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Targeting of endoplasmic reticulum-associated proteins to axons and dendrites in rotavirus-infected neurons

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ABSTRACT: To analyze sorting and compartmentalization of molecules in neuronal endomembranes, the distribution of endogenous proteins associated with the endoplasmic reticulum (ER), intermediate compartment, the Golgi apparatus in cultures of dorsal root ganglion (DRG), and hippocampal neurons was compared with that of newly synthesized ER-associated rotavirus proteins. The endogenous ER-retained immunoglobulin heavy chain binding protein, protein disulfide isomerase, and a peptide containing the KDEL amino acid sequence appeared in the soma and dendrites up to their first branching, but not in axons. However, two other endogenous ER-associated proteins, calreticulin and calnexin, occurred in axons as well as in the somatodendritic domains. The ER-associated rotavirus proteins, VP7 and NSP4, were widely distributed in cell bodies and dendrites. The former appeared also in axons and its localization partially overlapped with that of calreticulin and calnexin. One intermediate compartment protein, ER-Golgi-intermediate compartment-protein-53 (ERGIC-53), extended beyond the first division of the dendrites and did not, as the small guanosine 5'-triphosphate (GTP)-binding protein rab2, appear in axons. The location of rab2 to small vesicles was distinct from that of rotavirus VP7. *Cis*/medial Golgi cistern proteins were restricted to the cell bodies and proximal dendrites. This study emphasizes the marked heterogeneity in the targeting to axons and dendrites of proteins associated with ER and intermediate compartments. Therefore, the composition of axonal ER-retained molecules differs from that in the soma and this variation may reflect differences in functions between the ER compartments. Viral proteins are useful reporters for such heterogeneities and rotavirus VP7 may be a tool to reveal sorting signals for targeting of vesicular proteins to axons via a non-classical Golgi-independent mechanism. Such signals may also determine viral targeting to different regions of the brain. © 1998 Elsevier Science Inc.

KEY WORDS: Axonal transport, Chaperones, Nervous system, Virus.

INTRODUCTION

Correct delivery of newly synthesized molecules to axons and to the somatodendritic domain is fundamental for maintenance of the

structure and function of a neuron, but the mechanisms for intraneuronal targeting of proteins remain to be unraveled in greater detail. In epithelial cells, viral proteins, which utilize the cell's metabolic machinery, have provided efficient tools as markers for intracellular protein transport because they can be distinguished from the cell's endogenous proteins and, therefore, permit the tracing of a protein from its site of origin to its destination. Certain proteins of enveloped ribonucleic acid (RNA) viruses are targeted to the apical surface of polarized epithelial cells, while others to the basolateral domain. Such a selective viral protein distribution enables the study of protein sorting mechanisms [20,38].

Tracing of viral proteins is now also being used in neuroectodermal cells both *in vivo* and *in vitro* and in such studies asymmetries in transfer of viruses to the plasma domains have been revealed [5,8,19,22,23,27,49]. For these purposes, viruses that bud at the plasma membrane have commonly been used as reporters, but viruses that assemble on intracellular membranes are given increased interest [19]. In a series of studies using rotavirus, which assemble in the endoplasmic reticulum (ER), we have made the interesting observation that two viral proteins, which both are retained in the ER [28], show differences in their distribution in neurons. The nonstructural protein NSP4 remains in the cell body while the outer capsid protein VP7 appears also in axons. Although VP7 is a luminal ER-associated glycoprotein, its axonal transport could not be inhibited with brefeldin A [50], which is a drug that disrupts the organization of the Golgi apparatus [7], indicating that it can bypass the Golgi apparatus to reach the axons. The present study was undertaken with a view to compare the appearance of these newly formed viral proteins with the distribution of endogenous ER-retained proteins in neurons.

