Intracellular Routing of Wild-Type and Mutated Polymeric Immunoglobulin Receptor in Hippocampal Neurons in Culture

Meltsje de Hoop, Christine von Poser, Claudia Lange, Elina Ikonen, Walter Hunziker,* and Carlos G. Dotti
European Molecular Biology Laboratory, Cell Biology Program, 69012 Heidelberg, Germany; and *Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland

Abstract. Certain epithelial cells synthesize the polymeric immunoglobulin receptor (pIgR) to transport immunoglobulins (Igs) A and M into external secretions. In polarized epithelia, newly synthesized receptor is first delivered to the basolateral plasma membrane and then, after binding the Ig, transcytosed to the apical plasma membrane, where the receptor-ligand complex is released by proteolytic cleavage. In a previous work (Ikonen et al., 1993), we implied the existence of a dendro-axonal transcytotic pathway for the rabbit plgR expressed in hippocampal neurons via the Semliki Forest Virus (SFV) expression system. By labeling surface-exposed plgR in live neuronal cells, we now show (a) internalization of the receptor from the dendritic plasma membrane to the dendritic early endosomes, (b) redistribution of the receptor from the dendritic to the axonal domain, (c) inhibition of this movement by brefeldin A (BFA) and (d) stimulation by the activation of protein kinase C (PKC) via phorbol myristate acetate (PMA). In addition, we show that a mutant form of the receptor lacking the epithelial basolateral sorting signal is directly delivered to the axonal domain of hippocampal neurons. Although this mutant is internalized into early endosomes, no transcytosis to the dendrites could be observed. In epithelial Madin-Darby Canine Kidney (MDCK) cells, the mutant receptor could also be internalized into basolaterally derived early endosomes. These results suggest the existence of a dendro-axonal transcytotic pathway in neuronal cells which shares similarities with the basolateral to apical transcytosis in epithelial cells and constitute the basis for the future analysis of its physiological role.

In polarized cells, such as epithelial cells and neurons, the plasma membrane is divided into two domains that differ morphologically, functionally and biochemically. In most epithelial, and possibly also in neurons, the asymmetric organization of the plasma membrane is generated by the sorting of membrane constituents into distinct vesicles in the TGN (for reviews see Rodriguez-Boulan and Powell, 1992; de Hoop and Dotti, 1993; Craig and Banker, 1994). Besides direct sorting, certain polarized cells generate membrane asymmetry by another vesicular pathway called transcytosis (Bartles et al., 1987; for reviews see Rodman et al., 1990; Nelson, 1992; Mostov et al., 1992). Transcytosis is also used to transport substances from one domain to the other. This includes the transepithelial transport of transferrin (Soda and Tavassoli, 1984; Friden et al., 1991), low density lipoprotein (Hashida et al., 1986), serum albumin (Ghitescu and Bendayan, 1992), insulin (King and Johnson, 1985; Partridge, 1991), epidermal growth factor (Brändli et al., 1991; Gonnella et al., 1987), nerve growth factor (Siminoski et al., 1986), thyroglobulin (Herzog, 1983), and antibodies (Kraehenbuhl and Neutra, 1992; Hunziker and Mellman, 1989; Sooranna and Contractor, 1991).

The best characterized transcytotic molecule is the polymeric immunoglobulin receptor (pIgR). The pIgR has a large extracellular ligand-binding domain, called the secretory component (SC), a single transmembrane domain and an intracellular cytoplasmic tail of 103 amino acids (Mostov et al., 1984). In the polarized kidney epithelial cell line MDCK newly synthesized pIgR is sorted in the TGN to the basolateral plasma membrane where it can bind its ligand, dimeric IgA (dIgA). The receptor-ligand complexes, as well as unoccupied receptors are efficiently internalized into the basolateral early endosomes. Here, both the occupied and the unoccupied receptors are sorted into transcytotic vesicles, although some receptors recycle to the basolateral plasma membrane (Song et al., 1994; Breitfeld et al., 1989). Transcytotic vesicles are delivered to apical endocytic structures and from there to the apical plasma membrane (Apodaca et al., 1994; Cardone et al., 1994; Barroso and Sztul, 1994). Either during its intracellular transport or upon insertion into the plasma membrane,

1. Abbreviations used in this paper: BFA, brefeldin A; pIgR, polymeric immunoglobulin receptor; PKC, protein kinase C; SC, secretory component; SFV, Semliki Forest Virus.
the SC is proteolytically cleaved by a leupeptin-sensitive protease and released in complex with the bound ligand (Aroeti et al., 1992).

Several signals have been identified in the amino acid sequence of the receptor that regulate its complex intracellular routing. A sequence of 14 amino acids (from amino acid 655 to 668, bearing the sequence RHRRNVDVDRSICSY) in the cytoplasmic tail directs the receptor to the basolateral plasma membrane (Casanova et al., 1991). Deletion of this basolateral-sorting signal results in direct transport of the receptor to the apical plasma membrane. Two tyrosines present at positions 668 and 734 in the cytoplasmic tail mediate endocytosis, although mutating tyrosine 734 affects endocytosis more than mutations in tyrosine 668 (Okamoto et al., 1992). Transcytosis is regulated by various mechanisms. Although the unoccupied receptor is able to transcytose, transcytosis is stimulated by the binding of its natural ligand dlgA (Song et al., 1994). Phosphorylation of serine 664 and 726 enhances transcytosis (Okamoto et al., 1994; Casanova et al., 1990; Hirt et al., 1993). Although the identity of the kinases is unknown, the involvement of protein kinase A and C in transcytosis has been demonstrated (Cardone et al., 1994; Hansen and Casanova, 1994). Transcytosis is also controlled by heterotrimeric G proteins that have been shown to modulate many steps of the endocytic and exocytic traffic (Bomsel and Mostov, 1992, 1993; Barroso and Sztul, 1994; Hansen and Casanova, 1994). Finally it has been demonstrated that the drug brefeldin A (BFA) inhibits transcytosis, probably by interfering with the sorting of plgR into transcytotic vesicles in the basolateral early endosomes (Hunziker et al., 1991, 1992).

