The Polymeric Immunoglobulin Receptor Accumulates in Specialized Endosomes but Not Synaptic Vesicles within the Neurites of Transfected Neuroendocrine PC12 Cells

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Abstract. We have expressed in neuroendocrine PC12 cells the polymeric immunoglobulin receptor (pIgR), which is normally targeted from the basolateral to the apical surface of epithelial cells. In the presence of nerve growth factor, PC12 cells extend neurites which contain synaptic vesicle-like structures and regulated secretory granules. By immunofluorescence microscopy, pIgR, like the synaptic vesicle protein synaptophysin, accumulates in both the cell body and the neurites. On the other hand, the transferrin receptor, which normally recycles at the basolateral surface in epithelial cells, and the cation-independent mannose 6-phosphate receptor, a marker of late endosomes, are largely restricted to the cell body. pIgR internalizes ligand into endosomes within the cell body and the neurites, while uptake of ligand by the low density lipoprotein receptor occurs primarily into endosomes within the cell body. We conclude that transport of membrane proteins to PC12 neurites as well as to specialized endosomes within these processes is selective and appears to be governed by similar mechanisms that dictate sorting in epithelial cells. Additionally, two types of endosomes can be identified in polarized PC12 cells by the differential uptake of ligand, a housekeeping type in the cell bodies and a specialized endosome in the neurites. Recent findings suggest that specialized axonal endosomes in neurons are likely to give rise to synaptic vesicles (Mundigl, O., M. Matteoli, L. Daniell, A. Thomas-Reetz, A. Metcalf, R. Jahn, and P. De Camilli. 1993. *J. Cell Biol.* 122:1207–1221). Although pIgR reaches the specialized endosomes in the neurites of PC12 cells, we find by subcellular fractionation that under a variety of conditions it is efficiently excluded from synaptic vesicle-like structures as well as from secretory granules.

PLASMA membrane proteins that normally reside in axonal domains of neurons are selectively targeted to apical domains when expressed in epithelial cells, suggesting an overlap between epithelial and neuronal-targeting mechanisms (Dotti et al., 1991; Powell et al., 1991; Pietrini et al., 1994). The overlap may include endocytotic structures as well, since apical and axonal early endosomes do not accumulate internalized transferrin and so are different from the endosomes at the base of the epithelial cell and in the cell body of the neuron (Fuller and Simons, 1986; Hughson and Hopkins, 1990; Parton et al., 1992; Barroso and Sztul, 1994). Because the latter endosomes primarily recycle proteins involved in cell maintenance functions, we refer to them as housekeeping endosomes. Specialized endosomes, on the other hand, operate in specialized regions of cells, such as the sub-apical cytoplasm, and appear to give rise to small tubulovesicular structures that fuse with the cell surface. Examples of such apically recycling vesicles include those that contain the vasopressin-sensitive water channels in kidney collecting ductules (for review see Verkman, 1992), the gastrin-responsive H^+K^+-ATPase in gastric parietal cells (for review see Forte et al., 1989), and the polymeric immunoglobulin receptor (pIgR)[1] in hepatocytes and MDCK cells (Sztul et al., 1993; Barroso and Sztul, 1994; Apodaca et al., 1994). A characteristic shared by these specialized endosomal pathways is that the rate of recycling is responsive to hormones, such as vasopressin or gastrin, or to activators of protein kinase C (Cardone et al., 1994).

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1. *Abbreviations used in this paper*: dIgA, dimeric immunoglobulin A; ECL, enhanced chemiluminescence; LDL-R, low density lipoprotein receptor; MPR, cation-independent mannose 6-phosphate receptor; pIgR, polymeric immunoglobulin receptor; TfR, transferrin receptor; TX-114, Triton X-114.

Axons also have a regulated endocytotic recycling pathway since synaptic vesicles are recycling endocytotic vesicles whose rate of fusion and perhaps rate of formation by endocytosis can be regulated (for reviews see De Camilli and Jahn, 1990; Jahn and Südhof, 1993; Kelly, 1993).

If targeting to axonal endosomes is related to targeting to apical endosomes, then a marker of apical endosomes should be preferentially targeted to axons. One protein that is targeted to apical endosomes is the pIgR, which mediates transport of polymeric IgA and IgM across epithelial surfaces (for reviews see Mostov et al., 1992; Mostov, 1994). Most of the newly synthesized pIgR is not delivered directly to the apical cell surface but goes via the basolateral surface. In this indirect pathway, the receptor is initially targeted from the TGN to the basolateral cell surface where it binds ligand and is internalized into basolateral endosomes. The receptor/ligand complex is then transported to a specialized apical endosome that gives rise to vesicles which eventually fuse with the apical plasma membrane (Sztul et al., 1993; Barroso and Sztul, 1994; Apodaca et al., 1994). Unlike the transferrin receptor (TfR), the pIgR should be a marker of both basolateral and apical endosomes.

We found by immunofluorescence microscopy that pIgR was much more effectively targeted to endosomes within the processes of neuroendocrine PC12 cells than were markers of housekeeping endosomes. We can conclude that proteins capable of being targeted to apical endosomes can be correctly targeted to specialized endosomes within PC12 neurites, which resemble, in this regard, axonal rather than dendritic processes.

It has been well established that although synaptic vesicles arise by endocytosis (for review see Kelly and Grote, 1993), they exclude endosomal markers such as low density lipoprotein receptor (LDL-R) and TfR (Linstedt and Kelly, 1991; Cameron et al., 1991). Therefore, it has been argued that synaptic vesicles are not simply transport vesicles involved in recycling between the cell surface and the early endosome. TfR and LDL-R, however, are marker proteins for housekeeping endosomes which we now know to be absent from the axon (Cameron et al., 1991; Parton et al., 1992) where assembly of mature synaptic vesicles is likely to occur (Mundigl et al., 1993). If synaptic vesicle proteins recycle through specialized axonal endosomes (Mundigl et al., 1993), it is more appropriate to ask if markers of specialized endosomes are excluded from synaptic vesicles. We found that although pIgR accumulates in specialized endosomes within the neurites, it was efficiently excluded from purified synaptic vesicle-like structures.

Materials and Methods

Materials

Matrigel was obtained from Collaborative Research (Bedford, MA). NGF-β (nerve growth factor), *n*-butyric acid, and poly-D-lysine were obtained from Sigma Chem. Co. (St. Louis, MO). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Corp. (Arlington Heights, IL). Gene pulser electroporation cuvettes with a 0.4-cm electrode gap were purchased from BioRad Labs. (Richmond, CA). Lipofection reagents included Lipofectin (GIBCO BRL, Gaithersburg, MD) and DOTAP (Boehringer Mannheim Corp., Indianapolis, IN). Cell culture reagents were obtained through the University of California (San Francisco, CA) Cell Culture facility. G418 was from GIBCO BRL. Permanox eight chamber slides were manufactured by Nunc, Inc. (Naperville, IL) and purchased from Applied Scientific (San Francisco, CA). Miscellaneous chemical reagents were acquired from Sigma Chem. Co. and Fisher Biochemicals (Santa Clara, CA).

Antibodies

Purified human dimeric IgA (dIgA) was generously provided by J. P. Vaerman. Goat anti-human dIgA, rabbit anti-guinea pig, goat anti-mouse, rabbit anti-mouse, rabbit anti-sheep, and goat anti-rabbit IgG conjugated to HRP, Texas-red, or FITC were obtained from Cappel (West Chester, PA). SC166, a monoclonal antibody against the cytoplasmic domain of the pIgR has been described previously (Solari et al., 1985). Guinea pig polyclonal antiserum against the lumenal domain of the pIgR was generated as described (Breitfeld et al., 1989*a*). Affinity purified sheep polyclonal antibodies directed against the lumenal domain of the pIgR were used in the uptake experiments (see below). Monoclonal antibodies directed against the cytoplasmic domain of synaptophysin were purchased from Sigma Chem. Co. (SVP-38). Ascites fluid for C7-IgG anti-human LDL-R (Beisiegel et al., 1981) was produced as described (Green et al., 1994). Mouse ascites fluid containing monoclonal antibodies against the cytoplasmic domain of the human TfR (H68.4, White et al., 1992) was the kind gift of Ian S. Trowbridge (Salk Institute, La Jolla, CA). Antiserum against rat synaptotagmin was kindly provided by Richard Scheller (Stanford University, Palo Alto, CA). IgG purified from polyclonal rabbit antiserum raised against rat cation-independent mannose 6-phosphate receptor (MPR) was the kind gift of William Brown (Cornell University, Ithaca, NY).

Cell Culture and Transfection

PC12 cells were grown in DME-H21 medium supplemented with 10% horse serum, 5% FBS, 100 U/ml Penicillin and 100 μg/ml streptomycin in humidified incubators with 10% CO_2. cDNA encoding the wild-type (Mostov et al., 1984) or Asp^{664} mutant (Casanova et al., 1990) forms of the rabbit pIgR was subcloned into the cytomegalovirus-based expression vector, pCB6 (Brewer and Roth, 1991). cDNA encoding the human LDL-R (Davis et al., 1986) was subcloned into pCB6 as described (Green et al., 1994). G418-resistant clones stably expressing wild-type pIgR were obtained after transfection by lipofection according to the method described by Muller et al. (1990). Clones were screened by immunoprecipitation of cell lysates after metabolic labeling. Six clones were characterized further by cell fractionation and Western blot analysis (described below).

