# Protein Transport to the Dendritic Plasma Membrane of Cultured Neurons Is Regulated by rab8p

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*Abstract.* In the companion paper (Huber, L. A., S. W. Pimplikar, R. G. Parton, H. Virta, M. Zerial, and K. Simons. *J. Cell Biol.* 123:35–45) we reported that the small GTPase rab8p is involved in transport from the TGN to the basolateral plasma membrane in epithelia. In the present work we investigated the localization and function of rab8p in polarized hippocampal neurons. By immunofluorescence microscopy we found that rab8p localized preferentially in the somatodendritic domain, and was excluded from the axon. Double-labeling immunofluorescence showed that some of the rab8p co-localized in the dendrites with the Semliki Forest Virus glycoprotein $E_2$ (SFV-$E_2$). An antisense oligonucleotide approach was used to investigate the role of rab8p in dendritic transport of newly synthesized viral glycoproteins. Antisense oligonucleotides corresponding to the initiation region of the rab8 coding sequence were added to the cultured neurons for four days. This treatment resulted in a significant decrease in cellular levels of rab8p and transport of SFV-$E_2$ from the cell body to the dendrites was significantly reduced. However, no effect was observed on axonal transport of influenza HA. From these results we conclude that rab8p is involved in transport of proteins to the dendritic surface in neurons.

L IKE most neuronal cells, the cell surface of hippocampal neurons can be divided into two territories: the axonal and the somatodendritic domains. To generate and maintain surface polarity and to perform their different functions, neurons have to target proteins to their specific dendritic and axonal localization. Hippocampal neurons in culture undergo a defined sequence of events from a nonpolarized to a mature polarized state (Dotti et al., 1988). After 10–14 d in culture the axon and the dendrites of these hippocampal cells have acquired all the molecular and functional properties of mature neurons, forming a synaptic network on the culture dish (Banker and Waxman, 1988; Bartlett and Banker, 1984; Dotti and Banker, 1987). One strategy to analyze polarized membrane traffic in neurons has been to use enveloped RNA viruses such as Semliki Forest virus (SFV),[1] vesicular stomatitis virus (VSV), and influenza virus and follow the transport of the newly synthesized viral glycoproteins. This method has been used successfully in nonneuronal cells (Simons and Warren, 1984; Roman and Garoff, 1986; Rodriguez-Boulan and Sabatini, 1978). After infection of hippocampal neurons with VSV or SFV, the viral glycoproteins (VSV-G, SFV-$E_2$) were distributed from the Golgi complex in the cell body to the dendritic cell surface and to the plasma membrane of the perikaryon, but not into the axons. In contrast, the influenza Fowl Plague virus-Hemagglutinin (FPV-HA), was distributed from the Golgi complex to the axonal surface (Dotti and Simons, 1990; Dotti et al., 1993). Neurons are not the only cells that polarize their surfaces. Epithelia form cellular sheets that segregate compartments of the organism. The plasma domain of each cell within these sheets is divided into two domains, an apical domain facing the external milieu and a basolateral domain which is in contact with the internal milieu and the blood supply. These plasma membrane domains have different lipid and protein compositions (Rodriguez-Boulan and Salas, 1989; Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Although surface polarities in neurons and epithelia have little in common functionally, the molecular mechanisms of surface protein sorting may share common features. The influenza virus HA is routed to the apical surface of MDCK cells while the spike glycoproteins of VSV and SFV are basolateral (Rodriguez-Boulan and Pendergast, 1980). These results suggest that the apical and axonal domains are equivalent, whereas the basolateral surface would correspond to the somatodendritic domain. Moreover from recent studies with glycophosphatidylinositol (GPI)-anchored proteins, that are sorted to the apical surface in most epithelial cells (Lisanti et al., 1990) at least the Thy-1 protein is delivered to the axon of hippocampal cells (Dotti et al., 1991). The transferrin receptor, basolateral in most epithelial cell types (Fuller and Simons,

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1. *Abbreviations used in this paper:* ECL, enhanced chemoluminescence; FPV, Fowl Plague virus; GPI, glycophosphatidylinositol; HA, hemagglutinin; NSE, neuron-specific enolase; PCR, polymerase chain reaction; RT, reverse transcription; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus.

1986) is present in the somatodendritic domains of hippocampal neurons (Cameron et al., 1991; Parton et al., 1992). Besides similarities and dissimilarities in the sorting pathways of membrane proteins between neuronal and epithelial cells (reviewed in de Hoop and Dotti, 1993; Rodriguez-Boulan and Powell, 1992) little is known about the molecular composition of the machinery which confers specificity and directionality of vesicular transport either in MDCK cells or in hippocampal neurons. Recent studies have shown that ras-like small GTP-binding proteins play an important role in membrane traffic (reviewed in Pfeffer, 1992; Olkkonen et al., 1993). According to the rab protein paradigm, polarized cells like MDCK as well as hippocampal neurons would require different rab proteins to confer basolateral/apical and dendritic/axonal specificity. We have shown that in polarized MDCK cells rab8p is located along the basolateral transport route from the TGN to the plasma membrane (Huber et al., 1993). Using in vitro assays (Kobayashi et al., 1992; Pimplikar and Simons, 1993) in MDCK cells we further demonstrated that rab8p is a regulating component of the basolateral transport machinery (Huber et al., 1993). In this paper we show that rab8p is preferentially found in the somatodendritic domain of hippocampal neurons and that lack of rab8p results in impairment of dendritic transport of newly synthesized viral glycoproteins.

## Materials and Methods

### Cell Culture

Hippocampal cells were prepared as described by Bartlett and Banker (1984). Briefly, the hippocampi of 18-d old rat embryos were microscopically dissected, trypsinized (0.25% for 15 min), and further dissociated by repeated passages through a constricted Pasteur pipette. Cells were plated onto polylysine-coated coverslips in dishes containing MEM supplemented with 10% FCS and allowed to attach to the substratum for 4 h. The neuronal coverslips were then transferred to culture dishes containing a monolayer of astrocytes that had been kept in serum-free medium for at least 24 h. The coverslips were next put with the neurons upside down, in close proximity toward the glial monolayer. This coculture, neuron-glia, was previously shown to improve the long-term survival of the neurons. Cells were maintained in a humidified incubator at 37°C and 5% $CO_2$ for 10–14 days before virus infections or antisense-oligonucleotide experiments were performed (stage 5 neurons). For some experiments cells were kept in culture for only 24–48 h before virus infection (stage 2 and stage 3 neurons).

