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p180, a Novel Recycling Transmembrane Glycoprotein with Restricted Cell Type Expression

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A 180-kilodalton (kDa) protein (p180) was identified among the antigens for a panel of monoclonal antibodies raised against human fibroblast cell surface proteins. Binding studies with ^{125}I-Fab′ fragments of an anti-p180 monoclonal antibody demonstrated that 10 to 30% of p180 was located on the plasma membrane and that the remaining 70 to 90% was on intracellular membranes. p180 was rapidly internalized from the cell surface at 37°C, and kinetic analyses indicated that this was a constitutive process followed by the recycling of p180 back to the plasma membrane. Morphological studies demonstrated that on the cell surface p180 was concentrated in coated pits, whereas inside the cell it was found in endosomes as suggested by its colocalization with the transferrin receptor. Immunoblot analysis with a polyclonal antiserum raised against purified human protein showed that p180 has a restricted distribution with expression at high levels in fibroblast cultures and in tissues containing cells of mesodermal origin. A biochemical characterization of p180 showed it to be a transmembrane glycoprotein with an extracellular domain, which consists of ~30 kDa of complex oligosaccharides attached to at least 45 kDa of the protein core. The cytoplasmic domain of p180 was found to contain a serine residue(s) that was phosphorylated both in vivo and in vitro by activated protein kinase C. p180 was purified by subjecting solubilized membrane proteins from a human osteosarcoma cell line to immunoaffinity chromatography and gel filtration. The N-terminal sequence information obtained from the purified protein showed no homology to other known proteins. It was concluded that p180 may be a novel recycling receptor which is highly restricted in its expression to fibroblastlike cells.

Eucaryotic cells are surrounded by a plasma membrane. To receive information from the environment and to transfer essential molecules into the cell, this membrane must contain specific receptor proteins. A choice in the range of receptors expressed on the surface of a cell offers a mechanism for cells to maintain their individual character. One approach to the study of cell surfaces has been to identify and characterize plasma membrane proteins with extracellular domains on the basis of their antigenicity (7, 32). This approach has been particularly successful in the analysis of the lymphocyte cell surface. Over 70 such proteins have been identified, and the functions of a significant fraction of these molecules are now beginning to be understood. To learn more about the intercellular interactions among cells of nonhematopoietic lineages, a panel of monoclonal antibodies (MAbs) was raised against cell surface proteins present on human fibroblasts.

One of these MAbs recognized a 180-kilodalton (kDa) protein, p180. p180 was chosen for further study because it was distributed on the surface of cultured fibroblasts in a punctate pattern reminiscent of coated pits. Coated pits function by pinching off the cytoplasm to form intracellular vesicles containing selected membrane proteins that can then be transported to appropriate locations within the cell. This process of receptor-mediated endocytosis provides a mechanism for the internalization of a wide variety of nutrients, growth factors and hormones, toxins, immune complexes, viruses, and enzymes (reviewed in reference 12). The cell surface distribution of p180 suggested that it is a recycling receptor, and this possibility was examined by a combination of kinetic, morphological, and biochemical analyses.

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MATERIALS AND METHODS

Cells. Human cell lines were obtained from the following sources: AG1523 foreskin diploid fibroblasts (NIA Aging Cell Repository, Institute for Medical Research, Camden, N.J.); Flow 2000 embryonic lung diploid fibroblasts (Flow Laboratories, Inc., McLean, Va.); F1084 embryonic fibroblasts (Colin Hopkins, Imperial College, London); MG-63 osteosarcoma cells (Erkki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, Calif.). All cells were cultured in Dulbecco-Vogt modified Eagle medium (DME) supplemented with 10% fetal calf serum (FCS). For [^{35}S]methionine pulse-chase experiments, cells were cultured for 1 h in methionine-free DME plus 4% dialyzed FCS and then 300 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml was added for a further 15 min. The dishes were washed and incubated with DME containing 5% FCS and 3 mM methionine for 0 to 48 h. For [^{35}S]methionine labeling in the presence of tunicamycin, cells were incubated for 4 h in DME-methionine plus 4% dialyzed FCS with 0, 0.5, or 5 μg of tunicamycin (Sigma Chemical Co., St. Louis, Mo.) per ml and 300 μCi of [^{35}S]methionine per ml was added for the last 2 h of the incubation. For $^{32}P_i$ labeling, cells were incubated for 18 h in phosphate-free DME containing 4% dialyzed FCS and 1 mCi of $^{32}P_i$ (ICN Radiochemicals, Irvine, Calif.) per ml and 50 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) per ml was added for 10 min at the end of the labeling period. Lactoperoxidase-catalyzed cell surface radioiodination was performed by the method of Trowbridge et al. (31).

Antibodies. Monoclonal antibodies (MAbs) against p180 were generated by immunizing mice with either viable cells or crude membrane preparations from human fibroblasts. Hybridoma supernatants were screened for their ability to immunoprecipitate ^{125}I-proteins from cell surface-labeled

viable AG1523 human fibroblasts as described by Isacke et al. (19). To obtain Fab' fragments, MAb E1/183 was partially purified from mouse ascitic fluid by 50% $(NH_4)_2SO_4$ precipitation and digested at a mass ratio of antibody/pepsin of 33:1 in 0.1 M sodium acetate buffer (pH 4.2) for 18 h at 37°C. The same amount of pepsin was then added, and the digestion continued for a further 24 h. $F(ab')_2$ fragments were separated from undigested immunoglobulin G and other proteins by gel filtration on a Sephacryl S-200 column equilibrated with 10 mM EDTA–0.2 M Tris hydrochloride (pH 8.0). Fab' was obtained from $F(ab')_2$ by incubation at 37°C for 30 min with 5 mM cysteine followed by the addition of 10 mM iodacetamide and dialysis against phosphate-buffered saline. A polyclonal antiserum against p180 was raised by immunizing a rabbit with 40 μg of purified human p180 in complete Freund adjuvant into the popliteal lymph nodes (29) on week 1, followed by 40 μg of purified p180 with incomplete Freund adjuvant subcutaneously on weeks 5 and 7. The rabbit was bled out on week 10. Other antibodies have been described elsewhere: anti-transferrin receptor MAb (B3/25) (24) and rabbit anticlathrin polyclonal antiserum (6).

Binding assays. MAb E1/183 Fab' fragments and transferrin were ^{125}I labeled by the Iodogen method (10) and with chloramine-T (18), respectively. Binding experiments were performed as described in the figure legends with confluent 35-mm dishes of F1084 human fibroblasts (two or three dishes per point), binding buffer (DME, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 1 mg of bovine serum albumin per ml, pH 7.4), and either ^{125}I-anti-p180 Fab' (200,000 cpm) or ^{125}I-transferrin (600,000 cpm). At the end of the experiment, the incubation medium was collected, ^{125}I-ligand bound to the cell surface was removed by incubating the cells with 1 ml of ice-cold 0.1 M sodium acetate–0.1 M NaCl (pH 2.5) for 4 min at 4°C (16), and the cells were detached from the dishes with 0.125% trypsin. To determine the percentage of acid-soluble and -precipitable radioactivity in each fraction, trichloroacetic acid (TCA) was added to 20% (wt/vol), the samples were placed on ice for 1 h, and the precipitates were pelleted by centrifugation. In all experiments, nonspecific binding in the presence of 200-fold excess of unlabeled ligand was shown to be less than 15% of specific binding, and values from duplicate or triplicate samples varied less than 10%.

Microscopy. Cells cultured on glass cover slips were fixed for 10 min with 2% paraformaldehyde in phosphate-buffered saline containing 10 mM Ca^{2+} and 10 mM Mg^{2+}. All subsequent procedures employed a washing solution of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 0.5% bovine serum albumin and 0.2% gelatin with or without 0.2% saponin (Sigma). Cells were washed, incubated for 30 to 60 min with primary antibody diluted in washing buffer and then for 30 to 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Sera-Laboratories) or rhodamine-conjugated goat anti-rabbit immunoglobulin (Miles, ICN Biomedicals, Costa Mesa, Calif.), and mounted in glycerol containing 2.5% *N*-propyl gallate. For tissue sections, freshly isolated rat kidneys or human skin were coated in Tissue-Tek (O.C.T. compound; Miles) and frozen in precooled isopentane and 10-μm sections were cut at −20°C with a Bright Instruments cryostat. Sections were air dried for 30 min, fixed for 10 min in 2% paraformaldehyde, and then processed as described above for cover slips. For electron microscopy, AG1523 cells were processed as described above except that the second-layer antibody was rabbit anti-mouse immunoglobulin conjugated to 8-nm gold particles. Cells were then fixed with uranyl acetate and embedded, and 50-nm sections were cut. Gold particles were visualized in a Philips 301 transmission electron microscope as described by Hopkins (15).

Biochemical analysis. For immunoprecipitation, labeled cells were lysed in 1 ml of RIPA buffer (28) supplemented with 2 mM EDTA, 100 μM Na_3VO_4, and 50 mM NaF and p180 was immunoprecipitated as described by Isacke et al. (20). Rat tissues or cell lines were prepared for immunoblot analysis as described by Gould et al. (13), and immunoblotting was performed as described by Glenney (11). Purified human p180 (50 ng) was phosphorylated by protein kinase C (PKC) (J. R. Woodgett, The Salk Institute) (34) in a final volume of 20 μl containing phosphatidylserine, diolein, and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) by the method of Woodgett and Hunter (34). Excised phosphoproteins were digested with trypsin and resolved in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis at pH 4.7 for 30 min at 1 kV followed by chromatography as described previously (8, 17).