Human LDL-receptor and mutant Asp^{664} pIgR were expressed transiently in wild-type PC12 cells or those stably expressing pIgR by electroporation according to the method described by Muller et al. (1993). Rapidly dividing cells were washed, pelleted, and resuspended in electroporation buffer (137 mM NaCl, 5 mM KCL, 0.7 mM Na_2HPO_4, 6 mM Dextrose, 20 mM Hepes, pH 7.05) at $\sim 4 \times 10^7$ cells/ml. 30–60 μg of cDNA was mixed with 0.8 ml of cell suspension and added to each electroporation cuvette (Biorad Labs.). Cells were incubated on ice for 10 min, pulsed at 230 V, 500 μF, and then incubated for an additional 10–20 min on ice before plating onto tissue culture dishes. After 12–18 h, the medium was changed and the cells were differentiated by plating onto matrigel-coated tissue culture dishes or chamber slides and incubation in the presence of 5 ng/ml NGF-β for 48 h as described (Elferink et al., 1993). To overexpress pIgR, sodium butyrate was added to the medium at a final concentration of 6 mM for 12–18 h (Gorman et al., 1983).

Metabolic Labeling and Immunoprecipitation

PC12 cells were metabolically labeled overnight by incubating cells in DME-H21 medium depleted of cysteine and methionine but supplemented with 1% horse serum, 0.5% FBS and 100 μCi/ml of ^{35}S-Translabel (ICN Biomedicals, Irvine, CA). For the cell surface delivery assay (described below), cells were labeled for 15 min in serum-free medium containing ^{35}S-Translabel before transfer to label-free medium for various periods of chase. Immunoprecipitation of pIgR from labeled cell lysates was performed as described previously (Breitfeld et al., 1989*b*). After immunoprecipitation, samples were analyzed by SDS-PAGE on 8% gels. Gels were processed as described (Green and Kelly, 1992) and analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) or by fluorography.

Cell Surface Delivery Assay

A protease sensitivity assay was performed essentially as described earlier (Breitfeld et al., 1990). PC12 cells stably expressing pIgR were pulse labeled as described above and chased at 37°C, 10% CO_2, in medium con-

taining 25 μg/ml trypsin for 15 min to 2 h. Parallel time points were obtained by chasing in trypsin-free medium. At the end of each time point, cells were placed on ice, washed, and lysed immediately in the presence of excess FCS. Immunoprecipitation and analysis of full-length intracellular pIgR was as described above.

Immunofluorescence Microscopy

PC12 cells stably transfected with pIgR were differentiated in the presence of NGF on matrigel-coated chamber slides as described above. For some experiments, cells stably expressing pIgR were transiently transfected with cDNA encoding the human LDL-R before differentiation. Control experiments were performed on differentiated wild-type PC12 cells. For uptake experiments, cells were placed onto metal plates in either a 37°C water bath or on ice. Cells were washed with Eagle's MEM/0.6% BSA/20 mM Hepes/pH 7.4, before addition of ligand. The following ligands were used: Human dIgA; polyclonal sheep anti-pIgR lumenal domain; and C7-IgG (anti-LDL-R) in MEM/0.6% BSA/20 mM Hepes/pH 7.4. Cells were incubated for either 5 or 20 min at 37°C or on ice. At the end of the incubation period, cells were placed on ice and washed extensively with ice-cold medium and PBS. Fixation was in 4% paraformaldehyde in PBS for 10 min on ice and an additional 15 min at room temperature followed by quenching with PBS/25 mM glycine. Nonspecific-binding sites were then blocked with PBS/1% fish skin gelatin (Sigma Chem. Co.)/2% BSA. In some experiments, the blocking solution also contained 0.02% saponin in order to permeabilize the plasma membrane. Subsequent incubations with fluorescently labeled secondary antibodies were carried out in blocking solution either with or without saponin. For steady state labeling, cells were fixed and permeabilized as above before incubation with primary antibodies. All washes were performed in PBS/0.02% saponin/1% fish skin gelatin/2% BSA (permeabilized cells) or in PBS/1% fish skin gelatin/2% BSA without saponin (unpermeabilized cells). Cells were mounted in PBS containing 90% glycerol and 10 mg/ml *p*-diamino benzene. Conventional images were documented using a Zeiss axiophot or a Leitz photomicroscope. Confocal images were obtained using a BioRad MRC 600 confocal laser scanning microscope.

Semiquantitative analysis of the images was performed by scoring each cell according to the pattern of immunofluorescence. The identity of each chamber slide was kept hidden from the observer until after the analysis was complete. The amount of immunoreactivity in the processes as compared to the cell bodies was assessed by a subjective impression of the density and brightness of puncta in each region. Labeling of large lysosome-like structures in the cell bodies was not included in the comparison.

Quantitative analysis of the staining intensity was accomplished using the confocal microscope and Comos software: series of optical sections 1 μm apart along the Z-axis were acquired from cells probed for TfR or pIgR. Each Z-series was projected onto a single image such that every pixel of the resulting image represented the maximum intensity value found in any of the individual sections. We measured the average pixel intensity in the cell bodies, excluding the nuclei, and compared these values to the average pixel intensity in the tips of the neurites.

Uptake of dIgA before Subcellular Fractionation

Human dIgA was iodinated using a modification of the iodine monochloride method described by Goldstein et al. (1983) (Breitfeld et al., 1989*a*). Undifferentiated PC12 cells expressing pIgR were incubated on tissue culture dishes in Hepes-buffered MEM/3% BSA in the presence of [^{125}I]dIgA for 2 h 40 min at 37°C. Cells were washed extensively with label-free medium on ice, and then chased in label-free medium for an additional 20 min at 37°C. In parallel experiments, cells were incubated in medium containing unlabeled dIgA (25 μg/ml). After exposure to ligand, cells were fractionated by velocity sedimentation as described below. Fractions were analyzed using a gamma counter and by Western blot.

Subcellular Fractionation

All steps were performed at 4°C. A postnuclear supernatant (S1–1,000 *g* for 5 min) and a high-speed supernatant, enriched in synaptic vesicles (S2–27,000 *g* for 35 min), were prepared from PC12 cells and analyzed by velocity sedimentation essentially as described earlier (Clift-O'Grady et al., 1990). Homogenization of the cells was improved using eight passes through a ball-bearing homogenizer with 12 μm clearance (Cell Cracker, European Molecular Biology Laboratory, Heidelberg, Germany). The velocity sedimentation gradients (5–25% glycerol in 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, 10 mM Hepes, pH 7.4 on top of a buffered 50% sucrose pad) were centrifuged for 1 h at 40,000 rpm in a SW 55 Ti rotor (Beckman Instrs., Inc., Palo Alto, CA).

Analysis of a postnuclear supernatant from PC12 cells by equilibrium density gradient centrifugation on isosmotic metrizamide/sucrose gradients was performed as described (Green et al., 1994). Briefly, cells were harvested in PBS and homogenized in sucrose buffer (0.32 M sucrose, 0.5 mM EDTA, 4 mM Hepes, pH 7.4) with 10–15 strokes in a Dounce homogenizer. A postnuclear supernatant (740 *g* for 8 min) was layered on top of linear isosmotic metrizamide/sucrose gradients (10% metrizamide/0.23 M sucrose to 23% metrizamide/0.11 M sucrose on top of a buffered 35% metrizamide pad) and centrifuged for 2 h and 15 min at 55,000 rpm in a fixed angle 70.1 Ti rotor (Beckman Instrs.).

Immunoblotting

Samples were subjected to SDS-PAGE, transferred to nitrocellulose using a semidry blotter (E & K, Saratoga, CA), blocked in PBS/0.05% Tween/5% non-fat dry milk, and probed with the following primary antibodies: pIgR (SC166 or guinea-pig anti-SC), synaptophysin (SVP-38), TfR (H68.4), and synaptotagmin (rabbit polyclonal antiserum). Secondary antibodies used were HRP-conjugated goat anti-mouse and goat anti-rabbit IgG. Bands were visualized using the ECL system and quantified by optical densitometry.

Flotation Gradient Analysis and Triton X-114 Phase Extractions

Velocity gradient fractions and postnuclear supernatants were analyzed by flotation gradient centrifugation. Samples (a postnuclear supernatant and the top three fractions from a velocity gradient) were taken up in 1.5 M sucrose (final concentration) and overlayed with a sucrose step gradient: 1.0 M sucrose and 0.4 M sucrose, covered with 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, and a layer of paraffin oil; all solutions contained 10 mM Hepes, pH 7.4, and protease inhibitors. Gradients were centrifuged in an SW 55 Ti rotor for 12 h at 40,000 rpm. Samples were subsequently collected from each interface and analyzed by SDS-PAGE and Western blot.

Triton X-114 (TX-114) phase extractions were performed on gradient fractions and whole cell lysates according to the method of Bordier (1981). TX-114 was added to a final concentration of 1% to the bottom 4 and the top 3 fractions from a velocity gradient on ice. Cells were lysed in ice-cold PBS/1% TX-114. Phase separation was achieved by warming samples to 37°C followed by centrifugation at 1,000 *g* for 5 min. Analysis of aqueous and detergent phases was by SDS-PAGE and Western blot as described above.