### Infection with Fowl Plague Virus and Semliki Forest Virus

Stocks of FPV and SFV were obtained as described previously (Matlin and Simons, 1984; Fuller et al., 1985a). Coverslips with neurons were removed from the culture dishes, rinsed in HBSS, and infected. FPV was used at a concentration of 80–100 pfu per cell and SFV at a concentration of 10 pfu per cell. After allowing viral entry for 1 h (37°C, 5% $Co_2$), the cells were washed in culture medium and the infection continued for 6 h. At the end of infection, the coverslips were rinsed in HBSS and processed for double-immunofluorescence microscopy for detection of FPV/SFV glycoprotein complexes.

### Immunofluorescence and Antibodies

Cells were washed once with PBS and permeabilized with 0.01% Saponin (Sigma Chemical GmbH, Munich, Germany) in 80 mM K-Pipes (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$ for 30 s. Cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 20 min, free aldehyde groups were quenched with 50 mM $NH_4Cl$ in PBS for 20 min. Unspecific primary antibody binding was prevented by a 30-min incubation in PBS containing 10% horse serum. The cells were then incubated in the presence of primary antibodies for ei-
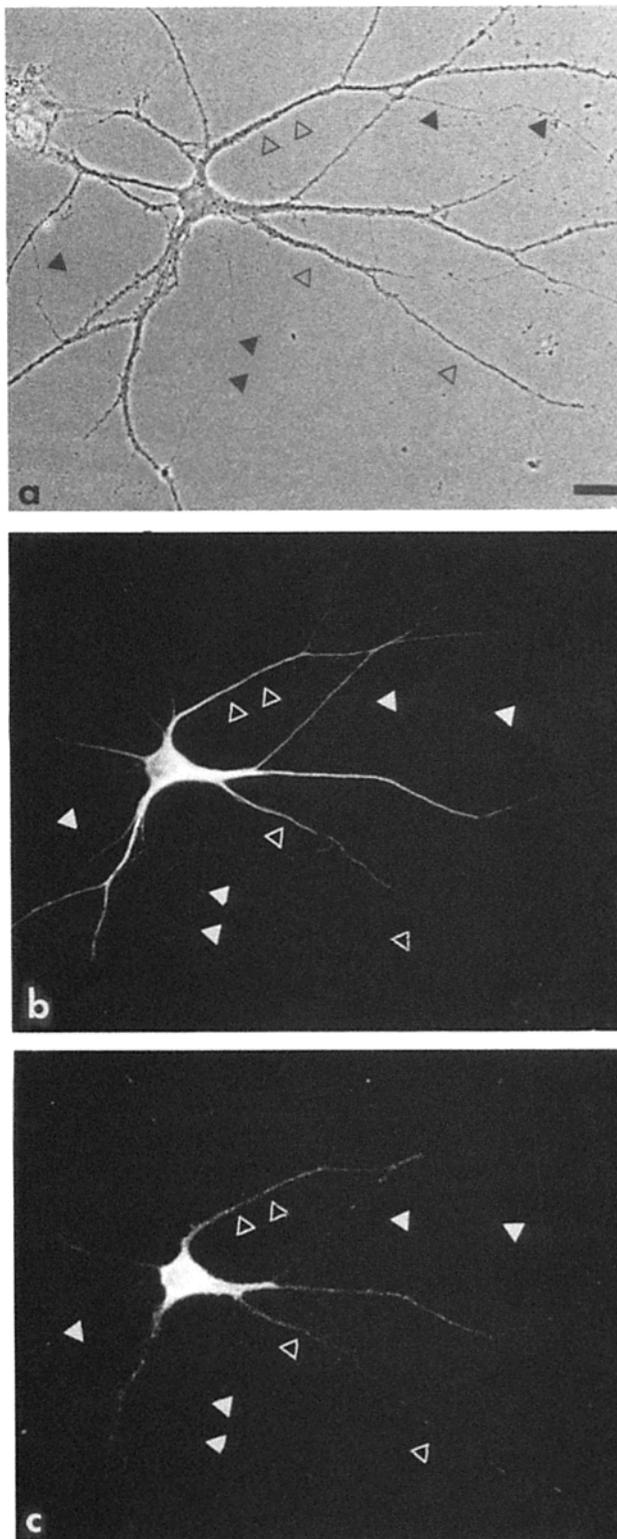