In our previous work we showed the direct delivery of the wild-type plgR to the dendrites of cultured hippocampal neurons and its subsequent appearance in the axons upon addition of dlgA (Ikonen et al., 1993). In the present paper we describe the direct sorting of the wild-type plgR to the dendritic membrane, and by following the movement of the antibody-labeled receptor in living cells, we demonstrate its transcytotic movement to the axonal domain. This transcytotic movement is inhibited by BFA and stimulated by PMA which activates PKC. We also show that, in contrast to the wild-type receptor, a mutant form of the receptor lacking the epithelial basolateral targeting signal (Casanova et al., 1991) is delivered directly to the axons.

**Materials and Methods**

**Chemicals**

Phorbol 12-myristate 13-acetate (PMA), also called 12-0-tetradecanoylphorbol 13-acetate (TPA), was stored as a 0.1 mg/ml stock solution in ethanol at -20°C and used at a final concentration of 2 μM. Brefeldin A was stored as a stock solution of 5 mg/ml in ethanol, and used at 10 μg/ml. Leupeptine was dissolved in water at 50 mg/ml, neutralized, and stored at -20°C until use at a concentration of 100 μg/ml. All these chemicals were purchased from Sigma (Sigma Chem. Co., St. Louis, MO).

**Antibodies**

The monoclonal antibody against the membrane and cytoplasmic domain of the rabbit plgR (SC166) has been described (Kühn and Kraehenbühl, 1983; Solari et al., 1985). A goat polyclonal antibody against the secretory component of the rabbit plgR was obtained from Cooper Biomedical (Malvern, PA). The polyclonal antibody against MAP 2 was generously provided by Dr. Diez-Guerra, Centro de Biología Molecular (CBM) (Madrid, Spain).

**DNA Constructs and Production of Recombinant Semliki Forest Virus**

The cloning of the pSFV1-plgR wild-type and the preparation of recombinant SFV has been described (Ikonen et al., 1993; Olkkonen et al., 1993). To obtain the plgR Δ655-668 mutant, the cDNA encoding wild-type plgR (Mostov et al., 1984) was subcloned from plasmid pCB6 (Brown et al., 1989) into Bluescript KS, resulting in the plasmid KS-plgR in which the 5' end of the plgR gene faces the T3 promoter. This plasmid was amplified with the 5' mutagenic primer (5' GCC ATG GCC GTG ATC A TA GCA AGC AGG AGA GAC ATT AGC ATG TCA GAC) and the 3' primer (5' GCT CCT CTA TCT CGA GGA GTG TCC). The PCR product was digested with MseI and XbaI and ligated into the MscI and XbaI-cut Bluescript KS-plgR. The MscI site is in the plgR transmembrane domain, XbaI in the Bluescript polylinker; this cloning procedure essentially exchanged the wild-type cytoplasmic tail for the mutated cytoplasmic tail. The region amplified by PCR was sequenced. plgR Δ655-668 was excised with SpeI and EcoRV, made blunt ended with the Klenow fragment of DNA polymerase and cloned into the dephosphorylated Smal-cut plasmid pSFV1 (Liljestrom and Garoff, 1991).

Detailed protocols on the preparation and application of recombinant SFVs in hippocampal neurons have been published (Liljestrom and Garoff, 1991; Olkkonen et al., 1993; de Hoop et al., 1994).

**Cell Culture and Infections**

Primary cultures of rat hippocampal neurons were prepared as described (Goslin and Banker, 1991). Cells were grown in N2 medium (Bottenstein and Sato, 1979) supplemented with 1 ng/ml basic fibroblast growth factor (Boehringer Mannheim Biochemica, Mannheim, Germany). Unless stated otherwise, neurons used had been cultured for 9-14 d (stage 5 cells; Dotti et al., 1988). Hippocampal neurons were infected with recombinant SFV as described (de Hoop et al., 1994). After 1 h infection, cells were incubated for 3-5 h to allow SFV-driven protein synthesis. In some experiments cycloheximide (Sigma Chem. Co.) was added (20 μg/ml) during the last 2 h of expression.

MDCK cells expressing either wild-type (Hunziker et al., 1990; Mostov and Deitcher, 1986) or plgR Δ655-668 (Casanova et al., 1991) under control of the CMV promoter were cultured at 37°C and 5% CO2 in MEM with 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml genetin (G418; Gibco BRL Life Technologies Inc., Grand Island, NY) for cells expressing the mutant Δ655-668 or 0.25 mg/ml hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) for cells expressing wild-type plgR.

**Immunofluorescence**

Neurons on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature followed by a 15-min incubation in 50 mM NH4Cl in PBS to quench excess paraformaldehyde. Cells were permeabilized via graded ethanol series. Nonspecific binding sites were blocked by incubation in 2% bovine serum albumin, 2% FCS and 0.2% cold-water fish-skin gelatin (Sigma Chem. Co.) in water for 30 min. The cells were then incubated with primary antibody for 1 h at room temperature and, after extensive washing with PBS, with species-specific secondary antibody (from Cappel, Durham, NC and from Dianova, Hamburg, Germany). The coverslips were mounted in Mowiol 4-88 (Calbiochem-Novabiochem Corp., La Jolla, CA) containing 2.5% DABCO (Sigma Chem. Co.) as an antifading agent and microscopy was performed with an Axioshot microscope (Zeiss, Oberkochen, Germany).