Results

Stable Expression of Rabbit Polymeric Immunoglobulin Receptor in PC12 Cells

PC12 cells were stably transfected with cDNA encoding the rabbit pIgR (Mostov et al., 1984). G418-resistant clones expressing the receptor were identified by immunoprecipitation of pIgR from cell lysates of metabolically labeled cells. Fig. 1 shows an autoradiogram of some of the immunoprecipitants after SDS-PAGE. All of the positive clones except for one displayed two characteristic bands of M_r 100–105 kD. These bands comigrated with pIgR isolated from transfected MDCK cells (data not shown). Previous work demonstrates that this pattern corresponds to the Endo H resistant, fully glycosylated form of the receptor (Mostov and Deitcher, 1986). The authors assume that the two species arise from variable processing of the oligosaccharides. A single clone (lane *3*) appeared to be truncated and was not detected when probed with an antibody that recognizes the cytoplasmic domain of the receptor. This clone was not characterized further.

To ensure that pIgR does not become arrested in a biosynthetic compartment, we determined the kinetics of delivery

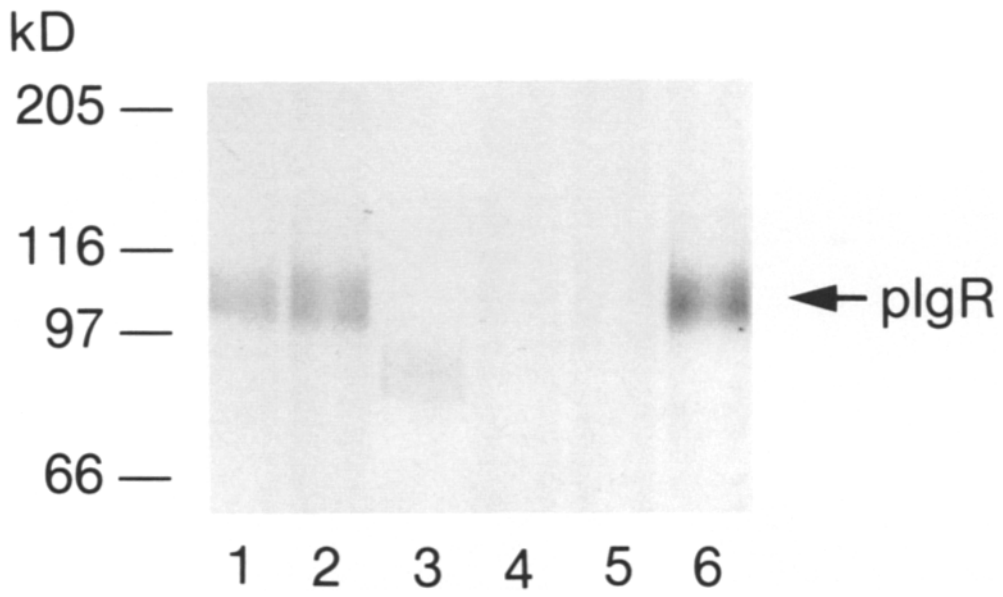


Figure 1. Expression of polymeric immunoglobulin receptor (pIgR) in stably transfected PC12 cells. cDNA encoding pIgR was introduced into PC12 cells by lipofection. G418-resistant clones were metabolically labeled overnight with ^{35}S-Translabel. pIgR was immunoprecipitated from cell lysates with antibodies directed against the lumenal domain of the receptor and analyzed by SDS-PAGE and fluorography. Positive clones with a pattern typical of fully glycosylated pIgR migrate at M_r 100–105 kD (lanes *1*, *2*, and *6*). A truncated form of pIgR lacking cytoplasmic portions of the receptor was observed in one clone (lane *3*). Some G418-resistant clones did not express detectable pIgR (lanes *4* and *5*).

of newly synthesized receptor to the cell surface using a protease sensitivity assay (Breitfeld et al., 1990). Transfected cells were pulse labeled with ^{35}S-Translabel and chased for various periods of time in medium containing trypsin, which cleaves the pIgR as it appears on the cell surface. Under these conditions, the receptor is delivered to the plasma membrane with a half time of less than 30 min (Fig. 2), which is comparable to the time course observed for delivery of the receptor to the basolateral surface in transfected MDCK cells (Breitfeld et al., 1990). These kinetics are consistent with constitutive delivery to the plasma membrane and suggest that little or no pIgR is targeted to regulated secretory granules. In the absence of trypsin, more than 80% of the labeled receptor survives a 2-h chase, implying little intracellular or surface protease activity (data not shown). This situation differs from hepatocytes (Musil and Baenzinger, 1987), and transfected fibroblasts (Deitcher et al., 1986) or MDCK cells (Breitfeld et al., 1989*a*), which possess an endogenous protease that efficiently cleaves the receptor at the cell surface.

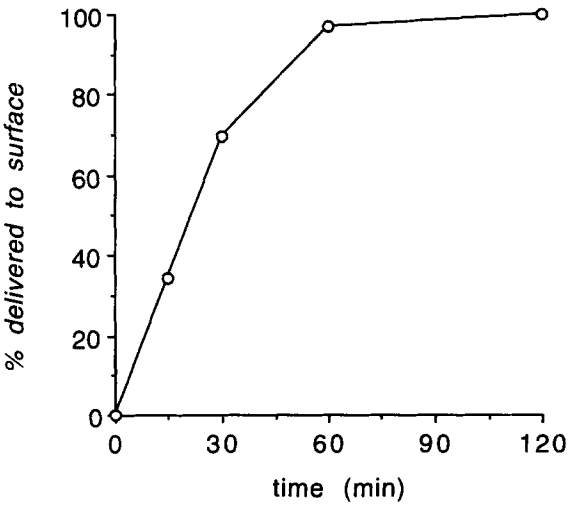
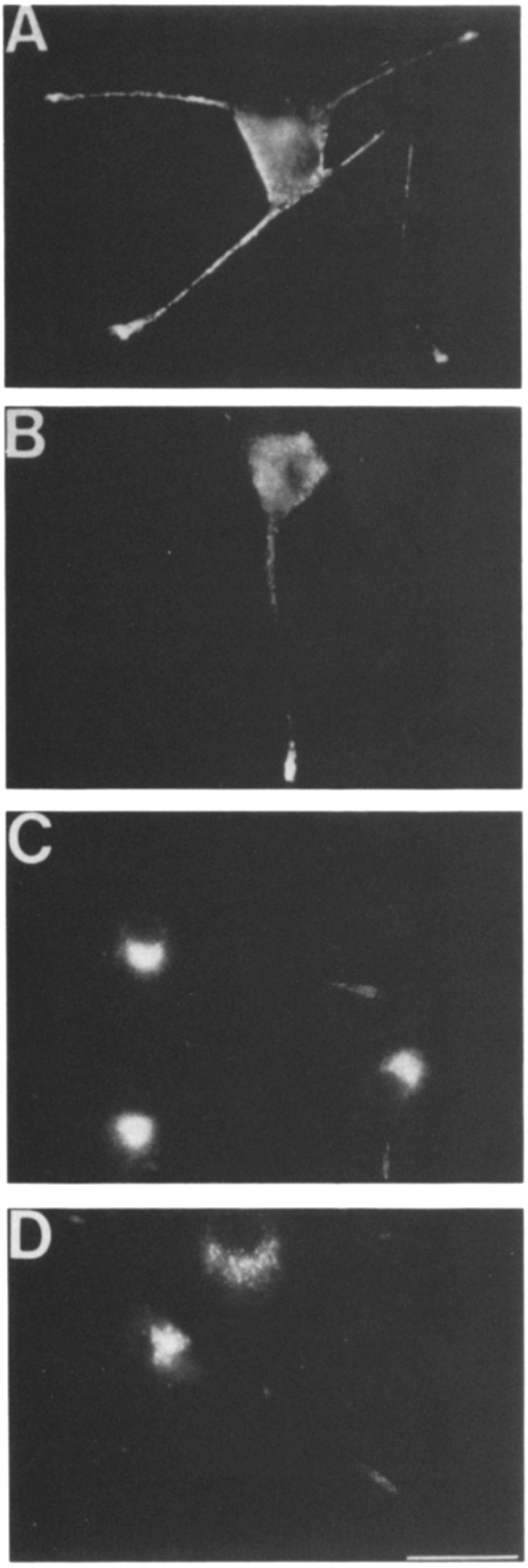


Figure 2. Delivery of newly synthesized pIgR to the cell surface. PC12 cells stably expressing pIgR were pulse labeled with ^{35}S-Translabel for 15 min at 37°C, and then chased for the indicated periods of time in medium containing 25 μg/ml trypsin effectively cleaving any receptor which reached the cell surface. At each time point cells were lysed on ice in the presence of excess serum. pIgR was immunoprecipitated and analyzed by SDS-PAGE. The full-length intracellular receptor was quantified using a phosphorimager. The percentage of labeled pIgR reaching the cell surface is plotted against time. Newly synthesized receptor is delivered to the cell surface with a $t_{1/2}$ of less than 30 min.