In epithelial cells, the ER and the intermediate compartment, which is involved in the transfer of molecules between the smooth ER and the *cis* Golgi cisterns, are heterogeneous in their compositions [12,33]. Certain soluble ER proteins have a KDEL sequence signal, which is considered to allow receptor-mediated retrieval of proteins from post-ER compartments, while other ER-retained proteins, such as the two rotavirus proteins described previously, do not contain this sequence [9,28]. In the present

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study we compare the distribution in neurons of different ER-retained proteins, which contain the KDEL sequence, with proteins that lack this sequence. The former included the luminal chaperones immunoglobulin heavy chains binding protein (BiP) [1,10,11], protein disulfide isomerase (PDI) [11] and calreticulin, which also is a major Ca^{2+} -binding protein in nonmuscle cells [29,30,42]. Calnexin is an ER-transmembrane protein with a cytoplasmic RKPRRE retrieval sequence [6,47].

Because the distribution of intercompartment proteins in neurons has only been subjected to limited studies [22], we also examined the localization of such proteins. ER-Golgi-intermediate compartment-protein-53 (ERGIC-53) is an integral membrane protein that recycles between the Golgi apparatus and the ER [40], and rab2 is a GTP-binding protein that catalyses the docking of vesicles and target membranes in a cell [35,46]. A focus of our studies on targeting of molecules to axons is given to the use of dorsal root ganglion (DRG) neurons, because their thick bundles of axons are easy to identify and analyze. To evaluate the appearance of proteins in dendrites, cultures of hippocampal neurons were used.

MATERIALS AND METHODS

Tissue Culture Technique

The DRG neurons were obtained from embryos of pregnant Sprague-Dawley rats (B&K, Stockholm, Sweden) taken on the 15th–18th day of gestation. Part of the study was performed on cultures that were prepared as described by Sotelo et al. [44]. In principle, the DRG were dissociated by several passages through a constricted Pasteur pipette and the cells seeded on collagen-coated (Collagen Corporation, Palo Alto, CA, USA) glass coverslips (G. Menzel, Braunschweig, Germany) attached to the bottom of sterile plastic Petri dishes (Costar, Cambridge, MA, USA). The culture medium consisted of minimum essential medium supplemented with 10% fetal bovine serum, 10% horse serum, 2% chicken embryo extract, gentamicin sulfate (15 $\mu\text{g}/\text{ml}$), L-glutamine (1 mM/ml; all obtained from Gibco, Paisley, Scotland), glucose (6 mg/ml), and nerve growth factor (NGF, 1 ng/ml; Sigma, St. Louis, MO, USA). The medium was changed three times a week. After 4–5 days, the cultures were exposed for 48 h to cytosine arabinoside (3 $\mu\text{g}/\text{ml}$; Sigma) to inhibit proliferation of nonneuronal cells. The same medium but devoid of 10% fetal bovine serum was then applied. For part of the study this technique was modified by coating the coverslips with Matrigel (Becton Dickinson, Bedford, MA, USA). The dissociated DRG were grown in a culture medium based on serum-free Neurobasal medium and B27 supplement, L-glutamine, gentamicin sulfate (15 $\mu\text{g}/\text{ml}$; all obtained from Gibco), and NGF (1 ng/ml; Sigma).

Hippocampal neurons were prepared from rat embryos, at 18–19 days of gestation, principally as described by Rothman [39]. The hippocampi were dissected, trypsinized (0.1% trypsin, Gibco) for 15 min at 37°C and dissociated by several passages through a constricted Pasteur pipette. Cell suspensions were seeded on poly-L-lysine hydrobromide (Sigma)-coated glass coverslips in Petri dishes. The culture medium, Dulbecco's MEM/Nutrient Mix F12 (Gibco) and 10% fetal bovine serum, was supplemented with glucose (1.2 mg/ml), 20 nM progesterone, 100 μM putrescine, 30 nM selenium dioxide (all obtained from Sigma), bovine insulin (5 $\mu\text{g}/\text{ml}$), and human transferrin (100 mg/ml; both obtained from Gibco). Penicillin and streptomycin (Gibco) were added to final concentrations of 20 units/ml and 20 $\mu\text{g}/\text{ml}$, respectively.