Filter-grown MDCK cells were fixed with 4% paraformaldehyde and permeabilized with saponin (0.025% in PBS containing 0.7% cold-water fish-skin gelatin) as described (Huber et al., 1993a). Cells were mounted with spacers in 50% glycerol containing 2.5% DABCO and viewed with the European Molecular Biology Laboratory Confocal Microscope.

**Identification and Quantitation of plgR Labeled Processes**

To precisely determine the axonal or dendritic distribution of the wild-
type or mutant receptor, low multiplicity of infection was used. In this way, very few cells per coverslip were infected and it was thus possible to unequivocally distinguish the origin, trajectory, and ending of the labeled processes. The identification of the processes as dendrites was done by double labeling with the dendritic marker MAP2 (Caceres et al., 1986). A process was identified as axon by its lack of MAP2 staining. The distribution was classified into the following pattern: soma and dendrites, soma and axons, and soma and both axons and dendrites. Several coverslips from different experiments were examined by two independent observers, the number of infected cells and process distribution averaged and the standard deviation calculated. Significance was determined by student's t test.

Internalization Experiments

The internalization experiments in neurons were performed as follows: FITC-coupled polyclonal antibodies against the secretory component of the rabbit pIgR were dissolved 1:100 in N2 medium. Coverslips with attached neurons were put on top of small droplets of prewarmed antibody-containing medium during 20 min at 5% CO2 and 36.5°C. Cells were then washed with cold HBSS containing 0.2% BSA, fixed with 4% paraformaldehyde in PBS and processed for immunofluorescence. To label the early endocytic compartment of neurons, Lucifer yellow-CH (dilithium salt, Sigma Chem. Co.) was added to the medium at 10 mg/ml. Neurons were incubated in Lucifer yellow-containing medium for 20 min, followed by a thorough wash with ice cold HBSS containing 0.2% BSA, fixed with 4% paraformaldehyde and processed for immunofluorescence.

To label the pIgRs present in the early endocytic compartment of filter-grown MDCK cells, the antibody against the secretory component of the rabbit pIgR (dissolved 1:40 in MEM containing 0.05% BSA and 10 mM Hepes-NaOH, pH 7.3; MEM-BSA) was added to the medium and the cells were incubated on ice for 45 min. Unbound antibody was removed by a quick wash in ice-cold MEM-BSA. Then, the basolateral side of the cells was exposed to 10 mg/ml lucifer yellow-CH (5 min at 37°C in MEM-BSA) to allow internalization of surface-bound antibodies and the fluid-phase ligand. Finally, the cells were thoroughly washed with ice-cold MEM-BSA, fixed with 4% paraformaldehyde and processed for immunofluorescence as described above.

Transcytosis Assay

Transcytosis of the pIgR in living cells was analyzed as follows: cells were infected for 1 h at 36.5°C, excess virus was removed and the cells were further incubated for 3 h in the presence of 100 μg/ml leupeptin to prevent the proteolytic removal of the secretory component (Apodaca et al., 1991). At the end of the 3-h expression period, the coverslips were incubated on ice for 30 min in small drops (= 100 μl) of air-MEM (MEM containing 4 mM NaHCO3, 10 mM Hepes, pH 7.3, 2 mM glutamine, 0.6% glucose, 110 μg/ml pyruvic acid) with 5% FCS, 100 μg/ml leupeptin and 1:100 diluted FITC-coupled anti-SC. Coverslips were subsequently washed in cold HBSS with 0.2% BSA and either fixed to examine the distribution of surface-bound antibodies against the ectodomain (the secretory component) or transferred to prewarmed maintenance medium containing BFA (10 μg/ml) or PMA (2 μM) or medium without additions for 20 min to 2 h at 36.5°C. Finally, the cells were fixed, washed, and mounted to examine the distribution of the anti-SC antibody by fluorescence microscopy.

Western Blot Analysis

Hippocampal neurons were plated directly onto polylysine-coated 6-cm culture dishes (Nunc, Roskilde, Denmark) at a density of 300,000-400,000 cells per dish and cultured for three days. 5 h after infection with approximately equal amounts of recombinant virus, carrying either the wild-type or Δ655-668 pIgR cDNA, the neurons were lysed (de Hoop et al., 1994) and the cell extracts were analyzed by Western blotting using the anti-CT monoclonal antibody SC166 (diluted 1:100), followed by ECL detection as recommended by the supplier (Amersham International, Buckinghamshire, England).

Results

Expression of the Polymeric Immunoglobulin Receptor in Hippocampal Neurons in Culture

In a previous work we showed by indirect immunofluorescence that infection of polarized hippocampal neurons in culture with recombinant SFV resulted in the appearance of the pIgR in the dendrites and, to a lesser extent, also in axons. The axonal labeling was increased 2–4-fold upon addition of ligand (Ikonen et al., 1993). The aim of the present work was to analyze the mechanisms of this transcytotic route and the sorting of the Δ655-668 mutant, which lacks a basolateral targeting signal.