Purification and sequencing of p180. Anti-p180 MAb E1/183 was coupled to cyanogen bromide-activated Sepharose CL-4B beads (Pharmacia, Inc., Piscataway, N.J.) by the method of Porath (26) with an efficiency of 91%. Between 100 and 120 dishes (15 cm) of confluent MG-63 cells cultured for 5 to 7 days in DME supplemented with 2.5% FCS and 5% calf serum plus one 15-cm dish of MG-63 cells labeled overnight with 10 ml of methionine- and cysteine-free DME–5% FCS and 3 mCi of [^{35}S]Translabel (containing [^{35}S]methionine and [^{35}S]cysteine; ICN) were washed twice with cold Tris-buffered saline and 5 ml of cold Ca^{2+}-, and Mg^{2+}-free phosphate-buffered saline containing 2% Lubrol PX (Sigma), 1% Nonidet P-40 (Sigma), 100 μM phenylmethylsulfonyl fluoride (Sigma), 0.5 μg of leupeptin per ml, 5 mM EDTA, and 2 mM EGTA was added to each dish. All subsequent procedures were done at 4°C. Cells were removed from the dishes with a rubber policeman, and the lysate (~700 ml) was stirred gently for 30 min and then vigorously for 15 s with a Polytron homogenizer. Nuclei and large cellular debris were removed by centrifugation for 20 min at 6,300 × *g*. This postnuclear supernatant was made 1% (wt/vol) with sodium deoxycholate (DOC), stirred gently for 10 min, and centrifuged at 100,000 × *g* for 1 h. The resulting supernatant containing solubilized membrane proteins was passed over two parallel 1-ml anti-p180 MAb affinity columns at the rate of 20 to 25 ml/h per column at 4°C. The columns were each washed sequentially with the following solutions: (i) 100 ml of 0.5% DOC in 10 mM Tris hydrochloride (pH 8.0) plus 100 μM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 5 mM EDTA, 2 mM EGTA; (ii) 50 to 80 ml of the same solution containing 150 mM NaCl; and (iii) 5 ml of 0.5% DOC in water. To elute protein, 0.75 ml of elution buffer (0.5% DOC, 50 mM diethylamine in water, pH 11.5) was added to each column and left for 5 min (DEA 1 in Fig. 9A). Then 3 ml of elution buffer was added to each column, and the eluates were collected and immediately neutralized by the addition of glycine to 50 mg/ml (DEA 2 in Fig. 9A). The columns were washed with 3 ml of 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–0.02% NaN_3 (DEA 3 in Fig. 9A) and then more extensively with the same solution and stored at 4°C.

The affinity column eluate fractions containing p180 were pooled and concentrated in Spectrapor 2 dialysis tubing (Sigma) surrounded by solid polyethylene glycol 6000. When the volume had been reduced to 10% of its original (2 to 4 h), the eluate was microcentrifuged for 10 min and resolved on a Superose 6 FPLC column (Pharmacia) at room tempera-

ture. The column was preequilibrated with the running buffer (0.2% DOC, 10 mM Tris hydrochloride [pH 8.0], 0.1 mM dithiothreitol), 200-μl samples were then applied, and the column was run at 0.33 ml/min for 70 min, collecting 0.5-ml fractions. For each large-scale preparation (i.e., 100 to 120 dishes [15 cm] of MG-63 cells), three Superose 6 column runs were performed. Peak fractions were pooled and dialyzed in Spectrapor 2 tubing against 100 mM NH_4CO_3 for 36 h and then against 100 mM acetic acid for a further 24 h. The dialyzed material was lyophilized on a Speed-Vac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.). At each stage in the purification procedure, samples were removed and (i) counted in 3 ml of Econolite scintillation fluid (Westchem, San Diego, Calif.) and (ii) resolved on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels which were subsequently enhanced with PPO, dried, and exposed to presensitized Kodak XAR film overnight or stained with Coomassie blue and then silver stained by the method of Ansorge (3).

Approximately 20 pmol of purified p180 protein was subjected to N-terminal sequencing in an Applied Biosystems model 470 gas-phase protein sequencer with an on-line phenylthiohydantoin analyzer from the same manufacturer. The N-terminal sequence was checked against the GenBank Nucleic Acid and Protein Databases in April 1989, and no similarities were found.

RESULTS

Distribution of p180 between plasma and intracellular membranes. The anti-p180 MAb, E1/183, was identified in a screen for MAbs that immunoprecipitate proteins from ^{125}I-surface-labeled human fibroblasts (19) (Fig. 1). To determine whether p180 was restricted in its expression to the plasma membrane and to study possible trafficking of p180, a ligand was required that would cause minimal perturbation of its cellular distribution. For this purpose, Fab′ fragments were generated from the anti-p180 MAb. Incubation of cultured fibroblasts with anti-p180 Fab′ did not substantially alter the half-life of the p180 protein (Fig. 2A). Anti-p180 Fab′ was then ^{125}I labeled and used to quantitate the level of p180 expression. From Scatchard analysis of binding assays, it was calculated that F1084 human fibroblasts have 2×10^5 to 3×10^5 molecules of p180 per cell on their surface (data not shown). In binding studies with saturating amounts of ^{125}I-ligand, the amount of Fab′ bound to nonpermeabilized F1084 cells was 20 to 30% of that bound to saponin-permeabilized cells (Fig. 2B). On this basis, it was estimated that F1084 fibroblasts express 7×10^5 to 15×10^5 molecules of p180 in total and that 70 to 80% of p180 is localized on intracellular membranes.

Trafficking of p180 in cultured cells. To examine the relationship between p180 on the plasma and intracellular membranes, ^{125}I-Fab′ was bound to human fibroblasts at 4°C, excess ligand was removed, and the cells were incubated for 0 to 15 min at 37°C. At various times, ^{125}I-Fab′ remaining on the cell surface was detached by acid stripping at pH 2.5 (see Materials and Methods) and the cells were removed from the dishes with trypsin to assess the distribution of counts in the medium, on the cell surface, and inside the cells. In the first 2 min of incubation at 37°C, there was an increase in the amount of cell-associated radioactivity that was resistant to an acid wash, indicating that 60% of the ^{125}I-Fab′ had been internalized from the plasma membrane. This fraction subsequently decreased to ~50% by 15 min, and during this time, the amount of radioactivity detected in the medium increased (Fig. 3A), indicating that at least some of the bound Fab′, or its degraded products, had been recycled back to the cell surface and released.

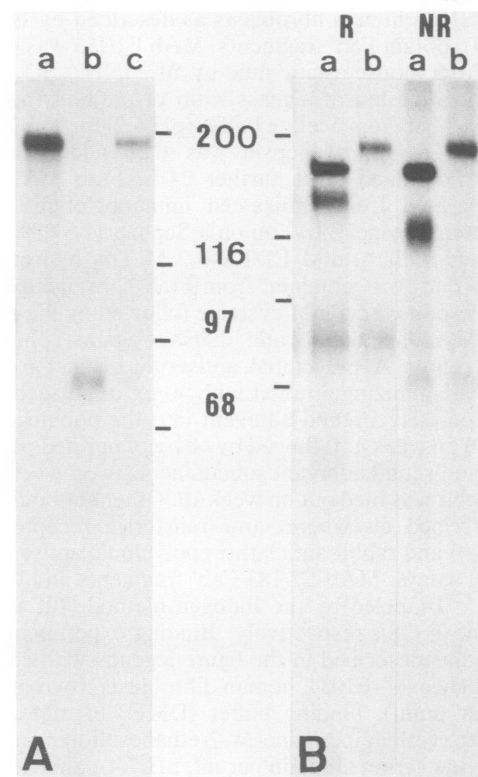

FIG. 1. Anti-p180 MAb immunoprecipitates a 180-kDa protein from ^{125}I-labeled human fibroblasts. (A) AG1523 fibroblasts were detached from dishes, ^{125}I labeled, and treated with or without 50 μg of trypsin per ml for 15 min at 22°C followed by 2 mg of ovomucoid per ml. Cells and supernatants were solubilized, immunoprecipitated with MAb E1/183, and resolved on a reducing SDS-10% polyacrylamide gel. Lanes: a, cells without trypsin treatment; b, supernatants from trypsin-treated cells; c, cells after trypsin treatment. (B) ^{125}I-labeled AG1523 fibroblasts were lysed and immunoprecipitated with either a control MAb (lanes a) which recognizes a member of the integrin family or anti-p180 MAb E1/183 (lanes b). Immunoprecipitates were resolved on reducing (R) or nonreducing (NR) SDS-10% polyacrylamide gels. Gels were exposed for 24 h. Molecular size markers are in kilodaltons.

