. (A) Example of a non-PDGF-treated cell sectioned for analysis into seven concentric regions. In most cells, the centrosome was confined to the first perinuclear ring (assigned number 2). The most peripheral regions of the cell, including sites of cell-cell contact, were encompassed in the last ring (assigned number 7). (B) Fluorescence intensity of each ring in 15 randomly selected nonstimulated (■) or PDGF-stimulated (■) cells. Plotted are the means ± standard errors of the means of the values obtained. This analysis was repeated once with an independent set of cells, and similar results were obtained.

dependent on the interaction of p85 or p85 enriched endosomes with the microtubule cytoskeleton.

DISCUSSION

In this study, we showed that following stimulation of cells with PDGF, tyrosine-autophosphorylated PDGF receptors can be found in isolated clathrin-coated vesicles. The kinetics of the association of activated receptors with clathrin-coated structures is consistent with a transient association of the receptor with these structures during its entry into the endocytic pathway. Although the entry of PDGF into the cell through coated pits has been previously documented, the results shown here provide the first indication that the PDGF receptor remains tyrosine phosphorylated during the endocytic process. Our studies also demonstrate that in response to PDGF, both the concentration and the specific catalytic activity of PI-3 kinase are increased by two- and threefold in isolated clathrin-coated structures. These results suggest that the binding of PI-3′ kinase to activated receptors is followed by its activation and entry with the receptor into the endocytic pathway. Thus, endosomes containing activated receptors may be highly enriched in 3′-phosphorylated lipids. These results raise questions about the mechanisms of activation of PI-3′ kinase by PDGF and about the significance of the presence of PI-3′ kinase and its products in the endocytic pathway.

Under some conditions, the p85 subunit of PI-3′ kinase can become tyrosine phosphorylated in response to growth factor receptor activation, and this modification may result in changes in its catalytic activity (16, 32). We cannot eliminate the possibility that tyrosine phosphorylation of p85 occurs at levels undetectable by our assays and that this modification plays a role in modulating the activity of PI-3′ kinase in clathrin coats. However, an alternative possibility is that the enzyme is activated allosterically by the PDGF receptor itself. Consistent with this view is the finding that tyrosine-phosphorylated peptides containing sequences that bind p85 activate PI-3′ kinase in vitro (2). Because our results do not involve addition of exogenous activators but result exclusively from the interactions among components isolated from intact cells, they support strongly the hypothesis that activation of PI-3′ kinase catalytic activity can occur in vivo.

The subcellular fractionation studies presented here indicate that a significant amount of PI-3′ kinase (20 to 30% of total cellular protein) remained in a particulate fraction of serum-starved 3T3-L1 fibroblasts, which includes all components that resist solubilization after homogenizing the cells in 0.05% Triton X-100. These results seem inconsistent with models of PI-3 kinase function (reviewed in references 4, 17, and 25), in which PI-3′ kinase is a principally cytoplasmic protein which is recruited to membranes quantitatively in response to receptor activation. We have found very little total phosphotyrosine in particulate fractions of serum-starved 3T3-L1 cells and a dramatic increase in response to stimulation by PDGF (not shown). These large changes in phosphotyrosine content are not reflected by the small (1.5-fold) increase in the amount of particulate p85 observed in response to PDGF stimulation (Fig. 5 and 9). Thus, our results are more consistent with a model in which p85 or PI-3′ kinase can be recruited to cellular membranes by several different mechanisms, a specific one of them being the association with phosphotyrosine residues via the SH2 domains of p85. Indeed, PIns(3)P is present in serum-starved intact cells (1), and even after mitogenic stimulation, PIns(3)P, and not newly formed PIns(3,4)P2 or PIns(3,4,5)P3, continues to represent the most abundant 3′ phosphoinositide in intact fibroblasts (1). These results are consistent with the hypothesis that PI-3′ kinase is active constitutively and may be involved in cellular processes that occur in the absence of mitogen stimulation, such as protein sorting.

A role for PI-3 kinase in the sorting of receptor tyrosine kinases has been suggested by the failure of mutant colony-