We first examined by Western blot the SFV-driven expression (Liljestrom and Garoff, 1991; Ollkonen et al., 1993) of wild-type and Δ655-668 mutant pIgR in polarized hippocampal neurons in culture. In neurons expressing wild-type pIgR, a band of ~120 kDa was visible (Fig. 1, lane 2) agreeing with the molecular weight of endogenous pIgR in liver (Sztul, 1992). In neurons expressing the Δ655-668 pIgR mutant (lane 3), a band with slightly higher electrophoretic mobility, due to the deletion, was observed. As expected, no expression was detected in control neurons and MDCK cells (lanes 1 and 4). The intensity of the 120-kD band differed slightly between neurons infected with truncated and wild-type pIgR. This is due to a difference in the efficiency of infection. The band of ~35 kDa visible in MDCK cells expressing wild-type pIgR (arrow in lane 5) represents the cleaved cytoplasmic tail and membrane domain of the receptor (Kühn and Kraehenbuhl, 1983; Solari et al., 1985).

Intracellular Distribution of Newly Synthesized Wild-Type and Mutant pIgR

To analyze the routing of newly synthesized pIgR, fully polarized hippocampal neurons were infected with recombinant virus which express the pIgR and the distribution of the receptor was determined by immunofluorescence microscopy at 3 h postinfection. This was the earliest time point at which pIgR expression was detectable. The wild-type receptor (Fig. 2 B) was predominantly found in the somatodendritic region. Quantitative analysis revealed exclusive cell body and dendritic labeling in 85% of the cells (Fig. 3 A). In the remaining cells (15%), the labeling was present in the cell body and both dendrites and axons. We did not find cells with cell body and axonal staining in the

![Figure 1. Western blot analysis of pIgR expression in infected hippocampal neurons. (Lane 1) Noninfected neurons; (lane 2) neurons infected with SFV-wild-type pIgR; (lane 3) neurons infected with SFV-mutant (Δ655-668) pIgR; (lane 4) control MDCK cells; (lane 5) stably transfected MDCK cells expressing wild-type pIgR. 40 μg protein was loaded in each lane. Positions of molecular weight markers are indicated on the right.](image-url)
Figure 2. Intracellular distribution of wild-type and Δ655-688 p1gR in polarized neurons at early time after infection. Hippocampal neurons infected with SFV encoding the wild-type p1gR (A–C) or p1gR Δ655-688 (D–F) were fixed at 3 h postinfection and the intracellular distribution of the expressed receptor was determined by immunofluorescence using the anti-CT antibody. The wild-type receptor is present in the dendrites (B, small arrows), identified by the presence of MAP2 (C). The axon (large arrows) is completely devoid of labeling. The mutant receptor is present in the axon (MAP2-negative neurite, large arrows in E), while the dendrites (small arrows, identified by MAP2 staining, panel F) are devoid of labeling. Bar, 20 μm.

absence of dendritic labeling. This result indicates the direct delivery of the wild-type p1gR from the cell body to the dendritic domain.

In contrast to the cells expressing wild-type p1gR, the majority (64%) of the cells expressing the mutant p1gR displayed cell body and axonal labeling (Fig. 2, D–F, Fig. 3 A). Occasionally, labeling was also observed in the proximal, but not the distal, part of the dendrites. This labeling might correspond to receptor accumulated in the organelles of the secretory pathway which, in these cells, are found some distance into the dendrites (Krijnse-Locker, J., R. G. Parton, S. D. Fuller, G. Griffiths, and C. G. Dotti, 1995).
The Intracellular Distribution of Overexpressed pIgR Changes at Long Postinfection Times

In epithelial cells, the pIgR transcytoses from the basolateral plasma membrane to the apical surface even in the absence of ligand (Song et al., 1994). To determine whether or not the dendritically delivered pIgR would move to the axonal domain in the absence of ligand, we analyzed its distribution at long (5 h) postinfection times. At this time, 45% of the cells showed cell body and axonal and dendritic labeling (Fig. 3 B), a threefold increase in the number of cells with axonal labeling compared to that of 3 h postinfection (see above). Although not so dramatic, an increase (1.6-fold) in axonal labeling was still observed in the presence of cycloheximide during the last 2 h of the postinfection time (not shown), suggesting either a transcytotic redistribution of wild-type pIgR from the dendritic to the axonal domain or missorting induced by the high expression obtained by the efficient viral expression system.

At long postinfection times (5 h), the percentage of cells expressing mutant pIgR with exclusive axonal staining had decreased to one fifth, from 64% (3 h postinfection) to 13% (5 h postinfection) (Fig. 3 B). This decrease in prevalent axonal staining was to a large extent prevented when cycloheximide was added during the last two hours of the postinfection period (data not shown), indicating that the dendritic labeling of the mutant pIgR might be predominantly caused by missorting due to overexpression rather than redistribution.

Overexpressed pIgR Is Internalized into Early Endosomes

A prerequisite for the transcytosis of pIgR in epithelial cells is internalization in basolateral early endosomes where sorting into transcytotic vesicles takes place (Hunziker et al., 1992). Hence, we analyzed next whether overexpressed wild-type pIgR would undergo internalization into the dendritic early endosomes. Infected neurons were incubated with FITC-labeled anti-SC antibody during the last 20 min of the postinfection period. After excess antibody was removed, the cells were fixed, permeabilized, labeled with anti-CT antibodies, and analyzed by immunofluorescence microscopy. Fig. 4 shows the results of such an experiment. The anti-SC antibody was found in vesicular structures present in the cell body and the dendrites (Fig. 4 C) which also labeled with the fluid-phase marker Lucifer yellow (not shown). The internalized antibody represented only a fraction of the total pool of wild-type pIgR as revealed by the anti-CT staining (Fig. 4 B). No antibody internalization was observed in noninfected cells, showing that the labeling with the anti-SC antibody was not caused by nonspecific endocytosis.