Virus Infection

The DRG and hippocampal cells were infected with plaque purified rhesus rotavirus [49] at a multiplicity of infection of 5.

Trypsin activation (10 $\mu\text{g}/\text{ml}$) for 30 min at 37°C was used to convert noninfectious rotaviruses into infectious ones. After 1 h incubation at 37°C, the inoculum was removed and the cells were washed once with Eagle's minimal essential medium and incubated in the culture medium without trypsin.

Antibodies

The following rabbit polyclonal antibodies were used: anti-GRP78 (BiP) antibody (Affinity BioReagents, Bolden, CO, USA), diluted 1:100; anti-KDEL antibody (Affinity BioReagents), diluted 1:50; anti-ERGIC-53 (G1/93) antibody [40] (kindly provided by Dr. H. P. Hauri, Department of Pharmacology, Biocenter of the University of Basel, Switzerland), diluted 1:100; anti-rab2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100; anti-calreticulin antibody (StressGen Biotechnologies Corp., Victoria, BC, Canada), diluted 1:50; and anti-calnexin antibody (StressGen), diluted 1:50. Furthermore, a rabbit antiserum raised against the native catalytic domain of rat liver Golgi alpha-mannosidase II [51] (kindly provided by Dr. M. G. Farquhar, Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, USA), diluted 1:300, was applied. Mouse monoclonal antibodies were used as follows: anti-PDI mAb (1D3 [48]; kindly provided by Dr. S. Fuller, European Molecular Biology Laboratory, Heidelberg, Germany), diluted 1:10 and anti-human PDI mAb (5B5; Dakopatts A/S, Copenhagen, Denmark), diluted 1:50. The rotavirus anti-NSP4 mAb (A54) and anti-VP7 mAb (M60) [50] (kindly supplied by Dr. H. B. Greenberg, Stanford University, School of Medicine, Stanford, CA, USA) were diluted 1:200 and 1:100, respectively. In addition, anti-MAP2 (2a + 2b) mAb (AP20; Sigma) diluted 1:100 and anti-tau-1 (PC1C6; Boehringer Mannheim, Indianapolis, IN, USA) diluted 1:100 were used.

Immunofluorescence and Confocal Microscopy Technique

Cultures were sampled at 24 and 48 h after infection. They were washed twice in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. They were permeabilized with 1% Triton X-100 (Eastman Kodak, Rochester, NY, USA) for 15 min and then incubated for 1.5 h at 37°C with the different antibodies diluted in PBS containing 0.3% Triton X-100. After washing in PBS, cells exposed to rabbit antisera were incubated for 45 min at 37°C with lissamine rhodamine (LRSC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:200 in PBS cells exposed to mouse monoclonal antibodies were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit antimouse IgG (Dakopatts), diluted 1:30. After rinsing, the cells were mounted in glycerol/PBS, pH 8.0, containing p-phenyldiamine. Double immunofluorescence labeling was performed using rabbit polyclonal antibodies against the different endogenous proteins and mouse monoclonal antibodies against the viral proteins NSP4 and VP7. The immunolabeling was visualized by LRSC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab.), diluted 1:200, and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (FITC), diluted 1:15, in 2% normal rat serum, respectively. The cultures were rinsed and mounted as described earlier. They were then examined both by epifluorescence light microscopy and by confocal microscopy (Sarastro).