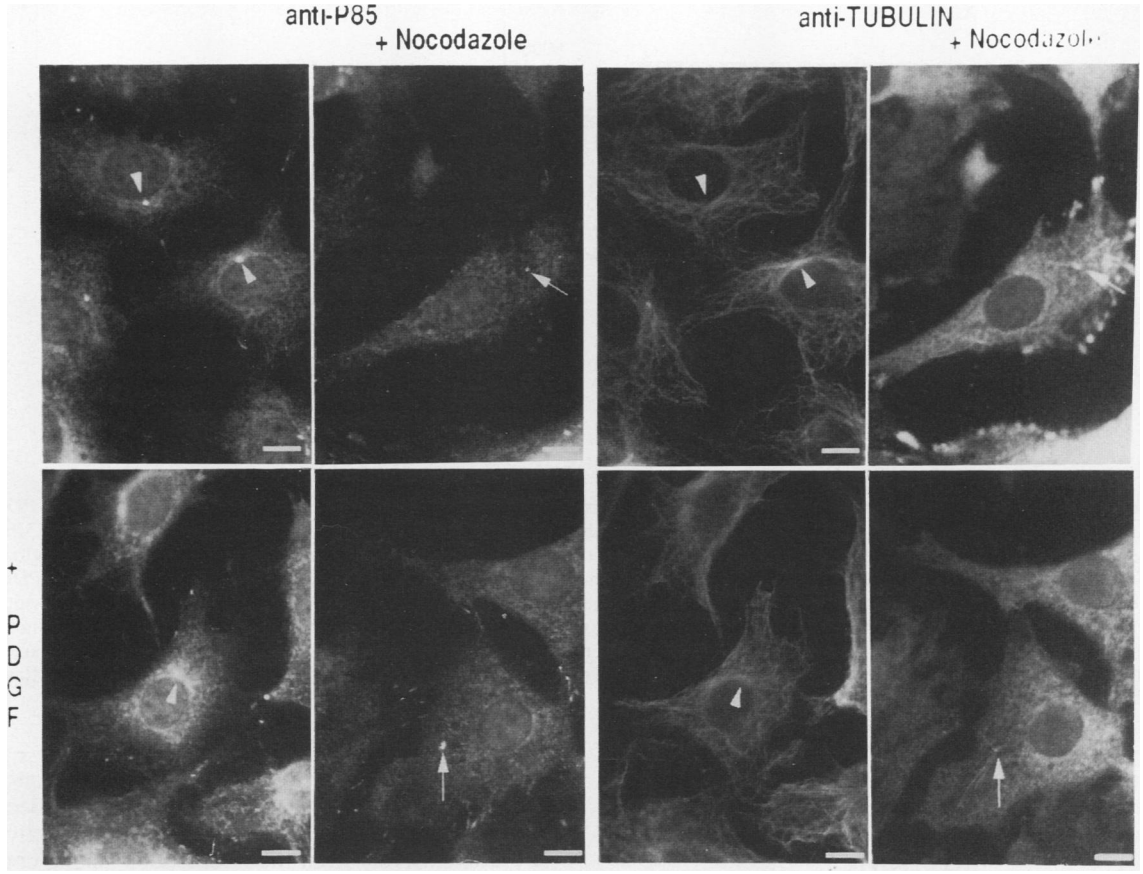


FIG. 11. Effects of PDGF in the absence or presence of nocodazole. Serum-starved cells were treated without or with 40 μg of nocodazole per ml for 60 min (+ Nocodazole) and then treated without or with 20 ng of AB-PDGF per ml for 10 min (+ PDGF). Cells were fixed and double stained with anti-p85 rabbit polyclonal antibody (anti-P85) or anti-tubulin mouse monoclonal antibody (anti-TUBULIN). The sites of high focal concentration of p85 and the position of the MTOC are indicated by arrowheads. Arrows point out the presence of high focal concentration of p85 at the remnants of the MTOC in nocodazole-treated cells.

stimulating factor receptors that lack PI-3 kinase binding sites to undergo lysosomal degradation following internalization (30). In addition, the principal phenotype ensuing from the absence or mutation of a protein similar to the p110 catalytic subunit of PI-3 kinase (Vps34p) in yeast cells is a severe defect in the sorting of proteins to the vacuole (14). In fibroblasts, PI-3′ kinase may play a role similar to that in yeast cells and be involved in multiple protein-sorting events, a specific one of which might be the sorting of activated receptor tyrosine kinases. Interestingly, the staining pattern of p85 is displayed by several proteins that bind to microtubules (6, 21) or that are involved in mediating the interactions between endosomes and microtubules (26, 35). These morphological results suggest a substantial degree of interaction between endosome containing p85 and the microtubule cytoskeleton. Further genetic and biochemical studies will be necessary to determine the functional relevance of these interactions.

Results shown in this study indicate that exposure of cells to PDGF results in an increase in concentration of p85 in the perinuclear region. These results are in agreement with those of Kelly et al. (20), which show an insulin-dependent increase in PI-3′ kinase activity manifested principally in intracellular membranes. An interpretation of these results is that PI-3 kinase remains associated with receptors as they internalize into endosomes and move from the plasma membrane toward the TGN. The complete abrogation this effect by the microtubule-depolymerizing drug nocodazole is consistent with this hypothesis and suggests that an intact microtubule cytoskeleton is required for this movement. An association between PDGF receptors and PI-3′ kinase during endocytosis is also consistent with the documented kinetics of formation of 3′-phosphorylated phospholipids in response to PDGF; in contrast to more transient effects of PDGF, such as PIns(4,5)P2 hydrolysis and calcium mobilization, which are initiated and end within the first 5 min of stimulation, the formation of D-3-phosphorylated lipids is sustained for 15 to 30 min (1). This time course parallels the time required to move activated receptors from the plasma membrane to the lysosome, as evidenced by the half-life of the activated receptor assessed in methionine pulse-chase experiments (18).

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