*Figure 1.* Intracellular distribution of rab8p in mature (stage 5) hippocampal neurons. (a) phase-contrast micrograph; (b) anti-MAP2 (1:20); (c) anti-rab8p (1:20). Dendrites (*open arrowheads*) and axons (*closed arrowheads*) are differentiated by their morphology and by MAP2 immunoreactivity (only dendrites are positive). Incubation with the affinity purified anti-rab8p antibody revealed labeling in the cell body and in dendrites (c). Axons (*closed arrowheads*) are devoid of labeling. Bar, 10 $\mu$m.
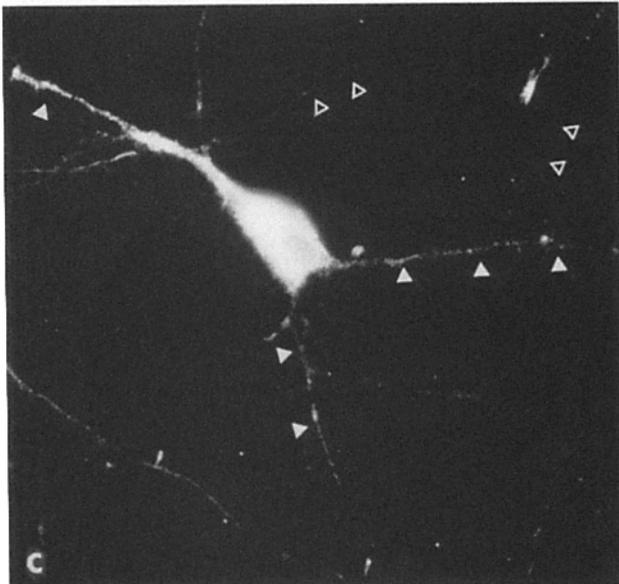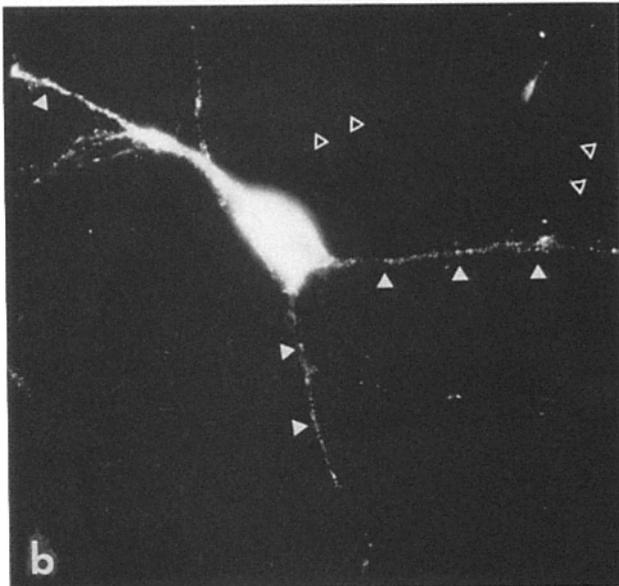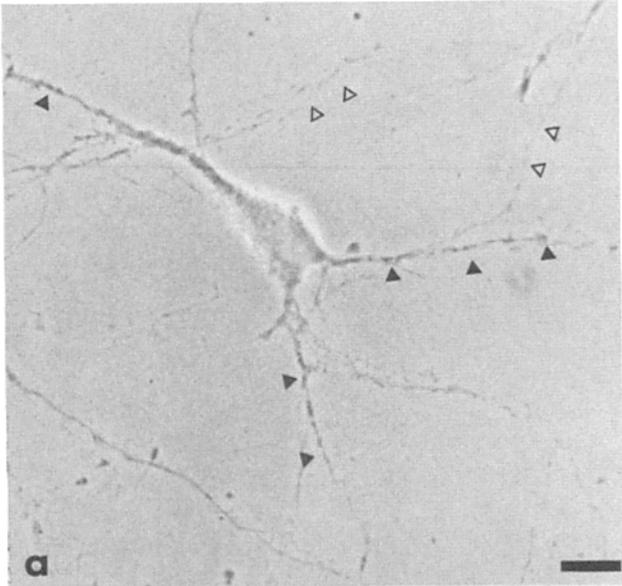
ther single or double immunofluorescence. The reaction was stopped by three 5-min rinses in PBS followed by a 30-min incubation with the corresponding secondary antibodies (FITC-conjugated goat anti–mouse IgG and RITC-conjugated goat anti–rabbit IgG; Dianova GmbH, Hamburg, Germany). For surface labeling of the FPV-HA glycoprotein, cells were processed as described in Dotti and Simons (1990). Briefly, cells were fixed with 4% formaldehyde and then reacted with an anti-HA mAb followed by species-specific FITC-conjugated secondary antibody. Cells were then permeabilized in Triton X-100 and rab8p localized as above. Immunofluorescence analysis was performed in an Axiophot fluorescence microscope from Zeiss (Oberkochen, Germany). Photographs were taken using high-sensitivity film (TMax 3200, Eastman Kodak Co., Rochester, NY).

Polyclonal antibodies were raised against synthetic peptides as described in Huber et al. 1993. Mouse monoclonal anti-MAP2 antibodies were from Boehringer Mannheim GmbH (Mannheim, Germany), mouse monoclonals anti–FPV HA and anti–SFV-$E_2$ have been previously described (Matlin et al., 1981; Fuller et al., 1985b).

## Preparation of Total Cellular Membrane Fractions

High-density cultures of mature (stage 5) hippocampal neurons (400,000 cells per 6 cm culture dish) were washed three times with ice cold PBS(+), scraped at 4°C by using a rubber policeman in 1 ml PBS(+)/50 mm dish and transferred to a centrifuge tube. Plates were then washed again with 1 ml PBS(+) and the pooled suspensions were centrifuged for 5 min, 4°C at 1,500 rpm. Buffer was changed and cell pellets equilibrated by a centrifugation (10 min, 4°C, 2000 rpm) with 2 ml of 250 mM sucrose, 3 mM imidazole (Serva, Heidelberg, Germany), pH 7.4. Pellets were resuspended in 0.5 ml of 250 mM sucrose, 3 mM imidazole, pH 7.4, plus 0.5 mM EDTA and homogenized by passing 10 times through a blue pipette tip (Gilson 1 ml) and 5–15 times through a 22 G needle. The homogenate was analyzed by microscopy and showed that about ∼90-95% of the cells had been disrupted whereas nuclei were still intact. The homogenate was then centrifuged for 10 min, 4°C at 3,000 rpm. The supernatant, referred to as the postnuclear supernatant (PNS) was then transferred to a TLA100.2 microfuge tube and centrifuged for 30 min, 4°C at 60,000 rpm. The membrane pellet was then resuspended in PBS. An aliquot was separated for protein determination (Micro BCA protein assay; Pierce, Beijerland, Holland) and the rest transferred into SDS-sample buffer and processed for SDS-PAGE analysis.

## Immunoblotting

For Western blots, protein samples were lyzed in standard SDS sample buffer and extracts were separated on 15% polyacrylamide gels. Equal amounts of protein (100 μg) were applied. Separated proteins were transferred onto nitrocellulose filters. Western blotting with an enhanced chemoluminescence (ECL) system (Amersham Buchler GmbH, Braunschweig, Germany) was performed as described in Huber et al. 1993. Bands were quantified using the LKB Ultrascan XL laser densitometer. For reprobing blots were stripped at 50°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL, pH 6.7).