To extend these observations and determine whether Fab′ was recycled intact, human fibroblasts were incubated with ^{125}I-Fab′ for 3 h at 37°C to label both the intracellular and cell surface pools of p180. The cells were then washed and incubated at 37°C for up to 60 min. At time zero, the amount of radioactivity associated with the cell surface (i.e., acid dissociable) was between 10 and 25% and the remainder was acid resistant (i.e., intracellular). With incubation at 37°C, the amount of ^{125}I-Fab′ inside the cell and on the cell surface decreased and there was an increase in the amount of label found in the medium (Fig. 3B). The fractions stripped off the cell surface with acid and in the medium were precipitated with TCA, and in all experiments described here, >90% of the radioactivity present on the cell surface was TCA precipitable (data not shown). By contrast, ~70% of the radioactivity in the medium (i.e., ~30% of total Fab′ bound) at 60 min was TCA soluble (Fig. 3B), indicating that this proportion of the ^{125}I-Fab′ had been taken up into the cell, degraded, and then released. The TCA-precipitable material

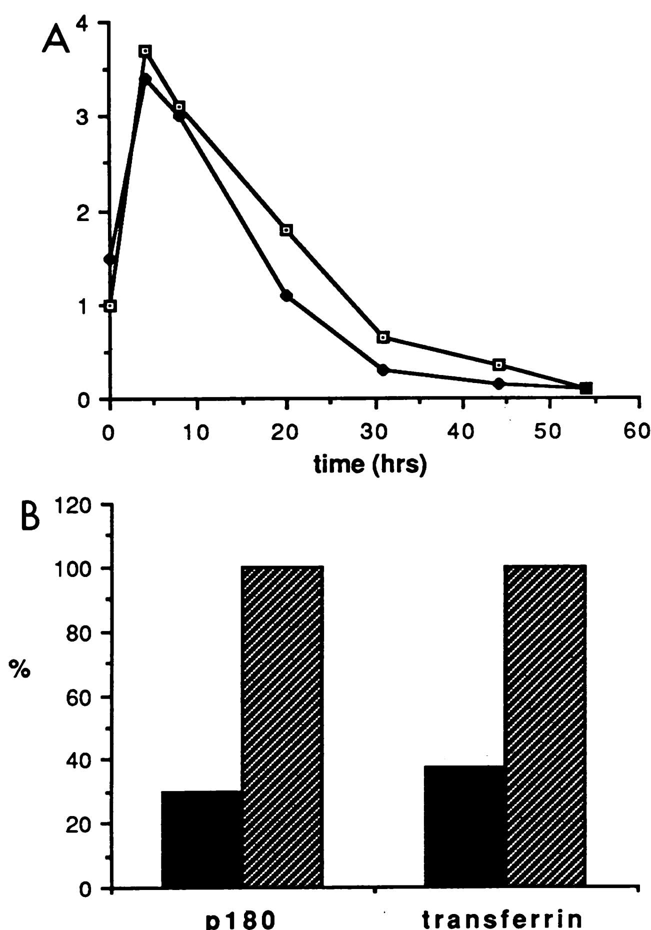

FIG. 2. Characterization of anti-p180 Fab' binding. (A) Dishes (35 mm) of F1084 fibroblasts were labeled with [^{35}S]methionine for 15 min and then chased in methionine-free medium as described in Materials and Methods for 54 h with (♦) or without (□) 20 μg of unlabeled anti-p180 Fab' per ml. Cells were lysed, immunoprecipitated with anti-p180 Mab, resolved on an SDS-7.5% polyacrylamide gel, and exposed to film for 10 days. The amount of p180 was quantitated by densitometric scanning of the autoradiogram, and the level of p180 in untreated cells at time zero is given as 1. (B) Comparison of binding to intact and permeabilized cells. ^{125}I-anti-p180 Fab' or ^{125}I-transferrin was bound to F1084 cells for 60 min on ice in the presence (▨) or absence (■) of 1% saponin. All cells were then extensively washed with binding buffer containing 1% saponin, detached, and counted. Control experiments showed that the presence of saponin in the wash buffer did not result in the release of any surface-bound ^{125}I-ligand. Binding of ^{125}I-ligand to permeabilized cells is given as 100%; values given were from duplicate samples which varied less than 10%. Similar results were obtained on three separate occasions.

in the medium and on the cell surface was found to represent intact Fab' by SDS-polyacrylamide gel analysis (data not shown).

From these ^{125}I-Fab' binding studies it was not possible to distinguish whether the intact Fab' released into the medium had been internalized and recycled or had dissociated from the cell surface directly without internalization during the 37°C incubation. To investigate this, dishes of cells replicating those shown in Fig. 3B were acid stripped after equilibrium binding so that at the start of the 37°C incubation period all the ^{125}I-Fab' associated with the cells was intracellular. At zero time, the distribution of label in the two sets of cells was identical except that 12% of the radioactivity originally on the cell surface had been detached in the acid-treated cells. After 60 min of incubation, label was found in the medium from both sets of cells and the proportion of this which was TCA precipitate was similar (i.e., 30% in untreated cells and 35% in treated cells) (Fig. 3B and C). This shows that intact Fab' detected in the medium is not the result of cell surface dissociation and that ^{125}I-Fab' can be internalized, recycled back to the cell surface, and secreted intact.

The presence of TCA-soluble radioactivity in the culture medium indicates that a fraction of the cell-bound Fab' has been degraded in the lysosomes and then released. This raises the question whether this is accompanied by the degradation of an equivalent fraction of p180 molecules or whether p180 is routed along a different pathway. Culturing fibroblasts in the presence of Fab' did not substantially affect the half-life of p180 as measured in a pulse-chase experiment (Fig. 2A). Since the data in Fig. 3B show that ~30% of total ^{125}I-Fab' bound to p180 under equilibrium conditions was degraded in 1 h, it is clear that the rate of degradation of p180 is much slower than that of the bound Fab'. This would be consistent with internalized p180 molecules, which carried bound Fab' into the cell, being able to recycle back to the cell surface. Further evidence to support this view comes from morphological observations showing that incubation of fibroblasts for various periods with Fab' did not alter the distribution of p180 as detected by fluorescence microscopy with a noncompetitive polyclonal anti-p180 antiserum (data not shown). Similarly, from double-labeling experiments in which epidermal growth factor (EGF)-EGF receptor complexes and p180 were analyzed together, there was no evidence that p180 was ever localized in lysosomes (data not shown).

Finally, the trafficking of anti-p180 Fab' was compared with that of transferrin as this is known to be a ligand for a constitutively recycling receptor. By binding ^{125}I-transferrin to nonpermeabilized and saponin-permeabilized F1084 cells, it was estimated that approximately 30% of the transferrin receptor was on the cell surface (Fig. 2B), which is consistent with published data (5, 16). When these cells were incubated with ^{125}I-transferrin for 3 h at 37°C, the same proportion of ligand was again detected on the cell surface. During the time course of the experiment, radioactivity was rapidly lost from the cell surface and from inside the cell and released into the medium. Essentially all the radioactivity in the medium was TCA precipitable, demonstrating that the transferrin is secreted intact (Fig. 3D).

Localization of p180 in cultured cells. To identify the pathways involved in the internalization of p180, its distribution in human fibroblasts was examined morphologically. In nonpermeabilized cells, p180 was located with a distinct punctate distribution in the plasma membrane (Fig. 4A and C). This pattern was reminiscent of that observed with antibodies against receptors such as transferrin (16, 33) or low-density lipoprotein (LDL) (2) that are constitutively concentrated in coated pits on the cell surface (reviewed in reference 12). Double-labeling experiments showed a strikingly similar cell surface distribution of p180, the transferrin receptor, and clathrin, the major protein component of coated pits (25) (Fig. 4A to D). The degree of colocalization suggested that the majority of p180 was associated on the cell surface with clathrin (~70%). However, not all clathrin-coated pits contained p180, and some p180 was found concentrated in areas that did not contain underlying clathrin. To improve the resolution of these observations, fibro-