The anti-SC antibody was also internalized in cells expressing the mutant pIgR. Fig. 4 F shows that the antibody accumulated intracellularly in vesicular structures. The total pool of the mutant receptor, as detected by the anti-CT antibody (Fig. 4 E), seemed to colocalize almost completely with the internalized antibodies. In addition, an almost overlapping staining was found between the total pool of mutant pIgR and Lucifer yellow, that had been internalized for 20 min (data not shown).

Considering that in MDCK cells more than 95% of the mutant pIgR is directly delivered to the apical surface (Casanova et al., 1991), the intense endosomal labeling, present also in the dendritic domain of the hippocampal neurons, was somewhat unexpected. Since the studies in MDCK cells accounted exclusively for the mutant protein that had reached the surface, it was still possible that in
Figure 4. Internalization of plgR in hippocampal neurons. Hippocampal neurons expressing either wild-type plgR (A–C) or Δ655-668 plgR (D–F) were incubated in the last 20 min of the postinfection period with FITC-conjugated anti-SC antibody, washed, and fixed. B and E show the total pool of plgR as revealed by labeling with the anti-CT antibody, and C and F, the endocytosed anti-SC antibodies. In cells expressing the wild-type plgR, the internalized antibody can be seen in the dendrites and in the cell body (small arrows), but not in any of the axons in the field (large arrows). The mutant plgR is present in regularly shaped structures in both the axon (large arrows) as well as in the dendrites (small arrows). Bar represents 20 μm.
these cells the Δ655-668 plgR was also present in the basolateral endosomes. To detect the plgR present in basolateral endosomes, MDCK cells expressing Δ655-668 plgR were incubated with anti-SC antibody and basolaterally added Lucifer yellow and processed for confocal immuno-fluorescence microscopy. We observed colocalization of Lucifer yellow (Fig. 5 B) with the internalized antibody (Fig. 5 A), indicating that in MDCK cells part of the mutant receptor is present in endosomal structures and also reaches the basolateral plasma membrane.

**The Transcytotic Movement of Anti-SC Antibody**

We examined next whether the wild-type plgR in the dendritic early endosomes would be transcytosed to the axonal domain. For that purpose we bound fluorescently labeled anti-SC antibody at 4°C at 3 h postinfection and analyzed its distribution immediately thereafter or after allowing internalization at 36.5°C for 2 h (see Materials and Methods and Fig. 6 A). In this way, we only look at the plgR that had reached the dendritic plasma membrane at this short postinfection time, and we thus avoid the interfering visualization of newly synthesized receptor. Directly after antibody binding, an intense uniform labeling was observed at the surface of the cell body and dendrites, but not at the axons (Fig. 7, A–C). After 2 h at 36.5°C, the staining pattern was vesicular and the axon was now intensively labeled (Fig. 7, D–F). These results show the change in the distribution of the receptor from the dendritic surface to the axonal domain. Using a similar experimental paradigm, we did not detect a change in the distribution of Δ655-668 plgR, from axonal to dendritic, suggesting lack of transcytosis of the mutant receptor. In addition, we were also unable to observe a change in the distribution of mutated plgR upon the addition of the dlgA (data not shown), which increases axonal labeling of the wild-type receptor (Ikonen et al., 1993).

**Transcytosis Is Inhibited by BFA and Stimulated by PMA**

The basolateral to apical transcytosis in MDCK cells is inhibited by the fungal metabolite BFA (Hunziker et al., 1991, 1992). To investigate whether transcytosis in hippocampal neurons is regulated by a similar mechanism, we analyzed the effect of BFA on the dendro-axonal transcytosis of plgR. Infected neurons were incubated for 30 min at 4°C with FITC-labeled anti-SC at the end of the 3-h postinfection period, excess unbound antibody removed and the cells warmed at 36.5°C for 2 h in the presence or absence of 10 μg/ml BFA (see Fig. 6 B). The results of these experiments are shown in Fig. 8 A. In the absence of BFA more than 50% of the infected neurons showed axonal anti-SC labeling at the end of the experiment. In the presence of BFA, on the contrary, axonal labeling did not occur and most cells retained the anti-SC labeling in the dendrites. The small percentage of cells with axonal labeling after BFA treatment was comparable to the percentage of axonal labeling observed at 3 h postinfection, before transcytosis was allowed to occur. These results show, similarly to the transcytosis of plgR in MDCK cells, the inhibitory effect of BFA in neuronal dendro-axonal transcytosis.

Recently it was shown that transcytosis of the plgR in MDCK cells is stimulated by phorbol esters (Cardone et al., 1994). Using the assay described above, we tested if the phorbol ester PMA would increase axonal transcytosis of the plgR. Anti-SC binding at 4°C was followed by a short incubation (20 min) at 36.5°C in the presence or absence of 2 μM PMA (see Fig. 6 C). At this short time after anti-SC binding, no significant axonal labeling was observed in the

![Figure 5](image-url)
Intracellular Routing of Wild-Type and A655-668 pIgR
in Immature Hippocampal Neurons

After 2–3 d in culture hippocampal neurons acquire morphological polarity; one axon and several short neurites ("stage 3 cells"); Dotti et al., 1988). We tested whether wild-type and the mutant Δ655-668 pIgR were sorted in a polarized fashion in these cells. Stage 3 neurons were infected with the recombinant viruses, fixed at 3 h postinfection and the distribution of the expressed receptors was analyzed by immunofluorescence microscopy (Fig. 9). In contrast to fully mature neurons ("stage 5 cells"), fixed at the same postinfection time, no selective delivery of pIgR receptors to any processes was observed in stage 3 cells.