## Antisense/Sense Oligonucleotides

Phosphorothioate oligodeoxynucleotides was synthesized via phosphoramidite chemistry by sulfurization with tetraethylthiuram disulfide in acetonitrile (Vu and Hirschbein, 1991), using an ABI 394 DNA/RNA synthesizer (Applied Biosystems). Since it has been found that antisense oligonucleotides are most effective when complementary to intron splice sites or initiation codons (for review see (Marcus-Sekura, 1988; Akhtar and Juliano, 1992) the sequence sites selected were centered on the initiation ATG (ATG-antisense) and on a nonoverlapping site located immediately downstream (inner-antisense), deduced from a canine rab8 cDNA (Chavrier et al., 1990). ATG-antisense, 5'CCATATTACACTCTC3'; inner-anti-

*Figure 2.* Distribution of SFV-$E_2$ glycoprotein and rab8p in infected mature (stage 5) hippocampal neurons. (*a*) Phase-contrast micrograph; (*b*) anti-SFV-$E_2$ (1:200); (*c*) anti-rab8p (1:20). The viral glycoprotein complex as well as rab8p are located exclusively in the somatodendritic territory. In the corresponding phase contrast micrograph (*a*) the lack of axonal labeling for both is evident (*arrowheads*). Bar, 10 μm.
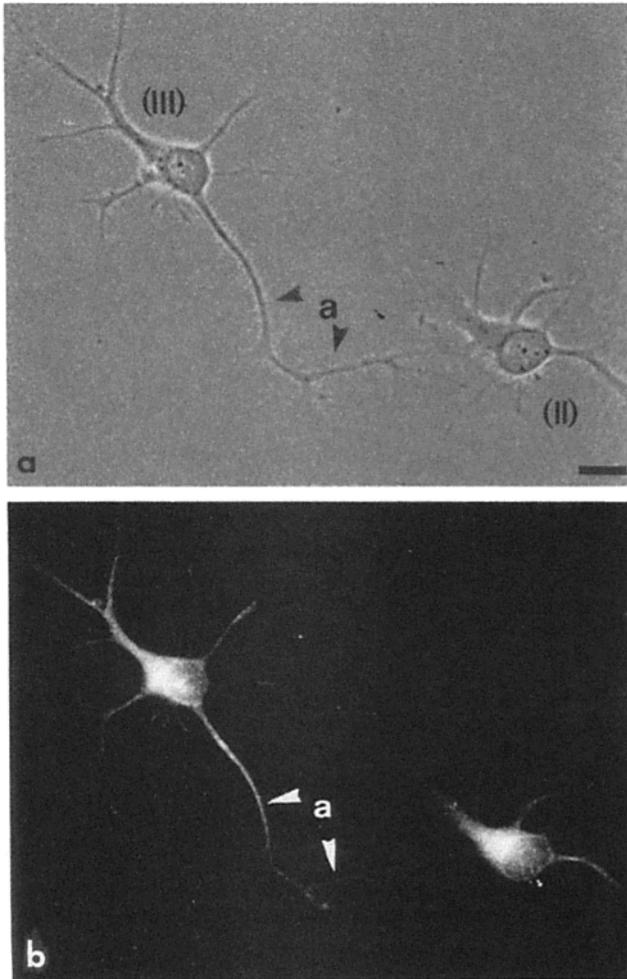
Figure 3. Immunofluorescence localization of rab8p in hippocampal neurons at stages 2 and 3 of development. (a) Phase-contrast micrograph; (b) anti-rab8p (1:20). Rab8p appears in the minor processes of stage 2 cells (II in a) and in the outgrowing axon and dendrites in stage 3 cells (III in a). Bar, 10 μm.

sense 5'AATCGTAGGTCTTCG3'. A reversed ATG-antisense (5'CTCTCA-CATTATACC 3') was synthesized as the control oligonucleotide.

Oligonucleotides were added in 10 μM final concentration in water directly to the culture medium of 10-d-old neurons. Cells were incubated for 72 h with either antisense (5 μM ATG antisense and 5 μM inner-antisense), reversed-antisense (5 μM) or were not supplemented with oligonucleotides. Fresh oligonucleotides were added every 24 h.

## Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were collected 5 h after the last oligonucleotide addition. Total RNA was extracted from equal amounts of cells (2 × 10⁵) in the presence of 20 μg *Escherichia coli* 5S RNA (Boehringer Mannnheim GmbH) as described (Chomczynski and Sacchi, 1987). RNA was reverse transcribed by 20 U AMV-RT (Promega, Heidelberg, Germany) and 0.5 μg oligo dT as primer for 1.5 h at 42°C and then heated for 5 min at 95°C to stop the reaction. 5% of the resulting cDNA was amplified in a total volume of 50 μl with 2.5 U Taq DNA polymerase (Boehringer Mannheim GmbH) in the presence of 0.25 μM dNTP and 50 pmole primers specific for rab8 and neuron-specific enolase. The primers (rab 8: 5' GTG AAC GAC AAG AGG CAG GTG [downstream] and 5'CCT TGT CCT CAC AGG AGA CTG [upstream]) [NSE: 5'GGA CAG CAA GAA AGA GGC TCC [downstream], 5'CTC TAC CAG GAC TTT GTC CGG [upstream]). The sequence of the oligonucleotides was based on published sequences (Elferink et al., 1992;
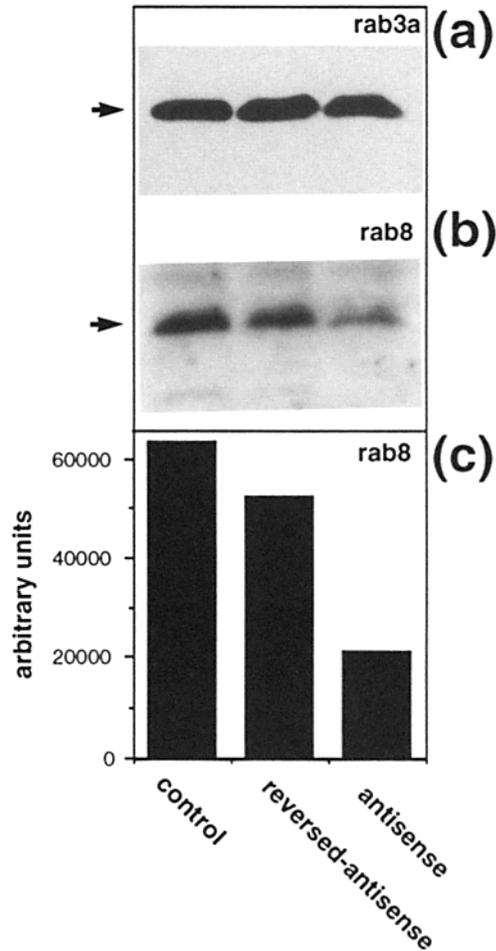


Figure 4. Western blot analysis of rab8 antisense-treated and -untreated cells. A Western blot of total membrane fractions first probed with affinity-purified anti-rab8p (1:5; b) was incubated with a secondary HRP-conjugated goat anti-rabbit antibody (1:3,000) and developed using ECL (Amersham). The blot was stripped and reprobed with a monoclonal anti-rab3ap (1:3,000; a) and developed in the same way with a secondary HRP-conjugated goat anti-mouse antibody (1:3,000). 200 μg of total membrane proteins were loaded in all lanes. Proteins are marked for identification. Histograms indicate relative intensities of rab8p bands (c) quantified with a LKB Ultrascan XL laser densitometer.