RESULTS

DRG Neurons

In this study we used two types of DRG cultures. One type was characterized by the presence of DRG neurons attached to a

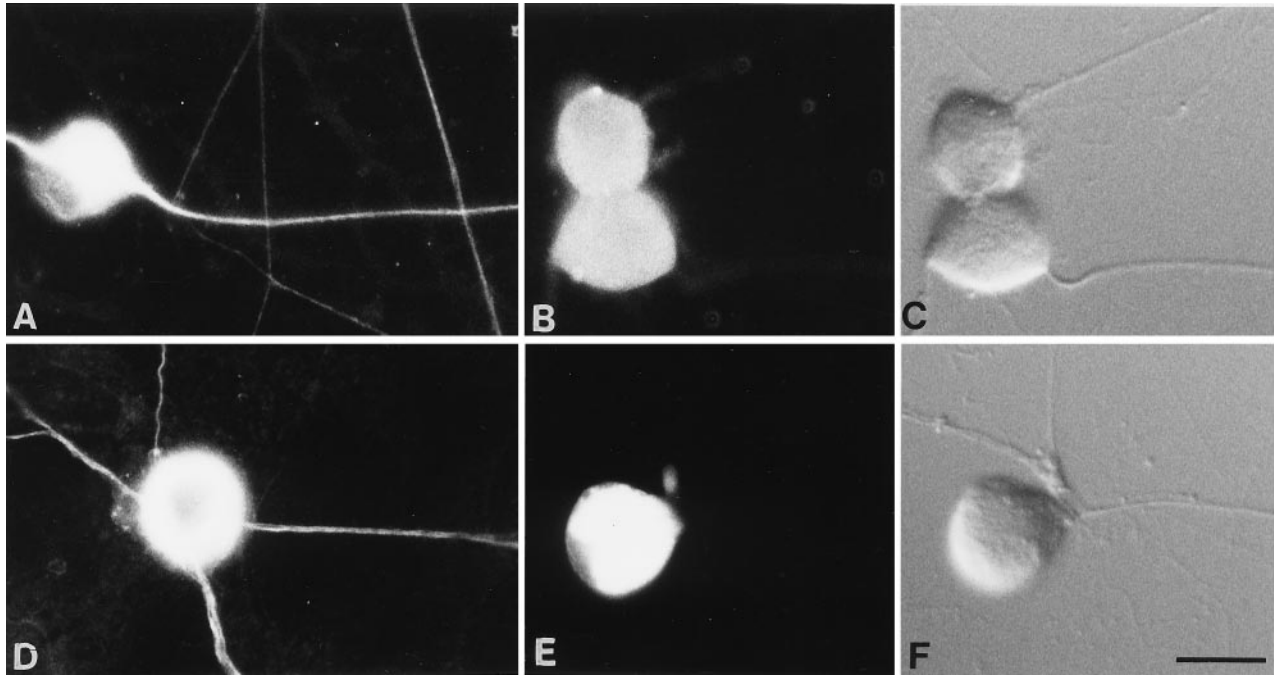


FIG. 1. The DRG neurons, 2 days in culture, showing immunopositivity for the tau protein in axons (A); while that for MAP2 is limited to cell bodies (B). In rotavirus-infected DRG neurons (24 h after infection) immunolabeling of the VP7 protein occurs both in cell bodies and axons (D). The rotavirus nonstructural protein NSP4 is confined to cell body (E). The DRG neurons that did not show immunolabeling of their axons (B and E) are visualized with Nomarski interference contrast microscopy (C and F). Scale bar = 25 μm .

collagen matrix and maintained in media complemented with sera. The neurons were well preserved for long periods of time and were used after 12–14 days in culture. However, in spite of the use of antimetabolic agents, non-neuronal cells tended to overgrow the neurons and their processes. In the other type of the DRG culture, the cells were seeded on the Matrigel substrate, which although effective in promoting the outgrowth of neuronal processes, could only maintain the cultures for a limited period of time. These cultures were maintained in serum-free medium, which diminished significantly the presence of nonneuronal cells and this allowed clear observations on axons. Such cultures were used for infection after 2 days in culture. In both types of cultures, the DRG neurons were characterized by the presence of well developed axons, which often formed thick bundles projecting from the cell bodies. The axonal processes were labeled with the antibody to tau (Fig. 1A), while immunolabeling with the MAP2 antibody was confined to the cell bodies in both types of cultures (Fig. 1B and C). After infection with rotavirus, the distribution of these microtubule-associated proteins was maintained. The rotavirus VP7 glycoprotein appeared in both cell bodies and axons (Fig. 1D), but the NSP4 protein was restricted to the cell bodies even at 48 h after infection (Fig. 1E and F).