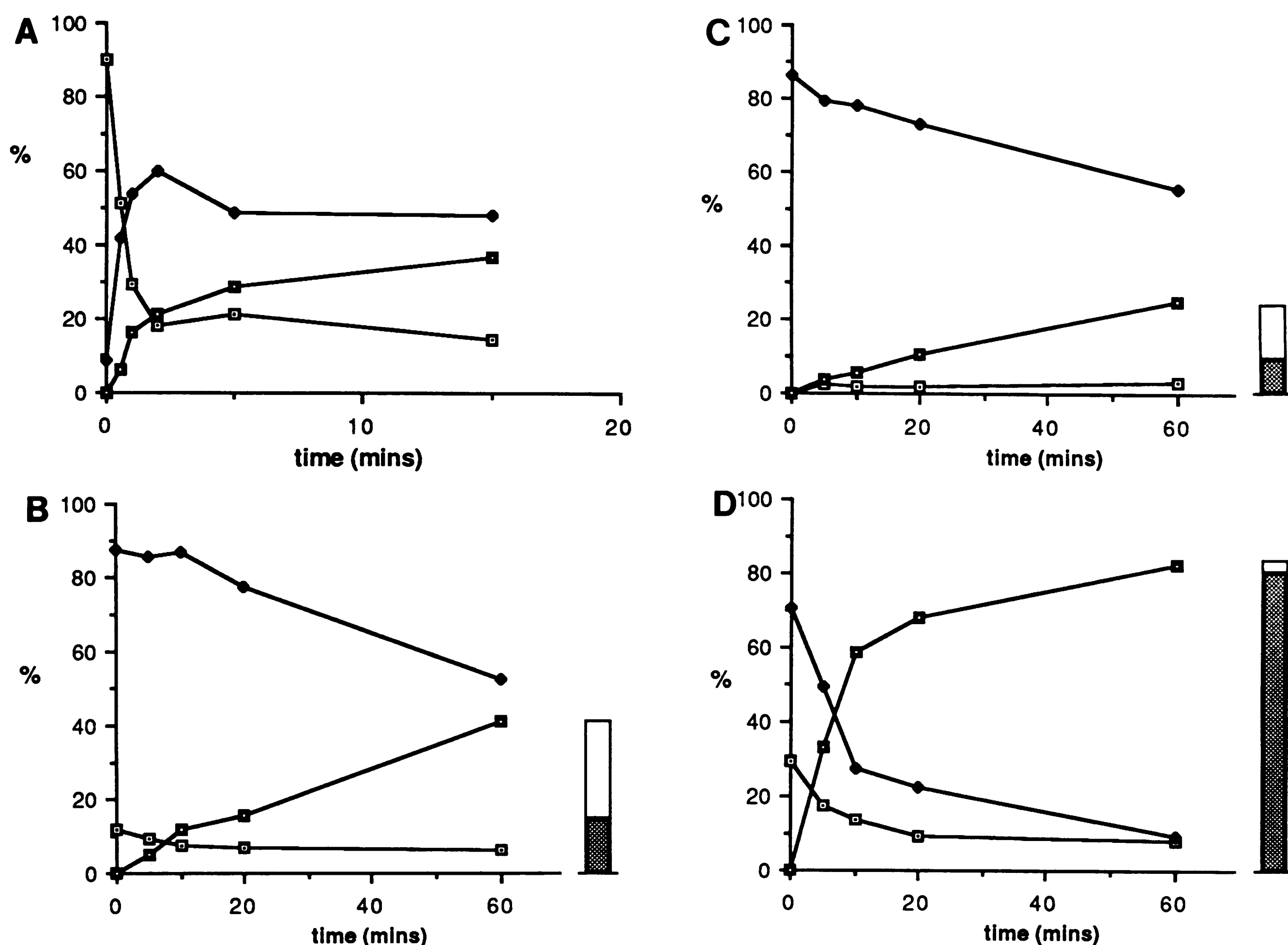

FIG. 3. Trafficking of ^{125}I-anti-p180 Fab' in cultured fibroblasts. (A) ^{125}I-Fab' was bound to F1084 cells for 60 min on ice. The cells were washed and incubated for 0 to 15 min with 1 ml of prewarmed binding buffer at 37°C. (B) F1084 cells were incubated for 3 h at 37°C with ^{125}I-Fab', washed, and then incubated for 0 to 60 min with 1 ml of binding buffer at 37°C. (C) Experiment parallel to that in panel B except that cells were stripped of surface-bound ligand before 60-min incubation at 37°C. (D) Experiment parallel to that in panel B except that cells were incubated with ^{125}I-transferrin. At the indicated times, the amount of ^{125}I-Fab' present in the incubation medium (■), attached to the cell surface, i.e., acid dissociable (□), and remaining cell associated (♦) was determined as described in Materials and Methods. ^{125}I-ligand in each of these three fractions is given as a percentage of the total amount of ^{125}I-ligand bound at the start of the incubation period. Bar shows the distribution of label in the medium at 60 min between TCA-precipitable (stippled) and TCA-soluble (open) counts. Values given were from duplicate samples, which varied less than 10%. Similar results were obtained on three separate occasions.

blasts that had been stained with the anti-p180 MAb followed by an anti-mouse immunoglobulin antiserum conjugated to 8-nm gold particles were embedded and sectioned and the gold particles were visualized by electron microscopy. p180 was predominantly found in coated pits (Fig. 5), indicating that this is the major route of p180 entry into the cell.

Binding assays demonstrated that a significant proportion of p180 is intracellular. Consistent with this observation, the staining of p180 in permeabilized fibroblasts was much more intense than in nonpermeabilized cells and was localized to vesicles that were distributed throughout the cytoplasm. To help identify these vesicles, permeabilized cells were double labeled with anti-p180 antiserum and anti-transferrin receptor MAb. The anti-transferrin receptor MAb gave a staining pattern characteristic of its intracellular distribution in endosomes (15, 16, 33), and there was an almost complete colocalization with p180 (Fig. 4E and F).

p180 shows a restricted cell and tissue distribution. To characterize p180 further, levels of expression in human cell lines and rodent tissues were measured by immunoblotting (Table 1) or quantitative immunoprecipitation from ^{125}I-labeled human cell lines. The only cell lines that expressed high levels of p180 ($\sim 10^6$ molecules per cell) were three diploid fibroblast cultures, two rodent fibroblast cell lines, and an osteosarcoma cell line, MG-63, which are all cells of mesodermal origin. Three other cell lines, HEp-2, KB, and SK-N-SH, showed a low level of p180 expression. Interestingly, with HEp-2 and KB cells, there was no detectable cell surface expression as monitored by immunoprecipitation from ^{125}I-surface-labeled cells or immunofluorescence staining of nonpermeabilized cells, but low levels of p180 were found on intracellular membranes by staining of permeabilized cells.

Of the tissues examined, lung and placenta had the largest amount of p180, with detectable amounts in kidney, bladder, spleen, and skin. No p180 was found in thymus, testes, brain, skeletal or heart muscle, liver, or small intestine. The distribution of p180 within tissues was further examined by

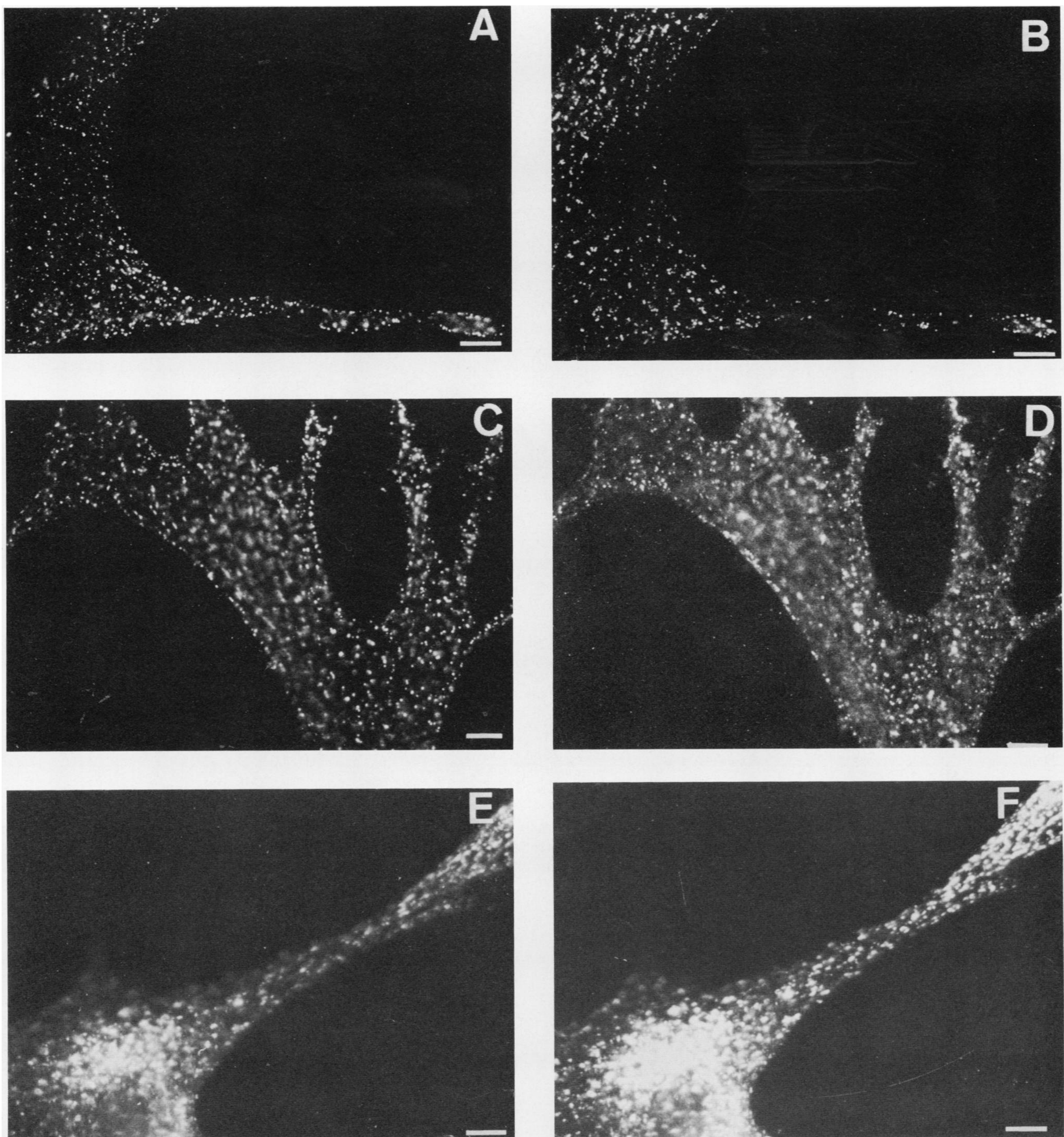

FIG. 4. Immunofluorescent localization of p180 in human fibroblasts. (A and B) Fibroblasts were fixed and stained with anti-p180 MAb E1/183 and FITC-conjugated anti-mouse immunoglobulin (A) and then permeabilized and stained with an anticlathrin polyclonal serum and rhodamine-conjugated anti-rabbit immunoglobulin (B). (C and D) Fibroblasts were fixed and stained with anti-p180 polyclonal antiserum and rhodamine-conjugated anti-rabbit immunoglobulin (C), followed by staining with anti-transferrin receptor MAb and FITC-conjugated anti-mouse immunoglobulin (D). (E and F) Fibroblasts were fixed, permeabilized, and stained with anti-p180 polyclonal antiserum and rhodamine-conjugated anti-rabbit immunoglobulin (E), followed by staining with anti-transferrin receptor MAb and FITC-conjugated anti-mouse immunoglobulin (F). Bar, 5 μm.