Discussion

Direct Sorting of Wild-Type and Mutant pIgR in Hippocampal Neurons

In MDCK cells, membrane proteins are sorted in the trans-Golgi network into distinct vesicles destined for either the apical or the basolateral plasma membrane (Wandinger-Ness et al., 1990). In polarized neurons, membrane proteins are sorted to the axonal and to the dendritic surfaces (for a review see Craig and Banker, 1994). In most cases membrane proteins, targeted to the apical surface of MDCK cells, are delivered to the axonal surface of neurons, whereas those with a basolateral fate in MDCK cells are delivered to the dendritic surface of neurons (for reviews see Rodriguez-Boulan and Powell, 1992; de Hoop and Dotti, 1993; Craig and Banker, 1994). In MDCK cells, the wild-type pIgR is delivered to the basolateral membrane whereas the mutant pIgR is preferentially delivered to the apical membrane (Mostov, 1993; Casanova et al., 1991).

Using the SFV-expression system in cultured rat hippocampal neurons, we observed a dendritic distribution of the wild-type receptor and axonal distribution of mutated receptor at 3 h postinfection, the earliest time at which any expression is detectable. This strengthens the case for homologous sorting mechanisms in epithelial and neuronal cells. Although we are analyzing the distribution of a heterologous protein in neurons, endogenous proteins expressed via the SFV system are appropriately sorted and distributed to the right compartments (Olkkonen et al., 1993; de Hoop et al., 1994; Simons et al., 1995). We therefore infer that the distribution of the pIgR at the earliest postinfection time reflects the domain to which direct sorting has taken place.

The Distribution of Wild-Type pIgR Changes at Longer Postinfection Times

With longer expression times, increasing amounts of the receptors were observed in the opposite domain; wild-type receptor appeared in the axons and the Δ655-668 mutant was detected in the dendrites. This change in distribution could be due to either transcytosis or missorting. We favor the idea that the late axonal appearance of the wild-type receptor is partly due to transcytosis. First, using an antibody binding and internalization assay, we noticed that wild-type receptors could migrate from dendrites to axons. Second, the labeling of axons was prevented by BFA and stimulated by PMA. Our previous results showing an increase of axonal pIgR upon addition of SIgA (Ikonen et al., 1993) further supports dendro-axonal transcytosis of pIgR. On the contrary, we consider the presence of the mutant receptor in the dendrites to be the consequence of missorting. Neither could we detect any change in intracellular distribution in the transcytosis assay nor had the addition of ligand any effect. The same mutant receptor expressed in MDCK cells does not transcytose (Casanova et al., 1991). In these cells, although most of the mutant protein is delivered apically, ~5% of the protein is detected on the basolateral surface (Casanova et al., 1991), also suggesting missorting. We found that in MDCK cells, part of the Δ665-668 receptor can internalize anti-SC antibody
Figure 7. Transcytosis of the plgR in neurons. Infected cells were incubated at 3 h postinfection with FITC-conjugated anti-SC for 30 min at 4°C. Excess antibody was removed, and the cells were fixed (A–C) or further incubated for 2 h at 36.5°C, and then fixed (D–F). Directly after the binding of the FITC-labeled anti-SC, a surface labeling of the dendrites and cell body is revealed (B; small arrows). Permeabilization after fixation permitted the labeling of the dendrites with antibodies against MAP2 (C and F). The axons (MAP2-negative; see C), visible in the phase contrast photograph in A, are unlabeled (large arrows). Fixation of the cells 2 h after binding and detection of the bound antibody reveals strong labeling in the axon (E; large arrows) as well as in the dendrites. In contrast to B, the staining in E appears to be predominantly punctate. Bar represents 20 μm.
Neuronal Transcytosis of Wild-Type plgR Is Sensitive to BFA and PMA

Transcytosis of the polymeric immunoglobulin receptor in MDCK cells appears to be sensitive to protein kinase C (stimulated via PMA) (Cardone et al., 1994) and brefeldin A (BFA) (Hunziker et al., 1992). We analyzed the effect of the compounds BFA and PMA to determine whether transcytosis of the plgR in neurons was affected in a way similar to MDCK cells. With our transcytosis assay we found that BFA inhibited the axonal migration of receptor that had been fluorescently labeled at the dendritic surface. In the presence of PMA, transcytosis from the dendrites to the axon was accelerated. This increase in axonal labeling could be observed in times as short as 20 min. Thus, these experiments suggest that the transcytotic pathway in neurons resembles that of MDCK cells; in the sense that it is inhibited by BFA and stimulated by PMA. Treatment of hippocampal neurons with BFA induces endosomal tubulation without affecting the uptake of fluid-phase ligands (Cid-Arregui et al., 1995). Therefore, as in MDCK cells (Hunziker et al., 1991), BFA might block the dendro-axonal transcytosis of plgR by specifically affecting the formation of transcytotic vesicles in the early endosomes. Whether BFA also affects the transport to the degradative compartment and/or the recycling to the plasma membrane in neurons is not known. In MDCK cells, however, BFA does not affect these pathways (Hunziker et al., 1991).

In MDCK cells PMA increases the movement of plgR from the apical endosomes to the apical surface and accelerates the rate of transcytosis from the basolateral endosomes (Cardone et al., 1994). Although we did not determine the effect of PMA on the release of IgA from the axonal endosomes, our work clearly shows that in neurons PMA increases dendro-axonal transcytosis by accelerating the migration of plgR from the dendritic early endosomes or from the dendritic plasma membrane to the axonal domain. It is believed that the effect of PMA on MDCK cells to stimulate transcytosis mimics the action of cholinergic agonists to stimulate the release of IgA by nasal and intestinal epithelia in vivo (Raphael et al., 1988; Freier et al., 1987; Wilson et al., 1992). Since hippocampal neurons in culture also appear to respond to PMA by increasing the rate of transcytosis, we envision that in vivo neurons may regulate the traffic of endogenous transcytosed molecules in response to different hormones and neurotransmitters.