The luminal endogenous ER-associated proteins showed marked heterogeneities in their distribution: calreticulin appeared both in the cell bodies and axons (Fig. 2A), while BiP (Fig. 2B and C) and PDI, as well as the peptide containing the KDEL sequence (Fig. 2E and F), were seen only in the cell bodies of the neurons. There was no difference in the immunolabeling using the two different PDI mAbs. The transmembrane ER-associated protein calnexin was seen both in the cell bodies and axons (Fig. 2D). This was also the case for the intercompartment protein rab2 immunopositivity, which was accentuated in distal axons (Fig. 2G). The other intercompartment protein, ERGIC-53, was, on the other

hand, restricted to the perikarya of the neurons (Fig. 2H and I). Antibodies to mannosidase II labeled stacks of cisterns within the cell bodies (see Fig. 4A).

In double-labeling experiments, VP7- and rab2-immunoreactive vesicles were distinct from each other and there was no overlap in the immunopositivities. In contrast, the immunopositivity to VP7 overlapped extensively with that to calreticulin and calnexin both in the nerve cell bodies and axons. Immunopositivities to BiP, PDI, the KDEL-peptide sequence, and ERGIC-53 were all restricted to the cell bodies and overlapped with that of the rotavirus NSP4 protein. None of these proteins appeared in axons even 48 h after infection.

Hippocampal Neurons

The hippocampal neurons showed an extensive network of both dendritic and axonal processes. The dendrites were prominent and could be identified by their tapering and branching at acute angles. Both the nerve cell soma and the dendrites were immunolabeled with the MAP2 antibody (see Fig. 4B). The axons were thin and, due to the overgrowth by glial cells, they were much more difficult to distinguish than the thick and prominent dendrites. In rotavirus-infected neurons, VP7-immunopositive material was seen as distinct and relatively large granules in the perikarya and far out into both dendrites (beyond the third division) and axons (Figs. 3A; 4C and D). The granules accumulated in larger groups at dendritic branching sites. The NSP4 immunolabeling was found in granules localized to both the cell body and dendrites, where they also extended beyond the third division (Fig. 3B), but it could not be detected in axon-like structures. Calreticulin immunopositivity extended equally far into dendrites and into axons (Fig. 3C), while that of the other proteins (BiP and PDI) and the peptide containing the KDEL sequence was restricted to the cell bodies and proximal