immunofluorescence staining of skin and kidney cryosections. The skin is composed of two layers, an outer ectodermally derived epidermis and an underlying mesodermally derived dermis. Only fibroblasts interspersed in the dermis were stained with anti-p180 antiserum (Fig. 6A to C). Transverse sections of the kidney reveal the outer cortex containing the proximal and distal convoluted tubules and glomeruli and the inner medulla containing the collecting ducts and

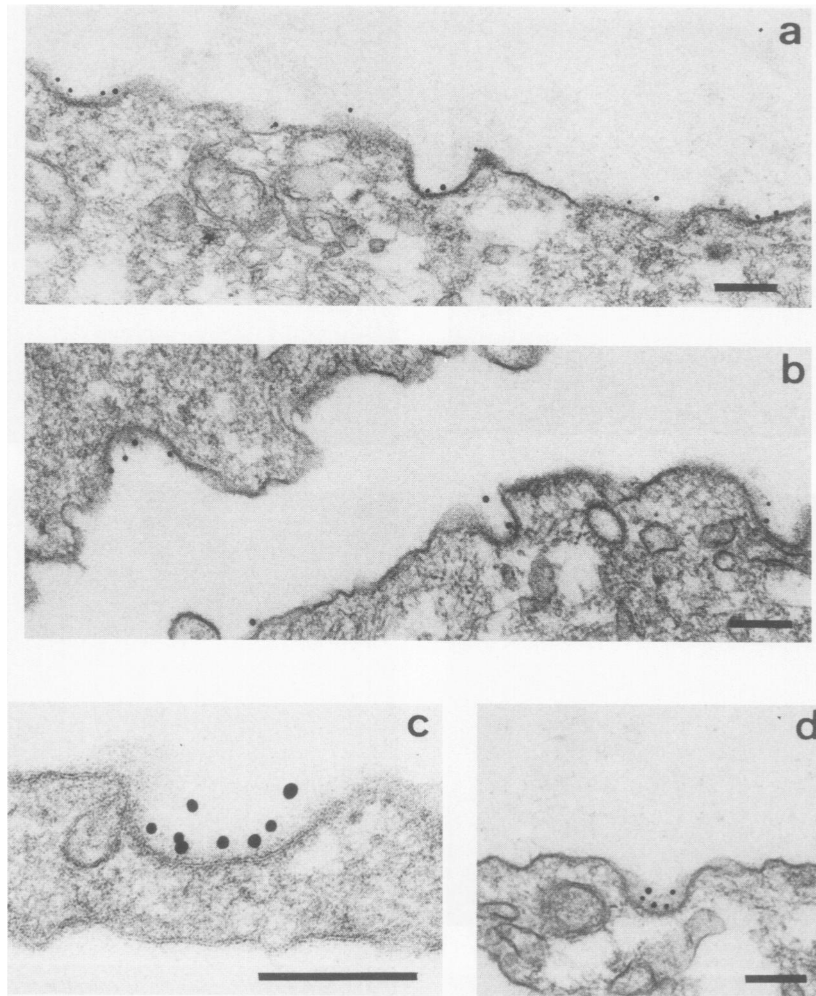

FIG. 5. Immunoelectron microscopic localization of p180 in human fibroblasts. Unpermeabilized cells were fixed, stained with MAb followed by anti-mouse immunoglobulin conjugated to 8-nm gold particles, and then sectioned and visualized in the electron microscope as described in Materials and Methods. Panels a to d show different sections through AG1523 cells stained with anti-p180 MAb E1/183. Bar, 0.1 μm.

thick and thin segments of Henle. Again, only fibroblasts in the connective tissue between the tubules expressed p180 (Fig. 6D to F). At higher magnifications, it could be seen that the p180 was localized within the cells in intracellular vesicles.

Biochemical characterization of p180. In a pulse-chase experiment, it was found that after 15 min of [^{35}S]methionine labeling, a single polypeptide of approximately 170 kDa was immunoprecipitated with the anti-p180 MAb. With increasing chase times, the amount of this immature 170-kDa form decreased with a concomitant appearance of the mature 180-kDa protein. This maturation took 4 h to complete, which suggests that during this period p180 is subject to posttranslational modification, most probably glycosylation (Fig. 7A). To investigate the extent of glycosylation of p180, fibroblasts were cultured in the presence of the antibiotic tunicamycin. Consistent with the pulse-chase experiments, a 2-h labeling period resulted in both the 170- and 180-kDa forms of p180 being immunoprecipitated in the absence of tunicamycin (Fig. 7B). Incubation with 5 μg of tunicamycin per ml for 4 h inhibited the formation of both these forms, and a precursor protein of 150 kDa was observed. At a lower concentration of tunicamycin (0.5 μg/ml), all three forms were seen (Fig. 7B), indicating that p180 has ~30 kDa of oligosaccharide attached to a 150-kDa protein core. Treatment of both 170- and 180-kDa forms of p180 with endoglycosidases suggested that this 30 kDa of carbohydrate is composed of 7 to 10 N-linked complex oligosaccharides and ~5 kDa of sialic acid residues (data not shown). After mild trypsin treatment of fibroblasts, a single 75-kDa immunoreactive fragment of p180 was released into the supernatant (Fig. 1A). As this most probably contains the carbohydrate moiety of p180, a minimum of 45 kDa of the 150-kDa protein core would be exposed on the outside of the cell. The apparent size of immunoprecipitated p180 resolved on a nonreducing gel was ~180 kDa, which shows that p180 does not exist as a dimer or covalently attached to heterologous subunits (Fig. 1B). However, this does not rule out the possibility that p180 is noncovalently associated with another protein(s). Finally, p180 is a relatively stable protein with a half life of 21 to 25 h (Fig. 2A and 7A).

Preliminary experiments had shown that p180 was constitutively phosphorylated when human fibroblasts were labeled to equilibrium with $^{32}P_i$. This initial observation led to two lines of investigation: (i) does p180 itself have intrinsic protein kinase activity, and (ii) is p180 a substrate for known

TABLE 1. p180 expression in tissues and cell lines[a]

Cell line	p180 expression	Tissue	p180 expression
A431 (epidermoid carcinoma)	−	Brain	−
Caco-2 (colonic adenocarcinoma)	−	Heart muscle	−
HeLa (epidermoid carcinoma)	−	Liver	−
HepG2 (hepatoma)	−	Skeletal muscle	−
HPT (colonic carcinoma)	−	Testes	−
HT-29 (colonic carcinoma)	−	Thymus	−
IM9 (B-cell lymphoma)	−		
K562 (erythroleukemia)	−		
RSV-A431 (virally transformed A431)	−		
ST-A431 (virally transformed A431)	−		
1321N1 (astrocytoma)	−		
HEP-2 (epidermoid carcinoma)	+	Bladder	+
KB (epidermoid carcinoma)	+	Kidney	+
SK-N-SH (neuroblastoma)	+	Spleen	+
		Skin[b]	+
AG1523 (diploid fibroblast)	++	Lung	++
Flow 2000 (diploid fibroblast)	++	Placenta[b]	++
F1084 (diploid fibroblast)	++		
MG-63 (osteosarcoma)	++		
Rat-2 (rat fibroblast)	++		
Swiss 3T3 (murine fibroblast)	++		

[a] Cultured cells or homogenized rat tissues were lysed, and samples containing equal amounts (~30 μg) of protein were analyzed by immunoblotting with the anti-p180 polyclonal antiserum. Levels of expression were compared with lysates of MG-63 cells. A high level of expression (++) indicates tissues or cell lines that expressed p180 at the same level as MG-63 cells. Low-level expression (+) indicates detectable p180 expression that was 3- to 10-fold lower than that in control cells. No detectable expression (−) indicates that p180 was not detected in autoradiograms after 1 week of exposure or with prolonged development of the color reaction when a horseradish peroxidase-conjugated anti-rabbit immunoglobulin was used. Some cell lines were also analyzed by quantitative immunoprecipitation from ^{125}I-labeled cells; these results are described in the text.