Sakimura et al., 1985). All primers had equal length and GC content to prevent differential annealing. In pilot experiments conditions were established in which the amount of PCR product increased with increasing amounts of cDNA. Reaction mixtures were amplified for 20, 25, and 30 cycles, each cycle consisting of 30 s, denaturation at 94°, 90 s, annealing at 60°C and 30 s, polymerization at 72°C. 15-μl reaction product was analyzed on a 2.5% agarose gel and visualized with UV after ethidium bromide staining.

## Results

### Colocalization of rab8p with Dendritic Markers

In our first series of experiments, we analyzed the intracellular distribution of rab8p in fully polarized hippocampal neurons (stage 5). Double-immunofluorescence analysis with anti-MAP2 antibody, a dendritic marker, (Caceres et al., 1984) and anti-rab8p antibodies revealed colocalization of these proteins in the somatodendritic domain (Fig. 1). The labeling along dendrites of anti-rab8p had a vesicular ap-

pearance. Axons were rab8p negative. To determine if the vesicular pattern corresponded to labeled exocytic vesicles, we next analyzed the distribution of rab8p in cells infected with SFV. Viral glycoproteins of SFV have been shown to be exclusively located in the somatodendritic domain of mature hippocampal neurons (Dotti et al., 1993). SFV-infected neurons were fixed and processed for dual immunofluorescence analysis of SFV-E$_2$ glycoprotein and rab8p. Rab8p and SFV-E$_2$ were seen in the cell body and along the dendrites (Fig. 2, a–c, *closed arrowheads*) whereas axons (Fig. 2, a–c, *open arrowheads*) were devoid of immunoreactivity for both antibodies. These results show the polarized distribution of rab8p, along the somatodendritic domain of mature hippocampal neurons and the partial colocalization with a newly synthesized membrane protein.

Next we studied whether rab8p is already present at early stages of neuronal development. It was shown previously that in early stages of hippocampal development (stage 2 and stage 3 cells) no significant polarity in the delivery of VSV glycoproteins and HA takes place (Dotti and Simons, 1990). Typical stage 2 and 3 cells are shown in Fig. 3 (labeled *II* and *III*). Stage 2 cells show short cellular processes whereas in stage 3 cells one process has become the axon (Fig. 3, *a* and *b*, arrowheads). Immunofluorescence localization of rab8p revealed an equally distributed staining pattern along all cellular processes (Fig. 3, *a* and *b*) including the outgrowing axon. This result suggests that the polarized distribution of rab8 accompanies the development of molecular sorting.

## Antisense-oligonucleotide Effect on rab8 Expression

Given the dendritic location of rab8 and the known role in basolateral transport in MDCK cells we analyzed next the role of rab8p in dendritic transport of newly synthesized proteins.

Antisense DNA-oligonucleotides were therefore tested for their ability to prevent rab8p expression and inhibit dendritic transport. Oligonucleotides that were 15-nucleotides long, were chosen because they are short enough to enter living cells, and long enough to be sequence specific (Marcus-Sekura, 1988; Akhtar and Juliano, 1992). A pair of oligonucleotides was designed from rab8 cDNA covering the ATG initiation codon (ATG-antisense, inner-antisense). A control oligonucleotide was designed by reversing the sequence of the ATG oligonucleotide (reversed ATG-antisense). We used phosphorothioate oligonucleotides that have been shown to be effective in selectively inhibiting expression of a variety of genes including ras p21 (Chiang et al., 1991; Chang et al., 1991).

Since rab proteins have a long halflife (Zerial, M., unpublished observation) high-density cultures of mature hippocampal neurons (400,000 cells per 6 cm culture dish) were incubated with or without oligonucleotides for 72 h; fresh oligonucleotides were added every 24 h. After treatment with the combination of the two antisense oligonucleotides the level of cellular rab8p was measured on Western blots of SDS-solubilized membrane fractions from neurons (Fig. 4). The antisense treatment markedly reduced the total amount of rab8p to approximately one third, whereas treatment with the control oligonucleotides (reversed ATG-antisense) did not affect rab8p expression (Fig. 4, *b* and *c*). The same blot was then stripped and reprobed with a mAb raised against rab3ap. The amount of rab3ap was identical in control cells
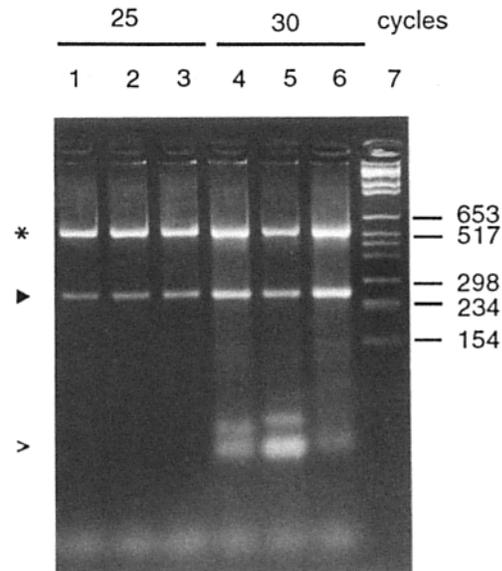


*Figure 5.* Detection of rab8 transcripts in reversed ATG antisense (lanes *1* and *4*), antisense (lanes *2* and *5*) and untreated neurons (lanes *3* and *6*). cDNA was amplified for 25 (lanes *1–3*) and 30 (lanes *4–6*) cycles in presence of oligonucleotides specific for rab8 and NSE. Lane *7* represents DNA molecular weight markers. Signals representing rab 8 (260 bp) are indicated by closed arrowhead, NSE signals (535 bp) by asterisk. The 260 bp was identified as a fragment containing rab8 sequences by EcoRV digestion. The presence of bands of equal intensities, representing NSE (in lanes *1–3* and *4–6*) shows that comparable amounts of RNA were isolated. Note that in lane *5* the total amount of PCR products is reduced due to primer-dimer formation (*open arrowhead*).

as well as in oligonucleotide treated cells (Fig. 4 *a*). This shows the specificity of the antisense-oligonucleotide treatment and confirms that equal amounts of cellular membrane proteins were applied to the analysis.