Polymeric Immunoglobulin Receptor Is Targeted to the Neurites of Polarized PC12 Cells

To determine where pIgR is targeted, we analyzed its subcellular distribution in NGF-differentiated, stably transfected PC12 cells by immunofluorescence microscopy. We compared the localization of pIgR at steady state to the following membrane proteins whose distribution has been established in neurons: the synaptic vesicle-specific protein synaptophysin, which is present in the cell body and is axonally transported to the nerve terminal (Bööj et al., 1989; Fletcher et al., 1991; Cameron et al., 1991); the TfR, a basolaterally recycling receptor which is poorly transcytosed in epithelial cells (Fuller and Simons, 1986; Hughson and Hopkins, 1990) and is restricted to the cell body of neurons (Cameron et al., 1991; Parton et al., 1992); and the cation-independent MPR, a marker of late endosomes which is also confined to the cell body of neurons (Parton et al., 1992).

Transfected cells were plated on matrigel-covered chamber slides and allowed to extend processes in the presence



of NGF. After 48 h, more than 95% of the cells had visible neurites. Many of the cells had multiple processes. pIgR staining was primarily intracellular and punctate throughout the cell. Many of the cells displayed an accumulation of the receptor in the perinuclear region. pIgR was detected along the entire length of the processes and often showed enrichment in the tips (Fig. 3 *A*). Synaptophysin had a similar overall distribution (Fig. 3 *B*), which resembled that described in cultured hippocampal neurons (Cameron et al., 1991; Fletcher et al., 1991). Punctate staining was again visible throughout the cell body and the neurites but there was a less pronounced perinuclear accumulation of the protein. In contrast, TfR and MPR appeared to be largely restricted to the cell body (Fig. 3, *C* and *D*). Both proteins demonstrated primarily a perinuclear-staining pattern. MPR puncta appeared larger than those of the other markers. In most cells, both endosomal marker proteins could be detected in the neurites, although the signal was minimal as compared to the amount of staining in the cell bodies.

To confirm our interpretation of the staining patterns, semiquantitative analysis was performed by counting and scoring cells in a double blind manner (Fig. 4). Each cell was scored according to the degree of staining in the tips of the neurites as compared to the cell bodies. Analysis of the steady state distribution of pIgR and synaptophysin revealed that for both markers more than 90% of the cells showed equal or enhanced staining of the tips of the processes as compared to the cell body and in no case were synaptophysin and pIgR excluded from the processes. In striking contrast, analysis of TfR and MPR immunoreactivities showed that these proteins are largely enriched in the cell body and more effectively excluded from the neurites. For TfR, only ~25% of the cells showed equal or enhanced staining in the neurites as compared to the cell body. Similarly, MPR scoring revealed that ~15% of the cells displayed equally intense staining in the neurites and the cell body and in no case was the receptor enriched in the processes.

The validity of the scoring system described above was confirmed by quantitative immunofluorescence using confocal microscopy and image analysis. The images of 10 cells

Figure 3. Steady-state distribution of pIgR, synaptophysin (p38), transferrin receptor (TfR), and the cation-independent mannose 6-phosphate receptor (MPR) in NGF-differentiated PC12 cells by immunofluorescence microscopy. PC12 cells stably expressing pIgR were induced to extend processes in the presence of NGF. The pattern of pIgR immunoreactivity (*A*) was determined by incubating fixed cells with antibodies against the cytoplasmic domain of the receptor (SC166, mouse monoclonal). P38 immunoreactivity (*B*) was obtained by incubation of cells with antibodies directed against the cytoplasmic domain of the protein (SVP-38, mouse monoclonal). TfR (*C*) was detected by a monoclonal antibody against the cytoplasmic domain of the receptor (H68.4, mouse monoclonal). MPR-containing structures (*D*) were visualized by incubating cells with an affinity purified IgG fraction from rabbit polyclonal antiserum (anti-rat MPR). Primary antibodies were visualized by incubation with Texas-red or FITC-conjugated secondary antibodies. Both pIgR (*A*) and p38 (*B*) show predominantly intracellular punctate staining in the cell body and the tips of the neurites. TfR and MPR immunoreactivity are mainly confined to the cell body. Bar, 10 μm.

probed for TfR and scored as tip staining less than cell body staining according to our semiquantitative assay, and 10 cells probed for pIgR and scored as either tip staining equal to cell body staining or tip staining greater than cell body staining were processed and analyzed as described in Materials and Methods. The mean ratios of average pixel intensity in the cell bodies to average pixel intensity in the tips of the neurites were 2.1 (SEM = 0.15) for TfR and 0.9 (SEM = 0.06) for pIgR. The difference between these values is statistically significant ($p < 0.001$; unpaired Student's t test). Although such quantitative analysis was not possible for the many hundreds of cells that were screened in this study, it was a useful confirmation that our visual assessments were correct.

Thus, PC12 cells, in the presence of NGF, appear to be functionally polarized with regard to the targeting of membrane proteins to their neurites. Although TfR and MPR are largely restricted to the cell body, pIgR, like the synaptic vesicle protein synaptophysin, is efficiently targeted to both the cell body and the neurites.

Polymeric Immunoglobulin Receptor Is Present in Specialized Endosomes within the Neurites

To determine whether the neurites of the differentiated PC12 cells contained functional endosomes and whether pIgR was targeted to them, a series of uptake experiments was performed. Endosomes containing pIgR were labeled by measuring uptake of the physiologic ligand, dIgA (Fig. 5 *A*). Cells were incubated for 20 min at 37°C in the presence of either 50 μg/ml or 150 μg/ml of human dIgA. Both concentrations of ligand gave indistinguishable results and similar results were also obtained when the uptake experiments were performed for 5 min. Endosomes containing dIgA were easily visible throughout the neurites and the cell body. Uptake of dIgA by untransfected cells was not detected indicating that the ligand was not internalized by fluid-phase endocytosis at these concentrations (data not shown).

To confirm that the staining pattern indeed reflected internalized dIgA rather than ligand bound to pIgR on the plasma membrane, cells were analyzed by confocal microscopy (see

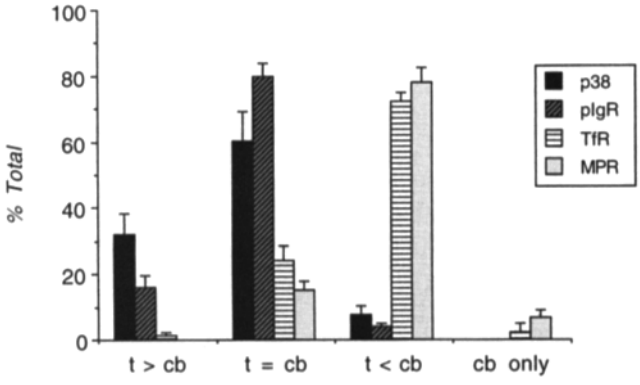


Figure 4. Semiquantitative analysis of the distribution of pIgR, p38, TfR, and MPR in NGF-differentiated PC12 cells processed by immunofluorescence microscopy. PC12 cells were processed as in Fig. 3. Cells were scored according to their pattern of immunoreactivity: predominant staining of the tips of the processes as compared to the cell body ($t > cb$); equivalent staining in both the tips of the neurites and the cell body ($t = cb$); diminished staining of the tips of the processes as compared to the cell body ($t < cb$); complete exclusion of immunoreactivity from the tips of the neurites (*cb* only). Approximately 200 cells were counted for each marker in each experiment. Shown are average values from two independent experiments; the error bars representing the range.

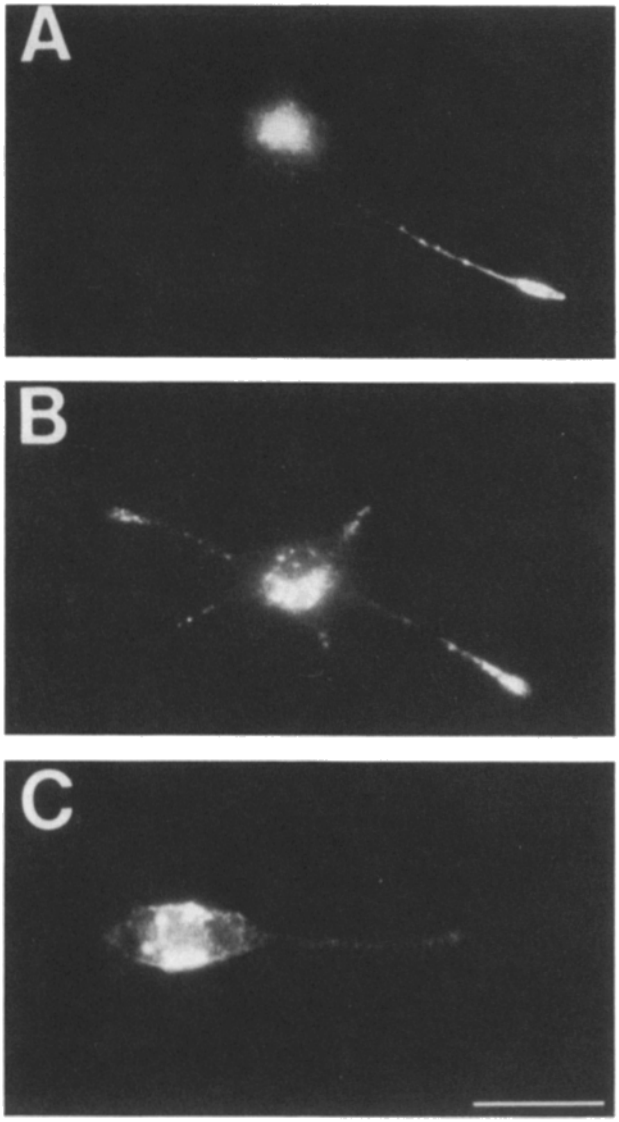


Figure 5. Uptake of ligand into endosomes in the neurites and cell body of polarized PC12 cells as assayed by immunofluorescence microscopy. PC12 cells stably expressing pIgR were transiently transfected with cDNA encoding the human LDL-R and induced to extend processes in the presence of NGF. Unfixed cells were incubated with ligand for 20 min at 37°C followed by extensive washing before fixation and detection with fluorescently conjugated secondary antibodies. Endosomes containing pIgR were labeled with either dimeric IgA (*dIgA*) (*A*) or a sheep polyclonal antibody (*anti-SC*) (*B*) directed against the lumenal domain of the receptor. Endosomes containing the human LDL-R were labeled with a monoclonal antibody directed against the lumenal domain of the receptor (*C7-IgG*) (*C*). Endosomes containing pIgR ligand are present throughout the cell in both the cell body and the neurites while endosomes containing LDL-R ligand appear to be largely confined to the cell body. Bar, 10 μm.