[b] Placenta and skin were of human origin, and the amount of p180 was quantitated by immunoprecipitation of ^{125}I-labeled membranes and immunofluorescence staining, respectively.

protein kinases? When purified p180 (see next section) or p180 immunoprecipitated from human fibroblasts was incubated with [γ-^{32}P]ATP, there was no evidence of autophosphorylation (Fig. 8A). In addition, p180 was not able to phosphorylate a wide range of protein-tyrosine and protein-serine-threonine kinase substrates under suitable conditions (data not shown). Although these experiments provided no evidence for p180 having an intrinsic kinase activity, it was observed that p180 is a substrate for PKC in vitro (Fig. 8A). To determine whether this reflects physiological events occurring in vivo, human fibroblasts were labeled to equilibrium with $^{32}P_i$ and then treated for 10 min with the phorbol ester TPA, which binds to and activates PKC. This resulted in a dramatic increase in the phosphorylation of p180 (Fig. 8B). p180 phosporylated in vitro by purified PKC or in vivo in TPA-treated cells resulted in the phosphorylation of two novel tryptic phosphopeptides (peptides 1 and 5) and greatly enhanced phosphorylation of another (peptide 2) (Fig. 8C). All these phosphorylation events were on serine residues (data not shown).

Purification and sequencing of p180. p180 was purified from MG-63 human osteosarcoma cells employing the high-affinity human-specific anti-p180 MAb for immunochromatography. In routine large-scale preparations, approximately 3 × 10^9 MG-63 cells were used as starting material, and to observe the purification at each stage, a dish of MG-63 cells labeled with [^{35}S]methionine-cysteine was processed along with the bulk of unlabeled cells. A lysate containing solubilized membrane proteins was passed over two parallel anti-p180 MAb affinity columns, and p180 eluted at high pH. Despite extensive washing of the affinity columns, the p180 protein eluted was always contaminated with other proteins, of which the major components appeared, from their molecular weights, to be actin and myosin (Fig. 9A). To obtain pure p180 protein, the affinity column eluates were fractionated on a FPLC column (Fig. 9B) from which p180 eluted at approximately the position expected for a 180-kDa monomer. To check the purity of p180, a portion was resolved on a reducing SDS-polyacrylamide gel and then stained with silver stain (Fig. 9C). The yield of p180 protein was ~1 μg of pure protein from a 15-cm confluent dish of MG-63 cells. Assuming ~10^6 molecules of p180 per MG-63 cell, this is a yield of 10 to 15%. Purified protein was sequenced from the N terminus on two separate occasions. The sequence obtained was X X P X D A L P E P N V/I F L I, where X indicates an unidentified residue and V/I indicates that on both occasions this residue was read as both valine and isoleucine.

DISCUSSION

p180 recycles via the endocytic pathway. Receptors that cluster in coated pits can be placed in two broad categories depending on whether they exhibit ligand-induced or constitutive receptor internalization. The first is exemplified by the EGF receptor, for which receptors are randomly distributed on the plasma membrane in nonstimulated cells but after the addition of ligand cluster into coated pits and are internalized. From the endosome, ligand-receptor complexes are targeted for degradation in lysosomes (22, 30; reviewed in reference 1). The second category is exemplified by the transferrin and LDL receptors for which, independent of ligand occupancy, receptors are internalized from coated pits on the cell surface into an endocytic pathway and subsequently recycled back to the plasma membrane (5, 15; reviewed in reference 12). Thus, when these receptor pro-

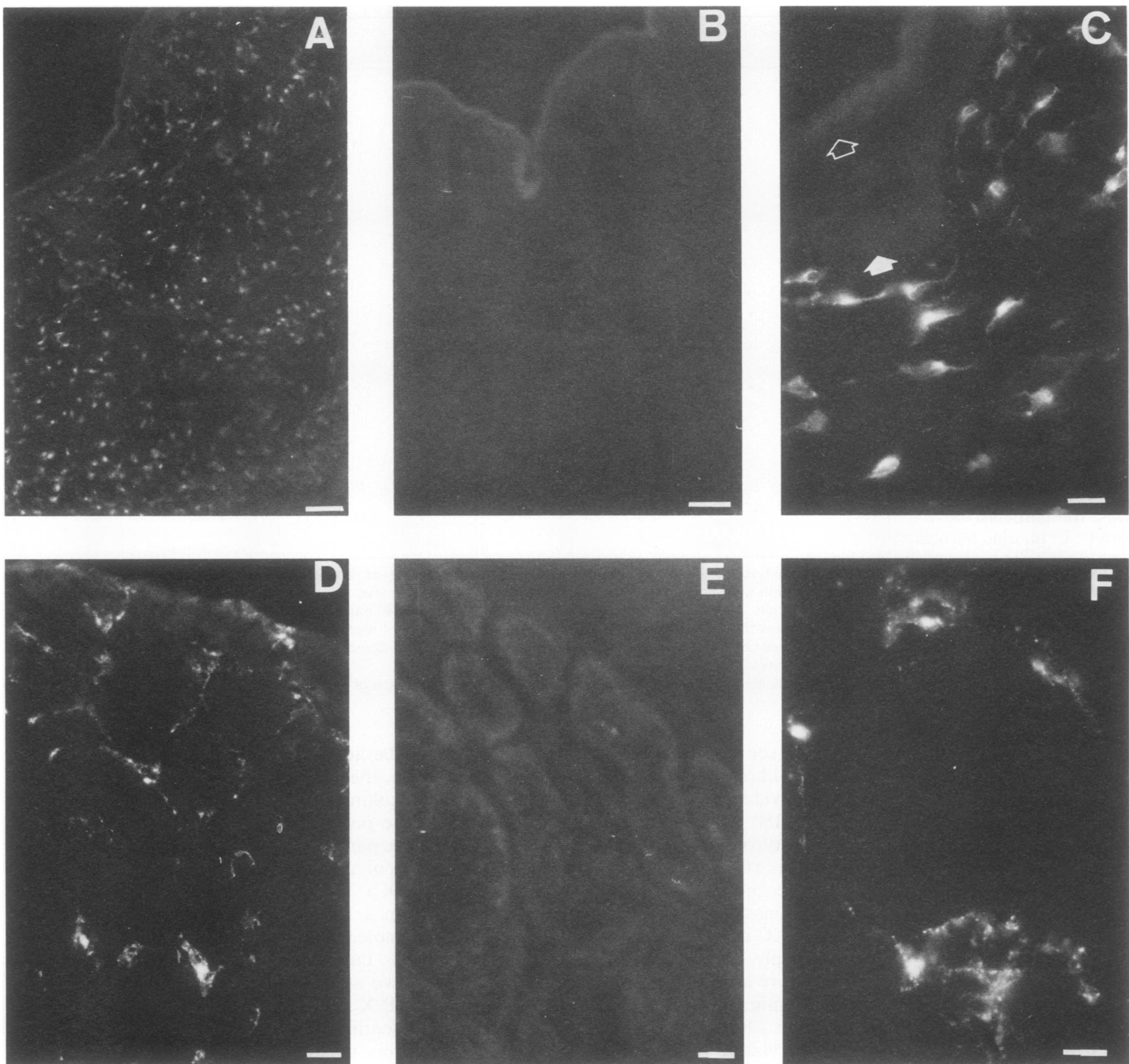

FIG. 6. Immunofluorescent localization of p180 in skin and kidney. Cryostat sections (10 μm) of human skin (A, B, and C) and rat kidney (D, E, and F) were cut and stained with anti-p180 polyclonal antiserum (A, C, D, and F) or with a control rabbit serum (B and E) and counterstained with rhodamine-conjugated anti-rabbit immunoglobulin. Bars, 80 μm (A and B), 20 μm (C, D, and E), and 10 μm (F). In panel C, the filled arrow marks the junction between the epidermis and the dermis, and the open arrow indicates the junction between the stratum corneum and the Malpighian layer.

teins are examined, their location on and in the cell is essentially independent of culture conditions. The results reported in the present study establish that p180 has properties characteristic of this latter class. This conclusion is based on two lines of evidence: (i) the dual distribution of mature p180 on the cell surface and within the cell; and (ii) the internalization of Fab′ fragments bound to p180 and their reappearance in the medium.