The mechanism causing reduction of protein levels by antisense inhibition is unknown. Possible mechanisms are either inhibition of transcription or of translation and/or interference with RNA stability (Akhtar and Juliano, 1992). We used the combination of reverse transcription (RT) of total RNA followed by the polymerase chain reaction (RT-PCR). This has been shown to be 100–10,000 times more sensitive than Northern blotting or RNA-protection assay (Siebert and Larrik, 1992). As a control for monitoring amounts of cDNA we co-amplified the RNA encoding neuron-specific enolase (NSE). All PCR reactions were performed under conditions where saturation in 25 and 30 cycles was not yet attained (Fig. 5). Two bands of 535 (NSE) and 260 bp (rab8) were visible. Despite antisense treatment, no obvious differences in rab 8 signals were obtained, indicating that, within the limit of our system (two- to threefold differences), no major differences in the amount of rab8 mRNA were detectable.

## Antisense Nucleotide Treatment Impairs Dendritic Transport

Once the inhibitory effect of the rab8 antisense treatment was proven at the protein level, we studied the effect of this treatment on viral glycoprotein transport. Hippocampal neurons
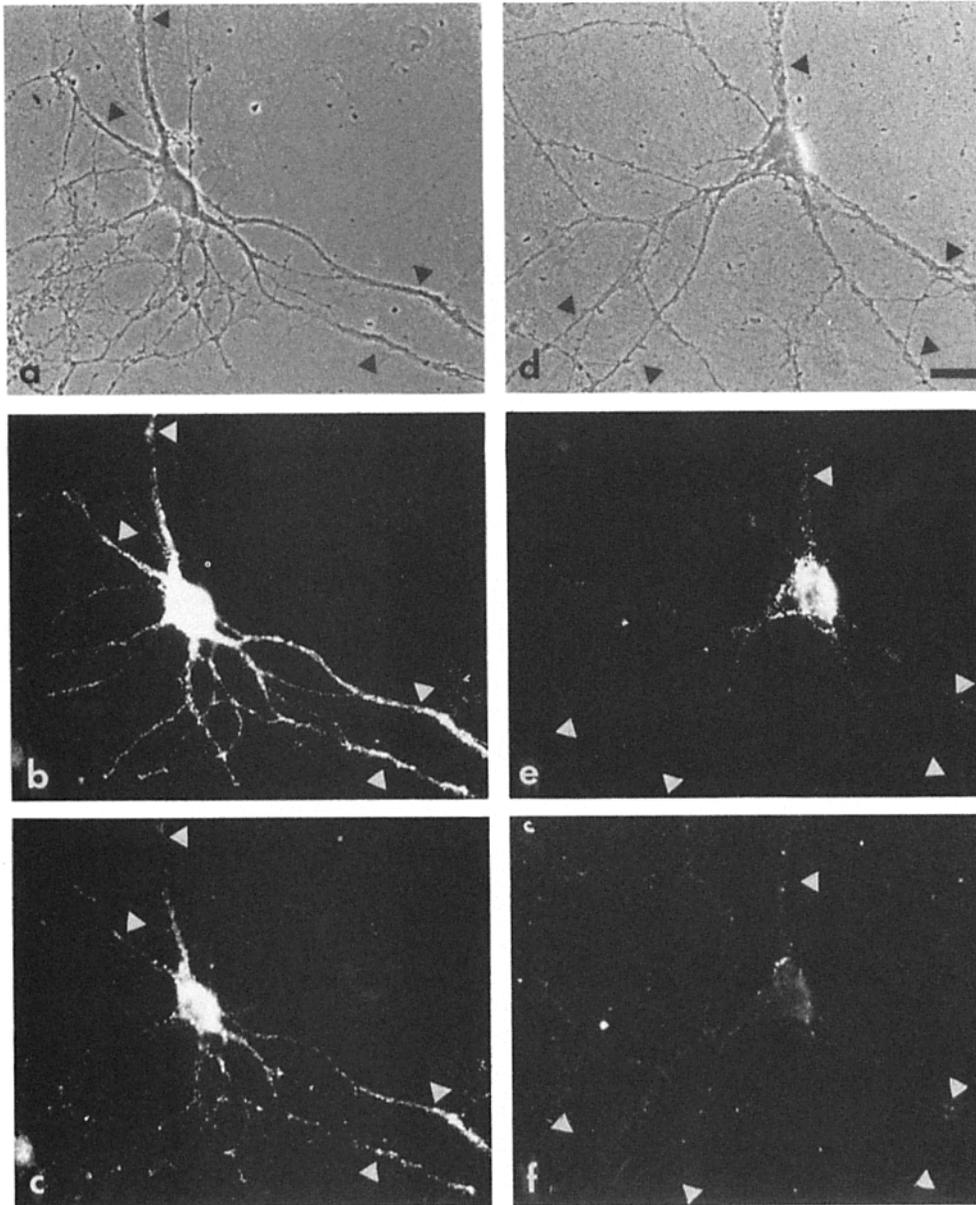
*Figure 6.* Dendritic delivery of SFV-E$_2$ in rab8 antisense oligonucleotide treated hippocampal neurons. Mature cells (stage 5) were treated for 72 h with either reversed ATG-antisense (*a–c*) or ATG-antisense oligonucleotides (*d–f*), infected with SFV, fixed, and prepared for double immunofluorescence analysis. (*a* and *d*) Phase contrast micrographs; (*b* and *e*) anti-SFV-E$_2$ (1:200); (*c* and *f*) anti-rab8p (1:20). Note: Dendritic delivery of SFV-E$_2$ glycoproteins is impaired in rab8p negative cells (*e* and *f*). Bar, 10 μm.