Fig. 7). A series of confocal sections obtained from cells after uptake of dIgA unambiguously demonstrates the intracellular localization of the ligand in both the cell body and the neurites (see Fig. 7, *A–D*). The dIgA pattern is clearly distinct from the staining observed under conditions where only ligand on the cell surface was accessible to the labeled anti-dIgA antibodies (see Fig. 7, *E–H*). Furthermore, no dIgA could be detected intracellularly when the uptake experiment was carried out at 0°C (data not shown).

To determine whether the pIgR-containing endosomes in the neurites were housekeeping or specialized endosomes, the human LDL-R, a marker of housekeeping endosomes, was transiently transfected into cells expressing pIgR before differentiation in the presence of NGF. Endosomes containing the LDL-R were then labeled by internalization of a monoclonal antibody (C7-IgG) directed against the lumenal domain of the human receptor. This antibody does not recognize the endogenous rat LDL-receptor. Trafficking of this antibody has previously been shown to closely parallel that of the physiologic ligand, LDL (Beisiegel et al., 1981). Cells were incubated in the presence of C7-IgG for 20 min at 37°C before fixation and immunofluorescence. In contrast to the results observed for dIgA, uptake of this ligand was confined primarily to the cell body. In most instances, endosomes containing the LDL-R were only marginally detectable in the neurites or were completely excluded (Fig. 5 *C*). Parallel experiments conducted at 0°C did not reveal any specific staining. No uptake was observed by untransfected cells indicating the absence of fluid-phase uptake of the C7-IgG.

Because C7-IgG is targeted to lysosomes (Beisiegel et al., 1981) while dIgA is not (Mostov and Deitcher, 1986), C7-IgG is found in large dense structures in addition to smaller punctate structures. To compare internalization of two anti-receptor antibodies, we also looked at uptake of a polyclonal anti-pIgR antibody. Staining of endosomes in both the neurites and the cell body was again visible (Fig. 5 *B*). In addition to small punctate structures, larger puncta were observed in the cell body presumably because of delivery of cross-linked structures to the lysosomal pathway. When parallel experiments were performed on ice or in untransfected cells, no specific intracellular staining was detected.

Semiquantitative analysis confirmed the segregation of endosomal classes: while more than 84% of the cells demonstrated at least equal uptake of pIgR ligand into neurite endosomes as compared to cell body endosomes, only ~10% of the cells internalized LDL-R ligand equally well into neurite and cell body endosomes (Fig. 6). Thus, two forms of endocytosis can be distinguished in PC12 cells because of their spatial resolution. Endosomes in the neurites (Fig. 7) differ from those in the cell body in that they appear to take up LDL-R ligand very poorly whereas pIgR seems to be internalized into both populations of endosomes equally well.

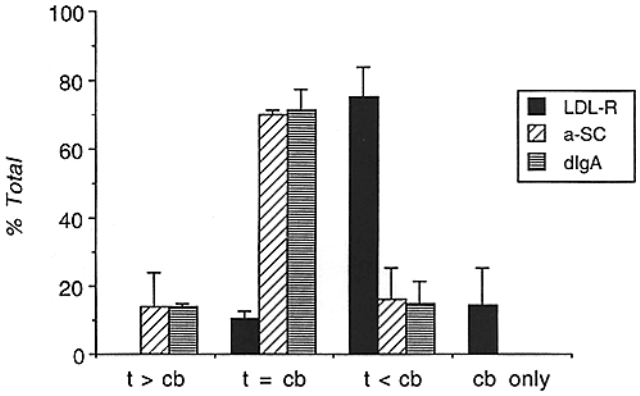


Figure 6. Semiquantitative analysis of the uptake of ligand into polarized PC12 cells. Cells were processed as in Fig. 5. Labeling of punctate structures in the cell body by dIgA, sheep anti-SC polyclonal antibodies, and C7-IgG anti-LDL-R monoclonal antibodies was compared to the labeling of the neurites and scored as described in Fig. 4. Lysosome-like structures were not included in the comparison. Approximately 200 cells were counted for each marker for each experiment. Shown are average values from two independent experiments; the error bars representing the range.

The Polymeric Immunoglobulin Receptor Is Excluded from Synaptic Vesicles

The above experiments confirm that pIgR selectively accumulates in specialized endosomes within the neurites of differentiated PC12 cells. Recent evidence suggests that synaptic vesicles may be derived from specialized endosomes within axons of hippocampal neurons (Mundigl et al., 1993). PC12 cells produce vesicles that have the same size, density, and composition as authentic neurotransmitter-containing synaptic vesicles found in brain (Navone et al., 1986; Wiedenmann et al., 1988; Johnston et al., 1989; Clift-O'Grady et al., 1990; Bauerfeind et al., 1993). To determine whether pIgR was targeted to synaptic vesicles, we fractionated NGF-differentiated transfected PC12 cells by differential centrifugation and velocity sedimentation as described previously (Clift-O'Grady et al., 1990). Postnuclear supernatants were subjected to centrifugation on glycerol gradients. Under these conditions synaptic vesicles, as identified by synaptophysin immunoreactivity, sedimented as a homogeneous population of 80-S particles in the upper third of the gradient (Fig. 8 *a*). Synaptophysin-containing membranes also accumulated on a dense pad at the bottom of the gradient as noted earlier (Clift-O'Grady et al., 1990; Linstedt and Kelly, 1991), as did TfR-enriched membranes, consistent with previous findings (Cameron et al., 1991). These fractions are known to contain endosomal and plasma membranes (Clift-O'Grady et al., 1990; Clift-O'Grady, E. Grote and R. B. Kelly, unpublished observations). For further purification of synaptic vesicles, large membranes were removed from the postnuclear supernatant by an additional centrifugation step before velocity sedimentation analysis (Fig. 8 *b*). In both instances pIgR-immunoreactivity was completely excluded from the synaptic vesicle peak (Fig. 8, *a* and *b*). Instead, the receptor was recovered with the TfR and the rapidly sedimenting synaptophysin-containing membranes on the dense pad at the bottom of the gradient.

If transfected PC12 cells were not treated with NGF, there was no appreciable change in the quantity of synaptic vesicles, nor was there a change in the distribution of pIgR. An identical distribution was observed in each of six stable clones analyzed under the same conditions (data not shown).

To label preferentially the endocytotically derived pool of pIgR, we incubated transfected PC12 cells with dIgA. Although the ligand is not required for transcytosis of the receptor in transfected MDCK cells, the rate of transcytosis is enhanced by dIgA binding (Hirt et al., 1993; Song et al., 1994). In hippocampal neurons, pIgR is detected more read-