Quantitative binding studies with anti-p180 Fab′ on intact and permeabilized cells showed that only 10 to 30% of the mature glycoprotein was on the cell surface (Fig. 2B), where it was predominantly concentrated in coated pits. The coated pits were identified by the underlying clathrin, as visualized by immunofluorescence, and by their structure, as visualized by electron microscopy. The 70 to 90% of p180 found inside the cell could be detected in intracellular vesicles identified as endosomes by the presence of transferrin receptor (Fig. 4 and 5). The immature and mature forms of p180 could be distinguished on the basis of size, and the bulk of p180 in the cell was found in the mature form (Fig. 7 and 9; and immunoblotting data not shown). Thus, although it has not been assessed directly, the large intracellular pool of p180 must be composed of mature molecules rather than being a population of newly synthesized molecules in transit

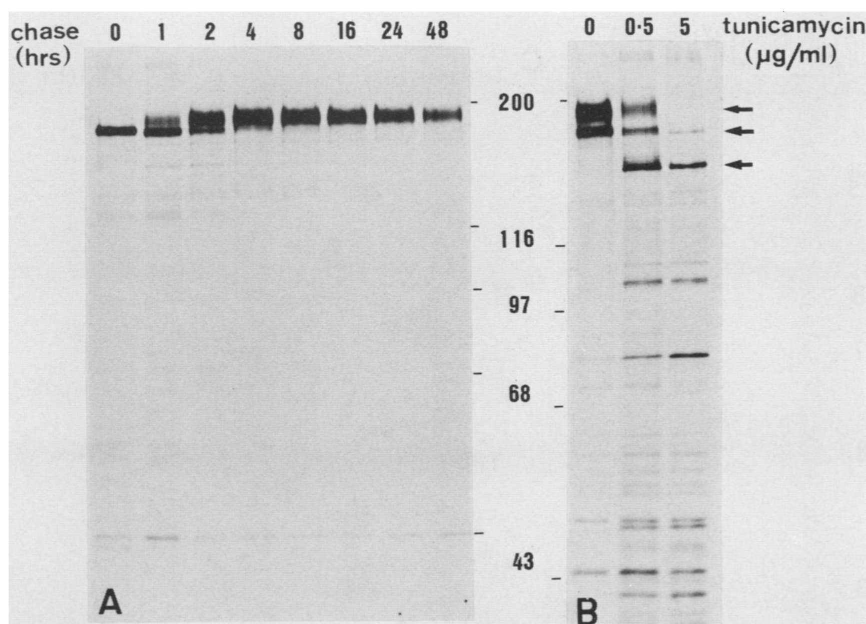

FIG. 7. Biosynthesis of p180 in cultured fibroblasts. Flow 2000 fibroblasts were labeled with [^{35}S]methionine in methionine-free DME for 15 min and then chased for 0 to 48 h in methionine-containing medium (A) or cultured for 4 h in the presence of 0, 0.5, or 5 μg of tunicamycin per ml and labeled with [^{35}S]methionine for the last 2 h (B). Lysates were immunoprecipitated with MAb E1/183, resolved on SDS-7.5% polyacrylamide gels, and exposed to film for 6 (A) or 4 (B) days. Arrowheads mark the three major forms of p180 at 150, 170, and 180 kDa. Numbers in middle show molecular size in kilodaltons.

to the cell surface. This in turn would be consistent with the intracellular p180 molecules having originated through internalization from the cell surface. To demonstrate this, ^{125}I-labeled Fab' fragments bound to p180 were used to follow the fate of p180 molecules on the surface of cells incubated at 37°C. It is clear that at least 60% of p180 molecules on the cell surface were internalized within 2 min at 37°C (Fig. 3A). This rapid rate of internalization is similar to that of receptors, such as the transferrin receptor (16), which are constitutively recycled.

As a first approach to determining the fate of p180 subsequent to internalization, cells were labeled to equilibrium with ^{125}I-Fab', washed, and then incubated without ligand for 0 to 60 min at 37°C. Even if all surface-bound ligand was removed before this 60-min incubation, intact Fab' was detected in the culture medium, showing that at least a proportion of the internalized ligand, presumably still bound to p180, was capable of recycling back to the cell surface (Fig. 3). However, ~70% of the label in the medium represented degraded Fab', raising the question of where in the cell dissociation of ligand might occur and whether degradation of Fab' is accompanied by an equivalent amount of p180 degradation. ^{125}I-Fab' bound to p180 on the cell surface dissociated from the cell surface between pH 5 and 5.5 (data not shown), which compares to other typical receptor ligands that dissociate at pHs between 5.5 and 6 (reviewed in reference 14). It is likely therefore that upon acidification of endosomes some of the Fab' bound to p180 will have dissociated and then have undergone degradation in lysosomes and secretion into the medium as TCA-soluble radioactivity. However, this is clearly not accompanied by an equivalent amount of p180 degradation, since the half-life of the protein only showed a slight decrease from 21 to 17 h in the presence of an excess of anti-p180 Fab' (Fig. 2A). This is in stark contrast to the dramatic reduction in the half-life of a receptor that is subject to ligand-induced receptor internalization and degradation such as the EFG receptor, for which the half-life decreases from 10 to 1.2 h in the presence of EGF (30).

Finally, the kinetics of anti-p180 Fab' internalization and release were compared with those of ^{125}I-transferrin, since the constitutive recycling of the transferrin receptor has been well documented. Morphologically, the distribution of p180 and transferrin receptor in cultured fibroblasts was essentially identical, suggesting that they share a recycling pathway (Fig. 4). The most obvious differences between the behavior of the two ligands was that the release of ^{125}I-anti-p180 Fab into the medium was slower than that of ^{125}I-transferrin and that ~60% of the released ^{125}I-anti-p180 Fab' was degraded, whereas >90% of transferrin was secreted intact. Both these effects may be due to the fact that Fab' is not the physiological ligand for p180. For instance, it has been reported that when A431 cells are incubated with ^{125}I-anti-transferrin receptor MAb, a proportion of the MAb is released in a degraded form (16), and that this release is slower than for transferrin (Clare Futter, Imperial College, personal communication). Alternatively, the ligand for p180 may normally be degraded in lysosomes, while p180 returns to the surface. It is known for a number of recycling receptors, such as the LDL, asialoglycoprotein, and α2-macroglobulin receptors, that the internalized ligand is dissociated and degraded in lysosomes, while the receptor recycles intact (reviewed in reference 12). For the LDL receptor, when cells are incubated at 37°C with an anti-LDL receptor MAb, this MAb is degraded in a manner similar to LDL (4). Whatever the fate of the Fab' ligand, from the results described here it is likely that a significant fraction of p180 molecules are internalized and return to the cell surface within 60 min.

p180 exhibits a restricted tissue and cell type distribution. In the cell lines examined (Table 1), p180 was only expressed at high levels in cultured fibroblasts and the mesodermally

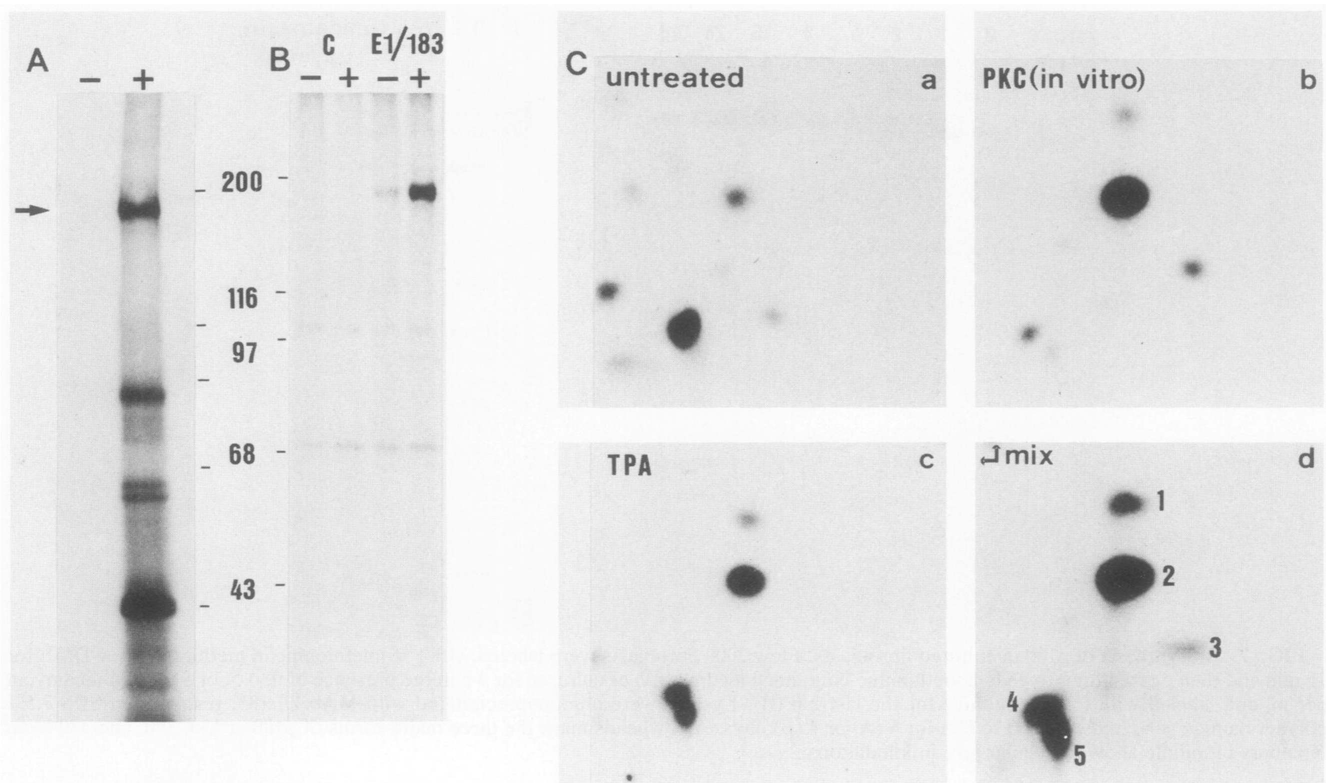

FIG. 8. p180 is a substrate for PKC. (A) Purified p180 was phosphorylated in vitro by purified PKC (+ lane) as described in Materials and Methods, together with a parallel reaction omitting only PKC (− lane). Reactions were resolved on an SDS-7.5% polyacrylamide gel. Arrowhead shows the position of p180. The bands lower than 180 kDa in the + lane were detected when PKC was incubated without p180 (data not shown) and are due to autophosphorylation of PKC and its degraded products. (B) $^{32}P_i$-labeled Flow 2000 human fibroblasts were treated for 10 min without (−) or with (+) TPA. Lysates were immunoprecipitated with an irrelevant control MAb (C) or anti-p180 MAb E1/183 (E1/183) and resolved on an SDS-7.5% polyacrylamide gel. Numbers in middle show molecular size in kilodaltons. (C) p180 was eluted from the gels shown in panels A and B and digested with trypsin, and the resulting phosphopeptides were separated in two dimensions. Electrophoresis was at pH 4.7 in the horizontal dimension (anode on the left, origin at the bottom left), and ascending chromatography was in the vertical dimension. Panels show tryptic peptide maps of p180 phosphorylated in vivo in control (a) and TPA-treated (c) cells. (b) p180 phosphorylated in vitro with PKC; (d) mix of b and c. Cerenkov counts per minute loaded onto each plate and times of exposure with an intensifying screen at −70°C were as follows: (a) 200 cpm, 7 days; (b) 1,000 cpm, 2 days; (c) 400 cpm, 7 days; (d) 1,000 cpm each of b and c, 2 days.