Sorting of the plgR Is Developmentally Regulated

In stage 3 hippocampal neurons morphological polarization is already visible, the longer neurite will develop into the futural axon (Dotti et al., 1988). This polarity is however not yet fixed; amputation of the futural axon results in the acquisition of axonal properties of one of the other neurites and the acquisition of dendritic properties of the amputated axon (Dotti and Banker, 1987; Goslin and Banker, 1989). Expression of either wild-type or mutant plgR in stage 3 hippocampal neurons, led to the nonpolarized distribution of the receptor. Accordingly, certain

from the basolateral surface to the basolateral early endosomes.
endogenous proteins, with polarized distribution in fully differentiated stage 5 hippocampal neurons, are evenly distributed in the stage 3 cells, despite their morphological polarity (Killisch et al., 1991; Craig et al., 1993; van Lookeren Campagne et al., 1992; Huber et al., 1993b; see however, Goslin et al., 1990 and Fletcher et al., 1991). Similarly, viral glycoproteins expressed in differentiated neurons are sorted to the axon or the dendrites of fully mature (stage 5) neurons, whereas the same proteins are not sorted in morphologically polarized stage 3 neurons (Dotti and Simons, 1990). Altogether, these results suggest the asynchronous development of the machinery responsible for the establishment of morphological polarity and polarized protein delivery. The establishment of the machinery for molecular sorting is also a late occurring event in epithelial cells. Two different types of epithelial cells, MDCK and FRT, show lack of protein sorting at early developmental stages (Wollner et al., 1992; Zurzolo et al., 1992). The mechanism which regulates the stabilization of neuronal polarity and molecular sorting remain to be identified.

**Implications of Neuronal Transcytosis**

Bearing in mind the similarities between epithelial MDCK cells and neurons, it was logical to hypothesize that neurons could make use of a dendro-axonal transcytotic route, either as a way to generate membrane asymmetry or to transport macromolecules. However, little is known about the existence of a transcellular vesicular transport pathway in neurons. Wheat germ agglutinin (WGA) has been shown to undergo dendritic to axonal transneuronal transport in retinal ganglion cells (LaVail and Margolis, 1987; Ruda and Coulter, 1982). However, it may not use the same transcytotic pathway as the plgR. Unlike plgR, after dendritic uptake WGA accumulates in the Golgi apparatus of most cells, including hippocampal neurons (Dotti and Banker, 1991). Therefore, the presence of WGA along vesicular profiles in axons of the visual system might represent exocytic rather than transcytotic vesicles. Horseradish peroxidase and fibroblast growth factor have also been shown to undergo anterograde transport in the optic nerve after uptake in the ganglion cells (Pickard and Silverman, 1981; Ferguson et al., 1990), possibly by a transcytotic mechanism similar to that of the plgR. There are also indications for a transneuronal axo-dendritic transport (Schwab and Thoenen, 1978; Schwab et al., 1979; Evinger and Erichsen, 1986; Fabian, 1991; Simons et al., 1995). Altogether our work reveals the existence of a transcytotic pathway in neurons and shows similarities of this pathway between epithelial and neuronal cells. To what extent transcytotic pathways are important for the establishment and maintenance of neuronal cell polarity or for transneuronal communication remains to be established. Transcytosis might be the pathway by which growth factors, like basic fibroblast growth factor, are transported during neuronal development (Ferguson et al., 1990), and
thus be an essential mode of transcellular communication. In support of this are the observations that an axonal to dendritic transcytotic process might be involved in the generation of motor neuron diseases and other neurological malfunctions (Fabian, 1991; LaRocca and Wiley, 1988) and that the amyloid precursor protein (APP) is transcytosed from axons to dendrites (Simons et al., 1995). However, the true in vivo physiological role of neuronal transcytosis remains to be identified.

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References

Apodaca, G., J. A. Katz, and K. E. Mostov. 1994. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. J. Cell Biol. 125: 57-68.

Apodaca, G., M. Bomsel, J. Arden, P. Breitfeld, K. Tang, and K. Mostov. 1991. The polymeric immunoglobulin receptor: a model protein to study transcytosis. J. Clin. Invest. 87:1877-1882.

Aroeti, B., J. Casanova, C. Okamoto, M. Cardone, A. Pollack, K. Tang, and K. Mostov. 1992. Polymeric immunoglobulin receptor. In: Rev. Cytol. 137B: 157-169.

Barroso, M., and E. Sztul. 1994. Basolateral to apical transcytosis in polarized epithelial cells is regulated by rab5 and that the amyloid precursor protein (APP) is transcytosed from axons to dendrites (Simons et al., 1995). How- ever, the true in vivo physiological role of neuronal transcytosis remains to be identified.
LaRocca, C. D., and R. G. Wiley. 1988. Monoclonal anti-Thyl antibody (OX7)

Kraehenbuhl, J.-P., and M. R. Neutra. 1992. Transepithelial transport and mu-

Mostov, K., G. Apodaca, B. Aroeti, and C. Okamoto. 1992. Plasma membrane

Mostov, K. E., and D. L. Deitcher. 1986. Polymeric immunoglobulin receptors

van Lookeren Campagne, M., C. G. Dotti, E. R. A. Jap Tjoen San, A. J. Verk-

LaVail, J., and T. P. Margolis. 1987. The anterograde axonal transport of wheat

Raphael, G. D., H. M. Druce, J. N. Baraniuk, and M. A. Kaliner. 1988. Patho-

Pickard, G. E., and A.-J. Silverman. 1981. Direct retinal projections to the hy-

Olkkonen, V. M., P. Liljestr6m, H. Garoff, K. Simons, and C. G. Dotti. 1993.