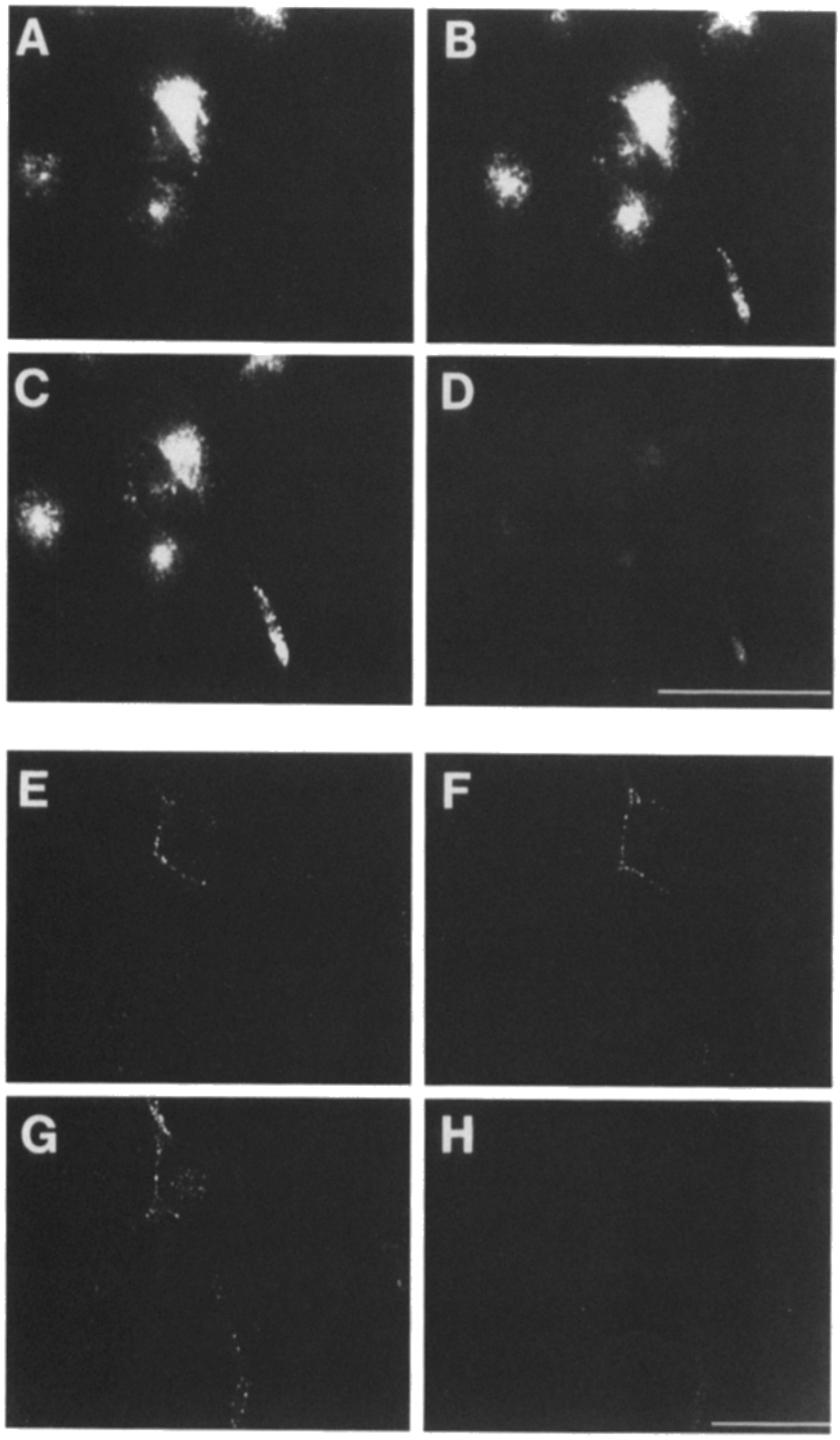


Figure 7. Series of confocal sections from polarized PC12 cells after internalization of dIgA. PC12 cells stably expressing pIgR were allowed to differentiate in the presence of NGF before dIgA-uptake experiments were performed as described in Fig. 5. The cells were either permeabilized with 0.02% saponin before incubation with the labeled anti-dIgA antibody (*A–D*) or left unpermeabilized and incubated with the labeled antibody in the absence of detergent (*E–H*). The distance between the planes of focus of successive sections is 2.4 μm (*A–D*) or 1.2 μm (*E–H*). In permeabilized cells strong intracellular staining is visible in the neurites as well as in the cell bodies. On the other hand, unpermeabilized cells display a distinct surface staining pattern and no intracellular label can be observed. Bars, 10 μm.

ily in axons after the addition of dIgA (Ikonen et al., 1993). [^{125}I]dIgA was bound to the surface of transfected PC12 cells, which were then warmed to allow internalization of ligand. Before fractionating as above, surface-associated [^{125}I]dIgA was removed by extensive washing. Greater than 85% of the internalized [^{125}I]dIgA was found at the bottom of the gradient in the fractions containing endosomes, but no label was recovered in synaptic vesicles (Fig. 9). (In contrast, when PC12 cells are incubated with labeled antibodies against a synaptic vesicle protein, with an epitope-tag as a lumenal domain, label appears in the synaptic vesicle peak as well as in the large membrane fraction [Grote, E., and R. B. Kelly, manuscript submitted for publication].) Incubation of cells with superphysiologic concentrations of unlabeled dIgA before fractionation, which might be expected to enhance delivery to a transcytotic pathway, also did not redirect pIgR to synaptic vesicles as judged by Western blot analysis (data not shown).

In the absence of ligand, transcytosis of pIgR in epithelial cells seems to depend upon phosphorylation of a serine resi-

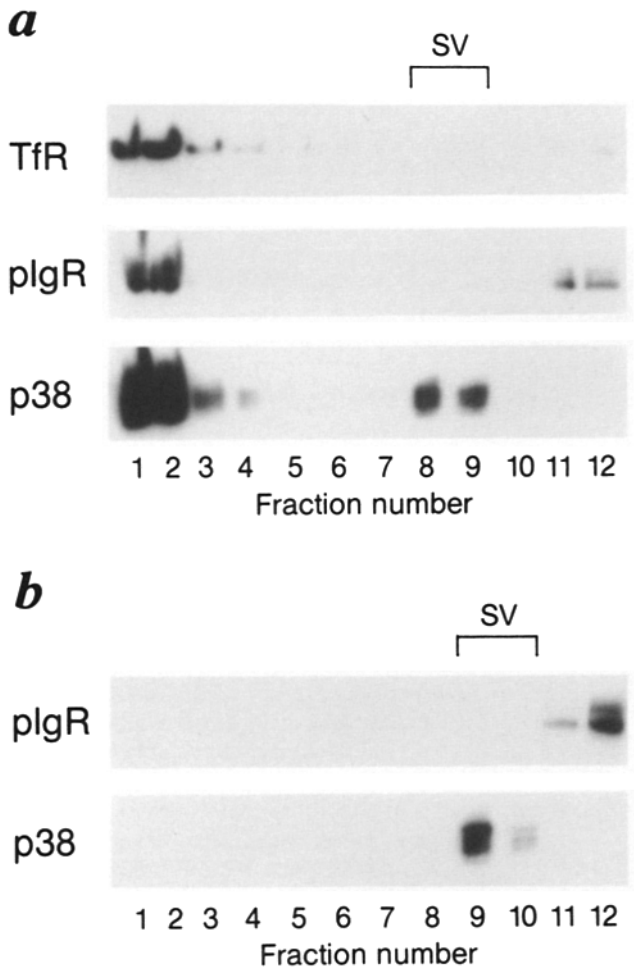


Figure 8. Subcellular fractionation of NGF-treated PC12 cells expressing pIgR by velocity sedimentation analysis. A postnuclear supernatant (*a*) and a high-speed supernatant (*b*) from NGF-differentiated PC12 cells stably transfected with pIgR were loaded onto 5–25% glycerol gradients over a 50% sucrose pad and centrifuged for 1 h at 152,000 *g*. Gradient fractions were subjected to SDS-PAGE and Western blot analysis. Immunoreactivities were determined with the following primary antibodies: *pIgR*, SC166, mouse monoclonal; *p38*, SVP-38, mouse monoclonal; transferrin receptor (*TfR*), H68.4, mouse monoclonal. Primary antibodies were detected by incubation with HRP-conjugated secondary antibodies and visualized using the ECL system. Fractions are numbered *1–12* from the bottom of the gradient where the largest membranes sediment to the top where soluble proteins are located. Note the absence of pIgR from the peak of synaptic vesicles (*SV*) as indicated by brackets in *a* and *b*.

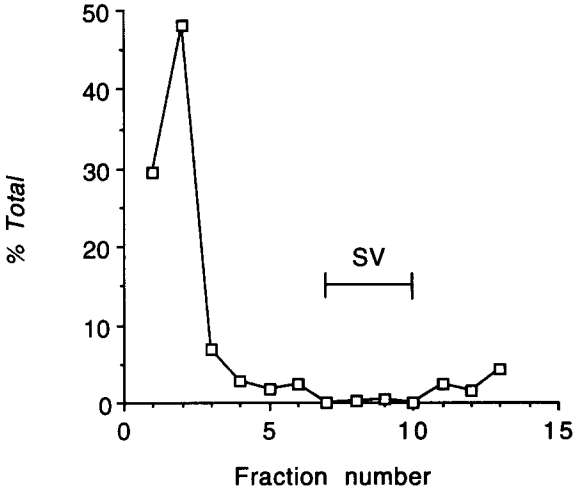


Figure 9. Internalized dIgA is excluded from synaptic vesicles. Undifferentiated transfected PC12 cells expressing pIgR were incubated in the presence of [^{125}I]dIgA for 2 h 40 min at 37°C, and then chased in label-free medium for 20 min at 37°C. After extensive washing, cells were homogenized and fractionated by velocity sedimentation analysis as described in Fig. 8. Fractions were then analyzed on a gamma counter. Shown is the percentage of the total cpm for each fraction. Fractions are numbered from the bottom of the gradient to the top. The synaptic vesicle peak (*SV*), as determined by synaptophysin immunoreactivity on an immunoassay, is indicated by brackets. Note the accumulation of labeled dIgA on the dense pad at the bottom of the gradient while it is nearly undetectable in the region of the gradient where synaptic vesicles sediment.

due at position 664 on the cytoplasmic tail of the receptor (Hirt et al., 1993; Song et al., 1994). A Ser to Asp mutation at position 664 is transcytosed even more efficiently than wild-type receptor without requiring specific phosphorylation (Casanova et al., 1990). Since PC12 cells may lack the kinase which phosphorylates the receptor, we also performed a series of subcellular fractionation experiments on cells transiently expressing the Asp^{664} mutant. Velocity gradients were assayed for the cosedimentation of the receptor with synaptophysin. The mutant receptor was excluded from synaptic vesicles and its distribution was the same as for the wild-type pIgR (data not shown).