were incubated for 72 h with antisense oligonucleotides and then infected with SFV as described (Dotti et al., 1993). The distribution of the SVF-E$_2$ glycoprotein and rab8p was analyzed by immunofluorescence microscopy. In 10 independently performed experiments 60–80% of SFV-infected cells were rab8p negative (Fig. 6 *f*). Double labeling with anti-SFV E$_2$ antibodies showed that in rab8p negative cells the viral glycoproteins were found to accumulate within the perikaryon without entering the dendrite (Fig. 6 *e*). However, untreated as well as reversed ATG-antisense oligonucleotide-treated cultures were unaffected and showed a normal distribution of SFV-E$_2$ in dendrites and cell body (Fig. 6, *b* and *c*). Quantification of several experiments (Fig. 7) revealed that 85% of control cells (no oligonucleotides added to the cultures) showed delivery of SFV E$_2$ to dendrites. Similarly ~80% of cells treated with reversed ATG-antisense oligonucleotides showed a dendritic distribution. In antisense oligonucleotide exposed cells the degree of normal dendritic glycoprotein delivery occurred in only 30% of the

cells (Fig. 7). We next analyzed the intracellular distribution of influenza HA glycoprotein under identical experimental conditions (Fig. 8, *a–f*). Immunofluorescence microscopy showed positive immunoreactivity with anti-HA antibodies for 97% of cells from both control and anti-sense cultures (Fig. 8, *b* and *c*). In antisense-treated cultures 69% of the cells were rab8p negative, and in 95% of the rab8p-depleted cells HA labeling was found in the cell body accompanied by the appearance of punctate HA immunoreactivity along the entire length of the axons (note the rab8p negative cell in Fig. 8 *e*). From these results we conclude that rab8-antisense treatment selectively inhibited dendritic transport of viral glycoproteins in polarized neurons, whereas the axonal route was not affected.

## Discussion

One important result of this study is that the small GTPase rab8p, that has been shown in MDCK cells to be involved
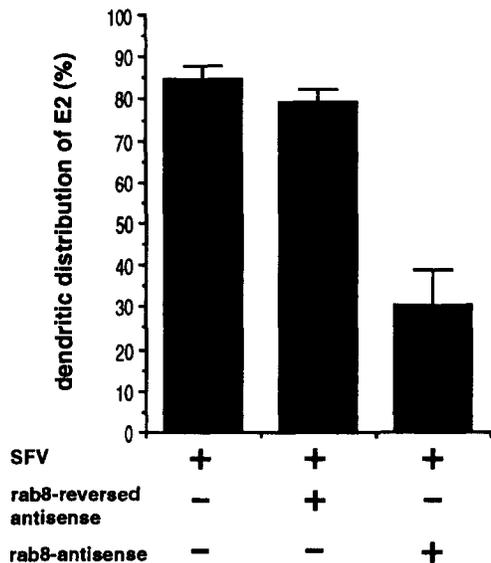
*Figure 7.* Dendritic distribution of SFV-E$_2$ was estimated from five independently performed experiments. Mature cells (stage 5) were treated for 72 h with either ATG-antisense, or reversed ATG-antisense oligonucleotides. No oligonucleotides were added to control cells. Cells were then infected with SFV, fixed, and prepared for immunofluorescence analysis with anti-SFV-E$_2$ antibodies. Dendritic distribution of SFV-E$_2$ is expressed as mean ± SE in percent from 100 cells, chosen from random fields for each experimental condition.

in basolateral membrane traffic from the TGN to the plasma membrane (Huber et al., 1993) is also involved in the dendritic delivery of viral glycoproteins in polarized neurons. We observed that in mature hippocampal neurons (stage 5) rab8p was limited to the cell body and to the dendrites, whereas there appeared to be very little, if any, rab8p in axons. In double immunofluorescence analysis of infected cells we observed a dendritic colocalization of rab8p with the viral glycoproteins of SFV. In contrast HA positive axons were clearly devoid of rab8p labeling.

To study hippocampal neurons deficient in rab8p we used synthetic oligonucleotides modified to prevent intracellular degradation. These oligonucleotide analogues are taken up intact by mammalian cells, possibly by endocytosis and/or by passive diffusion mechanism (Marcus-Sekura, 1988; Akhtar and Juliano, 1992). They have been shown to be effective in selectively inhibiting expression of a variety of genes including the small GTP binding protein ras p21 in Ha-ras 21 transformed NIH 3T3 fibroblasts (Chiang et al., 1991). Antisense oligonucleotides were also applied successfully for suppression of kinesin expression in cultured hippocampal neurons (Ferreira et al., 1992) and for inhibition of neuronal polarity by tau-antisense oligonucleotides in cerebellar primary cells (Caceres and Kosik, 1990).

Culturing hippocampal neurons for 72 h with rab8 antisense oligonucleotides caused a two third reduction in the level of cellular rab8p as detected by Western blotting. Several results indicate the specificity of our system. First, treatment for 72 h with either reversed ATG-antisense or antisense-oligonucleotides did not cause damage to the cells as appraised morphologically in phase contrast microscopy. Second, the reversed ATG-antisense oligonucleotide had no effect on rab8p expression. Third, as judged by immunofluo-

rescence microscopy two different antisense oligonucleotides complementary to two contiguous regions starting at the initiator ATG produced in parallel experiments similar reductions of rab8p (results not shown), but the combinational use of both of them was much more effective. Fourth, characteristic molecular markers of neurons are expressed in antisense-treated cells as they are in controls, including nerve-specific enolase mRNA and rab3a protein. Immunofluorescence analysis of low-density cultures revealed that in 60–80% of cells in antisense-treated cultures, immunoreactivity for rab8p was not detectable above background. The percentage of targeted cells in the cultures correlated well with the detected reduction of cellular rab8p by immunoblotting. There was good concordance between the lack of expression of rab8p and inhibiton of SFV glycoprotein delivery to the dendrites. On the other hand cells not expressing rab8p could still deliver the influenza HA to the axon. However, rab8 mRNA was not reduced to the same extent as was the rab8 protein. The observed reduction of rab8p after treatment with antisense oligonucleotides must therefore mainly be due to inhibition of translation. Similar results were obtained by others; Chiang et al. (1991) found that phosphorothioate oligonucleotides directed against the translation-initiation codon primarily interfered with translation, while oligonucleotides directed against the 3′-untranslated region reduced the amount of target mRNA. Also the reduction of MAP2 levels due to antisense treatment was predominantly caused by inibition of translation (Dinsmore and Solomon, 1991). Thus, the fact that antisense treatment did not result in a major reduction of rab8 mRNA level was not completely unexpected. The reduction in rab8p was reflected in a reduction of the number of neurons that could transport viral glycoproteins to the dendrites. Altogether these results demonstrate the functional role of rab8p in post Golgi transport in vivo in neurons. Whether rab8p is required for the formation of dendritic vesicles during the exit from the TGN or for the binding to specific dendritic microtubules or only to dock and/or fuse with the dendritic membrane remains to be established.