Pardridge, W. M. 1991. Advances in cell biology of blood-brain barrier trans-

Rodman, J. S., J. W. Mercer, and P. D. Stahl. 1990. Endocytosis and transcyto-

Rodriguez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neu-

Ruda, M., and J. D. Coulter. 1982. Axonal and transneuronal transport of wheat
germ agglutinin as a model for transcellular transport in neurons. In Axonal
Transport, R. S. Smith and M.A. Bisby, editors. Alan R. Liss, Inc., New
York. pp. 311-336.

LaRoca, C. D., and R. G.Wiley. 1988. Monoclonal anti-Thyl antibody (OX7)
Kraehenbuhl, J.-P., and M. R. Neutra. 1992. Transepithelial transport and mu-

Mostov, K. E., G. Blobel. 1984. The receptor for transepithelial transport of IgA

Mostov, K. E., M. Friedlander, and G. Blobel. 1984. The receptor for transepi-

Pardridge, W. M. 1991. Advances in cell biology of blood-brain barrier trans-

Sztul, E. 1992. Transcytotic vesicle fusion with plasma membrane. Methods En-
zymol. 219:44-52.

Wandinger-Ness, A., M. K. Bennett, C. Antony, and K. Simons. 1990. Distinct

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Simons, K., P. Gonella, J. Bernake, L. Owen, M. Neutra, and R. A. Murphy. 1986. Uptake and transmembraneous transport of nerve growth factor in suckling rat ileum. J. Cell Biol. 103:1979-1990.

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Ruda, M., and J. D. Coulter. 1982. Axonal and transneuronal transport of wheat
germ agglutinin as a model for transcellular transport in neurons. In Axonal
Transport, R. S. Smith and M.A. Bisby, editors. Alan R. Liss, Inc., New
York. pp. 311-336.

LaRoca, C. D., and R. G.Wiley. 1988. Monoclonal anti-Thyl antibody (OX7)
Kraehenbuhl, J.-P., and M. R. Neutra. 1992. Transepithelial transport and mu-

Mostov, K. E., G. Blobel. 1984. The receptor for transepithelial transport of IgA

Pardridge, W. M. 1991. Advances in cell biology of blood-brain barrier trans-

Sztul, E. 1992. Transcytotic vesicle fusion with plasma membrane. Methods En-
zymol. 219:44-52.

Wandinger-Ness, A., M. K. Bennett, C. Antony, and K. Simons. 1990. Distinct

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Simons, K., P. Gonella, J. Bernake, L. Owen, M. Neutra, and R. A. Murphy. 1986. Uptake and transmembraneous transport of nerve growth factor in suckling rat ileum. J. Cell Biol. 103:1979-1990.

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Ruda, M., and J. D. Coulter. 1982. Axonal and transneuronal transport of wheat
germ agglutinin as a model for transcellular transport in neurons. In Axonal
Transport, R. S. Smith and M.A. Bisby, editors. Alan R. Liss, Inc., New
York. pp. 311-336.

LaRoca, C. D., and R. G.Wiley. 1988. Monoclonal anti-Thyl antibody (OX7)
Kraehenbuhl, J.-P., and M. R. Neutra. 1992. Transepithelial transport and mu-

Mostov, K. E., G. Blobel. 1984. The receptor for transepithelial transport of IgA

Pardridge, W. M. 1991. Advances in cell biology of blood-brain barrier trans-

Sztul, E. 1992. Transcytotic vesicle fusion with plasma membrane. Methods En-
zymol. 219:44-52.

Wandinger-Ness, A., M. K. Bennett, C. Antony, and K. Simons. 1990. Distinct

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Simons, K., P. Gonella, J. Bernake, L. Owen, M. Neutra, and R. A. Murphy. 1986. Uptake and transmembraneous transport of nerve growth factor in suckling rat ileum. J. Cell Biol. 103:1979-1990.

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Ruda, M., and J. D. Coulter. 1982. Axonal and transneuronal transport of wheat
germ agglutinin as a model for transcellular transport in neurons. In Axonal
Transport, R. S. Smith and M.A. Bisby, editors. Alan R. Liss, Inc., New
York. pp. 311-336.

LaRoca, C. D., and R. G.Wiley. 1988. Monoclonal anti-Thyl antibody (OX7)
Kraehenbuhl, J.-P., and M. R. Neutra. 1992. Transepithelial transport and mu-

Mostov, K. E., G. Blobel. 1984. The receptor for transepithelial transport of IgA

Pardridge, W. M. 1991. Advances in cell biology of blood-brain barrier trans-

Sztul, E. 1992. Transcytotic vesicle fusion with plasma membrane. Methods En-
zymol. 219:44-52.

Wandinger-Ness, A., M. K. Bennett, C. Antony, and K. Simons. 1990. Distinct

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Simons, K., P. Gonella, J. Bernake, L. Owen, M. Neutra, and R. A. Murphy. 1986. Uptake and transmembraneous transport of nerve growth factor in suckling rat ileum. J. Cell Biol. 103:1979-1990.

Simons, M., E. Ikonen, P. J. Tienari, A. Cid-Arregui, U. Mönning, K. Bey-

Ruda, M., and J. D. Coulter. 1982. Axonal and transneuronal transport of wheat
germ agglutinin as a model for transcellular transport in neurons. In Axonal
Transport, R. S. Smith and M.A. Bisby, editors. Alan R. Liss, Inc., New
York. pp. 311-336.