To determine whether overexpression of pIgR would affect its targeting, PC12 cells stably expressing the wild-type receptor were fractionated after pretreatment with sodium butyrate which enhanced expression significantly over untreated cells. The receptor was again efficiently excluded from synaptic vesicles and primarily localized to rapidly sedimenting membranes at the bottom of the gradient (data not shown). Thus, the fidelity of sorting into PC12 synaptic vesicles appears to be quite high.

Slowly Sedimenting Polymeric Immunoglobulin Receptor

Under all of the above conditions, the antibody to the cytoplasmic domain of pIgR also bound to material that sedimented more slowly than synaptic vesicles (Fig. 8, *a* and *b*). The identity of this peak as pIgR was confirmed by reprobing the blot with a polyclonal antibody recognizing the lumenal domain of the receptor (data not shown). On equilibrium density flotation gradient centrifugation, the slowly sedimenting pIgR behaved as a soluble protein, and separated from membrane-associated proteins. To examine the solubility properties of pIgR, phase separation analysis in TX-114 was performed on slowly sedimenting material from the top of the velocity gradient, rapidly sedimenting material from the bottom, and whole cell lysates (Fig. 10). Each of these forms of pIgR segregated into the aqueous phase while synaptophysin, a well-characterized integral membrane protein, was extracted into the detergent phase as previously described (Jahn et al., 1985), verifying that the extraction procedure was working correctly. This is not the first example

of an integral membrane protein partitioning into the aqueous phase; the nicotinic acetylcholine receptor has been shown to behave similarly in the TX-114 phase separation assay (Maher and Singer, 1985). The most plausible explanation for the occurrence of slowly sedimenting pIgR is that the receptor is a very hydrophilic integral membrane protein that is much more readily solubilized into the aqueous phase than other integral membrane proteins. Presumably some detergent-like molecules liberated during homogenization solubilize a fraction of the pIgR. Consistent with this possibility, recovery of soluble pIgR depended on the homogenization conditions used.

The Polymeric Immunoglobulin Receptor Is Not Targeted to Dense Core Granules

The presence of pIgR in the tips of processes (Fig. 3) raised the question about the organelle in which it was located. Uptake experiments confirmed that pIgR was present in a population of specialized endosomes within the neurites (Figs. 5 and 7). Furthermore, we have clearly shown that pIgR is not in synaptic vesicles (Fig. 8). Secretory granules have also been identified in the tips of neurites of differentiated PC12 cells (Van Hooff et al., 1989; Elferink et al., 1993). It is unlikely that pIgR is targeted to secretory granules given the rapidity with which it is delivered to the cell surface (Fig. 2). However, the glucose transporter GLUT4 which, like pIgR, is a membrane protein of small, endocytotically derived vesicles that undergo regulated exocytosis (Slot et al., 1991), has been reported to be targeted to secretory granules when expressed in PC12 cells (Hudson et al., 1993).

To confirm the absence of pIgR from secretory granules of differentiated PC12 cells, a postnuclear supernatant was analyzed on isosmotic equilibrium density gradients. Using metrizamide/sucrose gradients, dense core granules, as identified by the soluble content marker secretogranin II, have a characteristic density of 1.10–1.13 g/cm^3 and are clearly separated from lighter organelles (Green et al., 1994). Under these conditions pIgR was enriched in a peak at 1.09–1.10 g/cm^3 (Fig. 11). The receptor was almost undetectable in the density range of granules, as identified by a peak of synaptotagmin, a membrane protein of both dense core granules and synaptic vesicles. pIgR also appeared to be excluded from lysosomes which have approximately the same buoyant density as secretory granules (Green et al., 1994). Similar results were obtained when undifferentiated PC12 cells were analyzed under identical conditions (data not shown). pIgR was not reproducibly separated from TfR- or synaptophysin-containing membranes on the basis of equilibrium buoyant density.

We conclude that, although pIgR accumulates within the neurites of differentiated PC12 cells, it resides in organelles other than secretory granules and synaptic vesicles. Its abundance there appears to reflect its presence in the plasma membrane of the processes and their specialized endosomes.

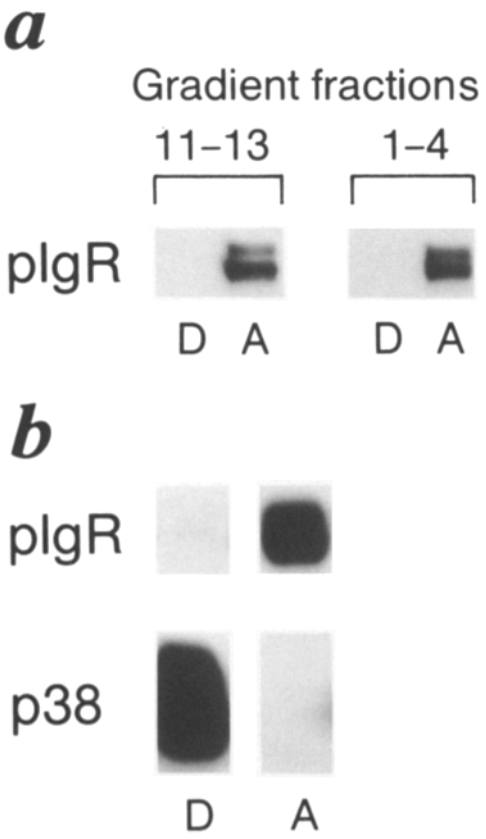


Figure 10. Triton X-114 (TX-114) phase extraction analysis of velocity gradient fractions and whole cell lysates of PC12 cells expressing pIgR. Phase separation of gradient fractions (*a*): to the bottom 4 fractions, *1–4* (*rapidly sedimenting pIgR*) and to the top 3 fractions, *11–13* (*slowly sedimenting pIgR*) from a velocity gradient as in Fig. 8, TX-114 was added to a final concentration of 1.0% at 4°C. Aqueous and detergent phases were collected after warming to 37°C and centrifugation at 1,000 *g* for 5 min. Samples were analyzed by SDS-PAGE and immunoblotting as described in Fig. 8. Phase separation of whole cell lysates (*b*): PC12 cells stably expressing pIgR were lysed in PBS/1% TX-114 and processed for phase separation as in *a*. pIgR was detected with SC166; p38 was detected with SVP-38. *D*, detergent phase; *A*, aqueous phase.

Discussion

Polarized Targeting of Membrane Proteins

Polarized cells such as neurons and epithelial cells have distinct domains of plasma membrane whose composition and function differ from one another. The axonal and nerve terminal plasma membranes of neurons are specialized for conduction of action potentials and neurotransmitter release. The somatodendritic surfaces carry out ion and metabolite transport activities required for cellular maintenance. Similarly, in epithelial cells, the basolateral surface is primarily involved in housekeeping functions while the apical surface has specializations that are appropriate for a membrane that communicates with the lumen of body cavities.

Transfection studies in epithelial cells and neurons have clearly shown a relationship between sorting mechanisms to the apical surface of epithelial cells and to the axon of neurons (for review see Rodriguez-Boulan and Powell, 1992). The viral glycoprotein influenza hemagglutinin, which is sorted to the apical surface in epithelial cells is primarily localized to the axon surface when expressed in neurons (Dotti and Simons, 1990). On the other hand, the vesicular stomatitis virus glycoprotein, which is targeted to the basolateral surface in epithelial cells, is found exclusively on

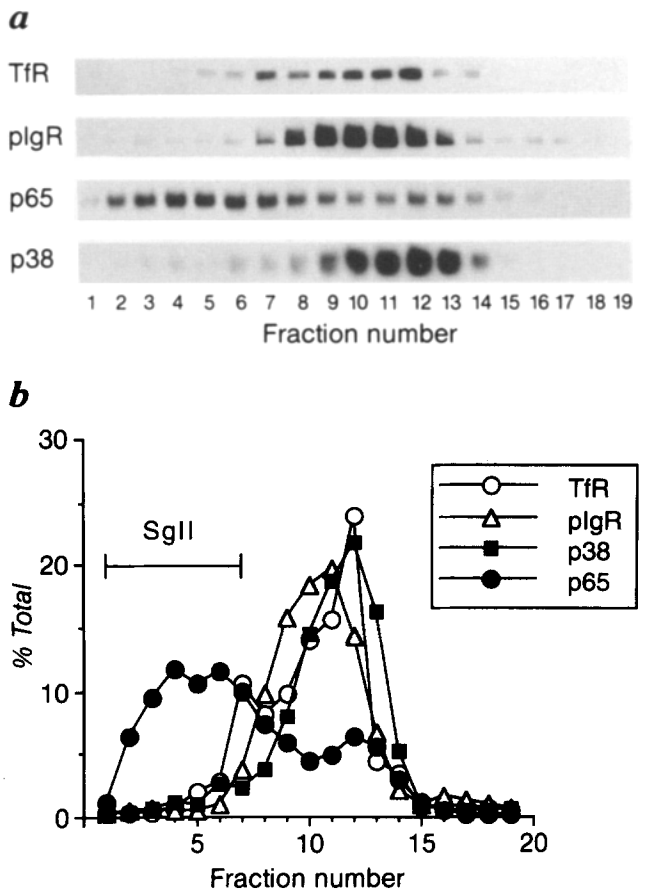


Figure 11. Subcellular fractionation of NGF-differentiated PC12 cells expressing pIgR by isosmotic equilibrium density gradient centrifugation. A postnuclear supernatant from PC12 cells stably transfected with pIgR was loaded onto linear isosmotic 10–23% metrizamide gradients over a 35% metrizamide pad and centrifuged to equilibrium density for 2 h 15 min at 207,000 *g*. Gradient fractions were analyzed by SDS-PAGE and Western blot as described in Fig. 8. Synaptotagmin (p65) was detected with rabbit polyclonal antiserum directed against the cytoplasmic tail of the protein. (*a*) Western blot analysis of gradient fractions. Fractions are numbered *1–19* from the bottom of the gradient where membranes of the highest density sediment to the top where soluble proteins are located. pIgR-containing membranes sediment in a single peak in fractions *8–12* (1.09–1.10 g/cm^3). (*b*) Densitometry of pIgR, p38, TfR, and p65 immunoreactivities from *a*. Note the separation of pIgR-containing membranes from p65-containing secretory granules. The position where the secretory granule content marker secretogranin II (SgII) sediments on parallel gradients is indicated by brackets.