One question that has to be answered is whether rab8p is already present in cells that are not molecularly and/or morphologically polarized (stage 2 and stage 3). The delivery of viral glycoproteins during establishment of neuronal polarity was studied previously (Dotti and Simons, 1990). No significant polarity in the delivery of VSV glycoproteins and HA could be observed in these cells (Dotti and Simons, 1990). In analogy our results showed positive rab8p staining in form of fine dots in all minor processes of stage 2 cells as well as in the single outgrowing axon of stage 3 cells. The preferential distribution of rab8p to the somatodendritic domain takes place later in differentiation. Thus, maturation would then need activation and synthesis of new proteins, including small GTP-binding proteins, necessary for transforming the TGN to a state where segregation and sorting of axonal and dendritic proteins into separate vesicles take place. An alternative mechanism to explain the maturation of polarized targeting would be to postulate that axonal and dendritic carrier vesicles are produced already in immature neurons but their targeting and docking mechanism polarize during maturation. Preliminary results with GTP-overlay after high-resolution 2D gel electrophoresis suggested that a subset of small GTPases is upregulated in mature neurons after synaptogenesis (Huber, L. A., and C. Dotti, unpub-
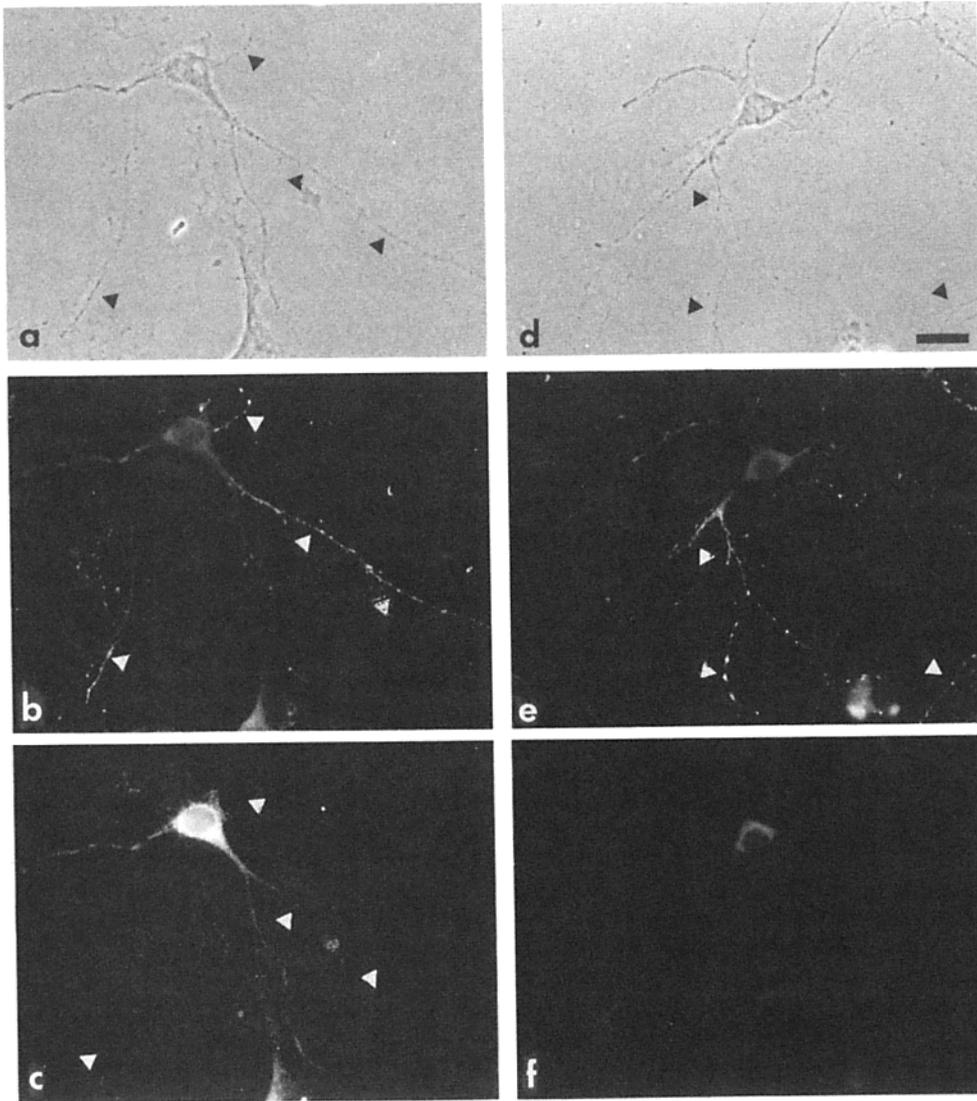
*Figure 8.* Axonal delivery of influenza HA in rab8 antisense oligonucleotide treated hippocampal neurons. Mature cells (stage 5) were treated for 72 h with either reversed ATG-antisense (*a–c*) or ATG-antisense oligonucleotides (*d–f*), infected with influenza virus, fixed and prepared for dual immunofluorescence analysis. The HA glycoprotein (*b* and *e*) is labeled on the neuronal surface. (*a* and *d*) phase contrast-micrographs; (*b* and *e*) anti-HA (1:400); (*c* and *f*) anti-rab8p (1:20). Note: All cells transport HA to the axon (*arrowheads* in *b* and *e*), including rab8p negative ones (*f*). Bar, 10 μm.

lished observation). Interestingly also rab8 protein as well as rab8 mRNA were found to be upregulated as detected by GTP-overlay and by quantitative PCR respectively (Huber, L. A., and C. Dotti, unpublished observation).

The possibility to create a "null" rab phenotype in cells that are exposed to antisense oligonucleotides immediately after plating provides a very promising approach to study neuronal development in hippocampal cultures.

These results show the specific involvement of rab8p in dendritic transport and further strengthen the hypothesis that part of the sorting machinery in neurons and epithelia may be similar (Simons et al., 1993).

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