derived human osteosarcoma cell line, MG-63. Interestingly, the low level of expression in the epidermally derived KB and HEp-2 cell lines was restricted to intracellular membranes. [^{35}S]methionine immunoprecipitations and immunoblotting data demonstrated that the p180 in these cells is essentially all in the mature 180-kDa form, and therefore, either the mature p180 is not transported to the cell surface or it is continually internalized from the cell surface. The apparently aberrant expression in these nonmesodermally derived tumor cell lines could be due to prolonged culture in vitro. However, this is not a general feature of tumor cell lines, since p180 was not found either on or in a number of other epithelioid carcinoma cell lines (HPT, HT-29, Caco-2, A431, HeLa) or two lines of virally transformed A431 cells (RSV- and ST-A431) or in two hematopoietic cell lines (IM9 and K562), a liver hepatoma (HepG2), or an astrocytoma (1321N1).

The distribution of p180 in vivo determined by immunoblotting a variety of rat tissues (Table 1) was consistent with cell line expression studies, with p180 found at the highest levels in those tissues with a high content of mesodermally derived cells (i.e., lung and placenta). Essentially, p180 was localized to fibroblastlike cells in fibroconnective tissue (e.g., in the dermis but not the epidermis of the skin and in the interstitial spaces between kidney tubules) (Fig. 6). In addition, staining of fibroblasts could be seen in the fibroelastic capsule surrounding the adrenal gland and in the lamina propria of the bladder (data not shown).

p180 is a novel protein and a physiological substrate for PKC. From experiments described here, it is proposed that p180 is single-chain transmembrane protein (Fig. 1) which is synthesized as a protein backbone of ~150 kDa yielding a 170-kDa glycosylated precursor as the first detectable form of p180. Maturation takes 2 to 4 h and results in a 180-kDa form bearing complex sugars and sialic acid residues (Fig. 7). Of this 180 kDa, at least 75 kDa is located extracellularly (Fig. 1).

In cultured fibroblasts, the phosphorylation of p180 was rapidly and greatly enhanced when cells were stimulated with TPA. The evidence that this phosphorylation event is directly mediated through PKC is that the same three phosphoserine-containing tryptic peptides were found when purified p180 was phosphorylated by purified PKC in vitro (Fig. 8). p180 joins a group of transmembrane proteins that are PKC substrates, many of which are also receptor proteins, including the transferrin receptor (reviewed in refer-

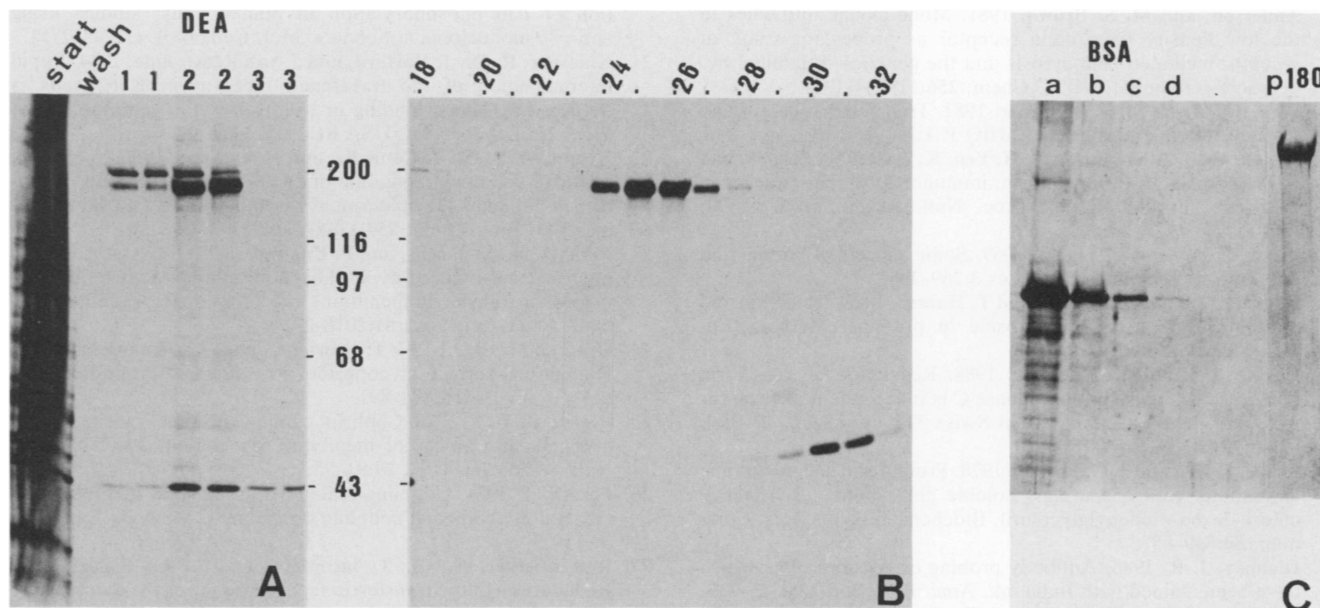

FIG. 9. Purification of p180. p180 was purified from 115 dishes (15 cm) of MG-63 cells as described in Materials and Methods. A tracer dish of cells labeled with [^{35}S]methionine-cysteine was included in the purification, and at each stage 30-μl samples were resolved on SDS-10% polyacrylamide gels. (A) Start, 700 ml of MG-63 cell lysate before loading onto the two anti-p180 MAb affinity columns; wash, 300 ml of wash solution pooled from the two affinity columns; DEA, consecutive fractions eluted with 50 mM diethylamine (DEA) (pH 11.5) from both columns (see Materials and Methods for details). (B) DEA fraction 2 from the two parallel affinity columns shown in gel A was pooled, neutralized, concentrated, and applied to a FPLC Superose 6 column. Fractions 18 to 32 were analyzed by SDS-polyacrylamide electrophoresis and autoradiography. The bulk of myosin eluted earlier than fraction 18; actin can be seen in fractions 30 and 31. Numbers in middle show molecular size in kilodaltons. (C) p180-containing fractions 24 to 27 shown in gel B were pooled, dialyzed, and lyophilized. A portion was resolved on a 10% polyacrylamide gel and silver stained. Purified bovine serum albumin (BSA) was run as a marker; lanes: a, 2 μg; b, 0.33 μg; c, 0.05 μg; d, 0.01 μg.

ence 35). The possible effects of PKC on the behavior of p180 are under investigation. One suggestion is that TPA would modulate the expression of p180 on the cell surface and/or its ability to recycle. However, although PKC-mediated phosphorylation of the transferrin receptor on serine 24 has been shown to result in the downregulation of receptors in some cells (21, 23), mutation of serine 24 does not appear to affect its behavior when transfected into mouse Ltk$^-$ cells (9, 27).

This biochemical analysis of p180 adds to the suggestion that it might be a receptor protein. One approach to determining whether this is the case is to obtain sequence information and to compare p180 structurally with known transmembrane receptors. p180 was purified to homogeneity from the human osteosarcoma cell line MG-63, resulting in an estimated yield of 10 to 15% (Fig. 9). The availability of purified protein has allowed a number of approaches to be taken toward determining the function of p180. For example, here it has been used to examine potential protein kinase activity and the significance of the TPA-induced phosphorylation events observed in vivo (Fig. 8). A polyclonal antiserum was raised against the protein which permitted the study of p180 to be extended to rodent species. Finally, purified protein was used to obtain sequence information from the N terminus. No homology was found with any other protein in the available databases, including several receptor proteins of this size.

The most reasonable explanation for the data on the cellular localization of p180 presented here is that p180 is a receptor which mediates the uptake of an unidentified ligand into fibroblasts and other cells of mesodermal origin and which recycles constitutively. A less likely possibility is that p180 plays some structural role in the formation of coated pits at the cell surface. If this was the case, a non-cross-reactive but functionally equivalent protein would have to perform this function in cells other than fibroblasts, since p180 is not ubiquitously expressed in cells that have coated pits. Identification of molecular clones for p180 and the putative ligand should clarify these issues.

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