the somatodendritic plasma membrane of infected neurons (Dotti and Simons, 1990). Endogenous membrane proteins appear to behave in a similar manner. When expressed in epithelial cells, the GPI-linked protein, Thy-1, which is restricted to the axonal plasma membrane in neurons (Dotti et al., 1991), is targeted to the apical surface (Powell et al., 1991). Similarly, the GABA transporter, GAT-1, normally localized in axons, is primarily targeted to the apical surface in transfected MDCK cells (Pietrini et al., 1994). Furthermore, targeting of proteins to the basolateral surface in epithelial cells seems to involve the same rab protein (rab 8) that is involved in the targeting of proteins to the somatodendritic region (Huber et al., 1993*a*,*b*).

Our results suggest that neuroendocrine PC12 cells, when induced to extend processes in the presence of NGF, are also functionally polarized. We have shown that the pIgR, targeted to the basolateral and apical surface of epithelial cells, is found in both the neurites and the cell body when expressed in PC12 cells. Conversely, the TfR, normally localized to the basolateral surface in epithelial cells, primarily accumulates in the cell body. Thus, sorting of membrane proteins to the neurites seems to occur by a similar mechanism that governs sorting of membrane proteins to the apical surface.

PC12 cells have been widely used as a model system to study neurite outgrowth. The neurites appear to be more like axons than dendrites. Like axons, they contain proteins specific for dense core secretory granules and synaptic vesicles (Elferink et al., 1993; Hudson et al., 1993). The neurites and especially their growth cones are also enriched in GAP-43 (for review see Costello et al., 1991), a marker protein of axonal growth cones (Goslin et al., 1988). Furthermore, the cytoskeletal protein, MAP2, which is predominantly associated with somatodendritic microtubules in neurons (Ginzburg, 1991), is also more concentrated in the cell body as compared to the neurites of differentiated PC12 cells (for review see Sano et al., 1990).

The selective enrichment of membrane proteins observed in neuronal axons and the neurites of PC12 cells could be due to selective targeting, or domain-specific retention or both. It is possible that pIgR and synaptophysin contain specific signals that permit their selective targeting to the neurites or are responsible for their selective retention within the processes. Similarly, TfR and MPR may either lack a neurite-targeting signal or contain a specific signal that confers selective retention in the cell body. In epithelial cells, when the basolateral localization signal of the LDL-R is weakened by mutagenesis, enhanced targeting of the receptor from basolateral endosomes to the apical cell surface is observed (Matter et al., 1993), suggesting that retention mechanisms normally play a role in its localization. To date, no axonal or neurite targeting or retention signals have been described in integral membrane proteins. Zuber et al. (1989) have identified an amino acid sequence in the NH_2 terminus of the cytoplasmic protein GAP-43 that confers targeting to the neurites of PC12 cells. Because of the ease with which PC12 cells can be grown and transfected, it should now be possible to look for domains conferring retention or targeting in other proteins.

Pathways of Targeting to Neurites

In epithelial cells, membrane proteins may reach the apical surface by two major routes: directly from the TGN and indirectly by transcytosis via the basolateral surface (Mostov et al., 1992). Of the two pathways, the indirect route is the only one common to all epithelial tissues (for review see Mostov, 1994). In neurons, where the membrane trafficking pathways have been less well characterized, axon-specific proteins are targeted to their final destination by axonal transport (for reviews see Sheetz et al., 1989; Vallee and Bloom, 1991; Craig et al., 1992). It is not clear whether axonally directed membrane proteins travel directly or in-

directly via the somatodendritic cell surface. There is some evidence for an indirect neuronal pathway. Lectins such as wheat germ agglutinin bind to the somatodendritic cell surface, are internalized into the cell body, and are eventually targeted to the nerve terminal by anterograde axonal transport where they can be released and taken up postsynaptically (Steindler and Deniau, 1980; Margolis et al., 1981; Ruda and Coulter, 1982). The targeting of pIgR (which reaches the apical surface indirectly via the basolateral surface) to the PC12 neurites suggests that the indirect pathway may also be present in PC12 cells.

A recently published study in which pIgR was expressed in primary hippocampal neurons suggested that pIgR reached the axon by transcytosis via the somatodendritic region (Ikonen et al., 1993). Under normal growth conditions the majority of the pIgR was recovered in the cell bodies, not the axonal processes, but selective accumulation in the axons could be induced by adding physiological ligand, dIgA, to the culture system. Although addition of ligand stimulates the rate of basolateral to apical transport of pIgR in MDCK cells, it is not normally required for transcytosis (Song et al., 1994). Alternatively, since dIgA binding stabilizes the receptor and protects against cleavage by the apical protease in epithelial cells (Mostov, K. E., unpublished observations), it is possible that the axonal domain of hippocampal neurons, unlike PC12 cells, contains a protease that effectively cleaves pIgR and prevents its detection in the absence of added ligand at steady state.

Polarity Extends to Endosomes

It has recently been shown that there are at least two distinct classes of early endosomes in neurons (Parton et al., 1992; Mundigl et al., 1993) which bear some resemblance to the two classes of endosomes found in polarized epithelial cells (Bomsel et al., 1989; Parton et al., 1989; Hughson and Hopkins, 1990). Like basolateral endosomes in epithelial cells, somatodendritic endosomes recycle TfR. Endosomes in the axon, like epithelial apical endosomes, do not accumulate the TfR (Cameron et al., 1991; Parton et al., 1992; Mundigl et al., 1993). They contain synaptic vesicle membrane proteins and may give rise to synaptic vesicles (Mundigl et al., 1993) which undergo regulated exocytosis in the nerve terminal. Our results suggest that targeting to specialized endosomes occurs by similar sorting mechanisms in both epithelial and neuroendocrine cells.

An unexpected implication of recent work is that two populations of endosomes with a different composition and function may be present even in unpolarized cells. In particular, PC12 cells may possess both housekeeping and specialized endosomes even when they are in an undifferentiated state before neurites are extended. Both NGF-differentiated and undifferentiated PC12 cells are capable of producing small vesicles that are indistinguishable from synaptic vesicles produced by neurons. If synaptic vesicles indeed form from specialized endosomes, then either undifferentiated PC12 cells are different from neurons and capable of making synaptic vesicles from housekeeping endosomes or they would have to have an "axonal" endosome capable of giving rise to synaptic vesicles, regardless of the apparent lack of cell polarity.

Sorting of Membrane Proteins during Synaptic Vesicle Biogenesis

Synaptic vesicles arise by endocytosis (for review see Grote and Kelly, 1993). When the synaptic vesicle membrane protein synaptophysin is expressed in nonneuronal cells it is found in housekeeping endosomes as identified by the presence of TfR and LDL-R (Johnston et al., 1989; Linstedt and Kelly, 1991; Cameron et al., 1991). Synaptophysin in neuronal cell bodies is also found in housekeeping endosomes (Mundigl et al., 1993). When synaptic vesicles are purified, they lack LDL-R or TfR (Linstedt and Kelly, 1991; Cameron et al., 1991) suggesting that synaptic vesicle proteins are sorted from other endosomal proteins during synaptic vesicle biogenesis. However, recent data show that only a subset of synaptic vesicle proteins are found in cell body endosomes, as opposed to specialized axonal endosomes which appear to contain all of the synaptic vesicle proteins (Mundigl et al., 1993). Assembly of synaptic vesicles, therefore, is likely to occur in the axon. In light of these findings, the exclusion of housekeeping endosomal markers from synaptic vesicles no longer seems surprising since proteins like TfR are retained in the cell body (Cameron et al., 1991; this study). The pIgR, on the other hand, is present in both housekeeping endosomes in the cell body and specialized endosomes in the neurites of PC12 cells. Its absence from synaptic vesicles, therefore, demonstrates that these vesicles are distinct from specialized endosomes and not just endosomal transport intermediates. Either synaptic vesicles arise directly by endocytosis from the plasma membrane, or, if they form from specialized endosomes, they exclude specialized endosome markers.

Although selective transport of proteins to axons is a well-established phenomenon, very little is known about the signals that dictate sorting from somatodendritic proteins. The advantage of studying a protein such as pIgR in neuroendocrine cells is the wealth of information that is already available on how its sorting behavior can be modified. Analysis of the many available sorting mutants (Aroeti et al., 1993; Mostov, 1994; Aroeti and Mostov, 1994) should yield valuable information regarding the molecular mechanisms that underly sorting to the neurite and the formation of specialized vesicles such as synaptic vesicles.

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