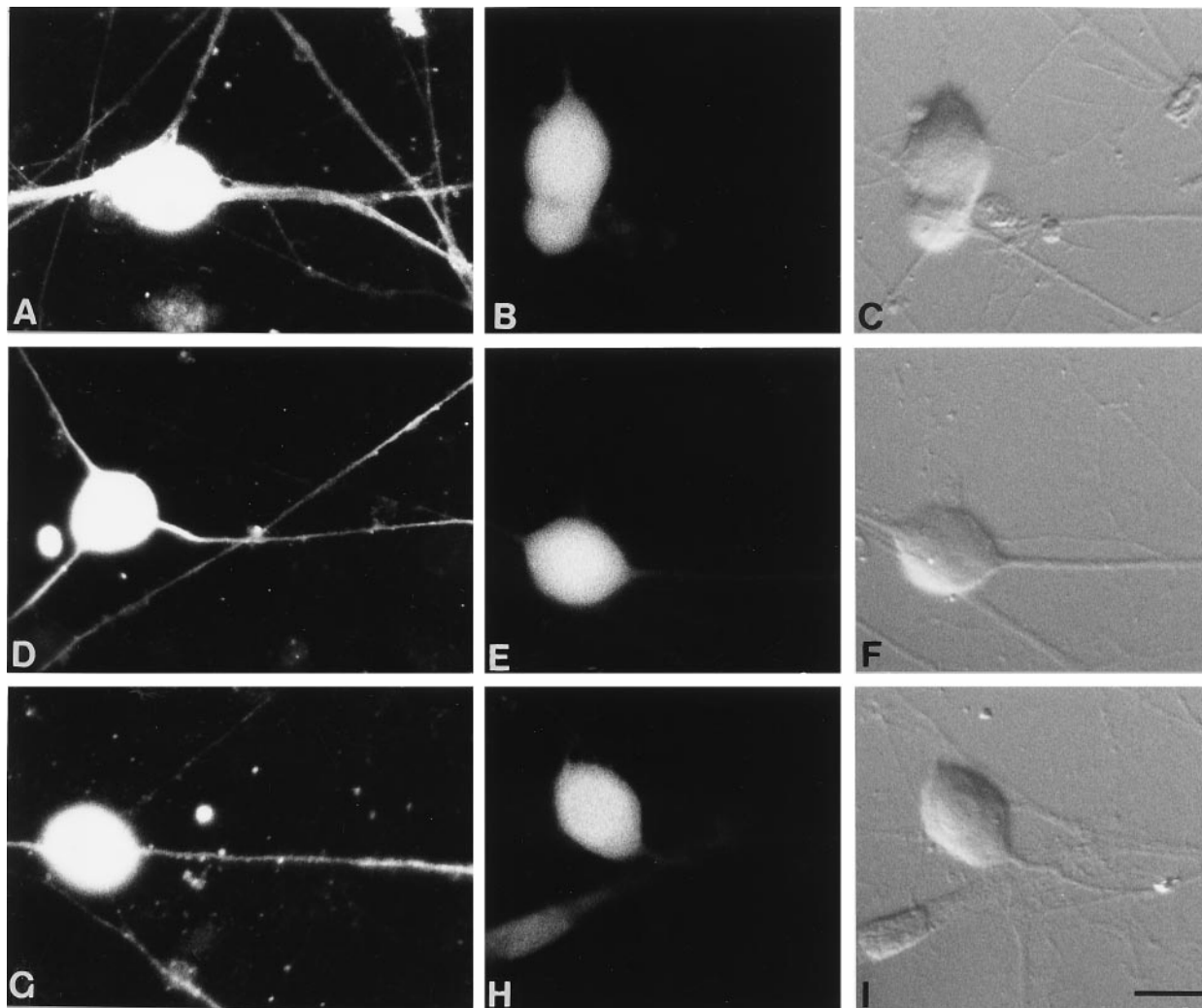


FIG. 2. Immunofluorescence of DRG neurons, 2 days in culture, showing localization of endogenous ER-associated and intercompartment proteins. Calreticulin (A), calnexin (D), and rab2 (G) appear both in cell bodies and axons. The KDEL-peptide sequence (B), BiP (E), and ERGIC-53 protein (H) are only seen in the perikarya; the axons of these neurons are visible in Nomarski microscopy (C, F, and I). Scale bar = 25 μ m.

parts of the dendrites. Immunolabeling with calnexin antibody showed a similar extension into dendrites and axons as calreticulin. The antibodies against intercompartment proteins rab2 and ERGIC-53 displayed distinct immunopositivity in the hippocampal neurons. The former one was found far beyond the third division of dendrites and in axons. The ERGIC-53 labeling appeared also beyond the first branching point of dendrites, but not in axons. Mannosidase II labeled cisterns mainly restricted to the cell bodies (Figs. 3D and 4B).

In double-labeling experiments the rotavirus VP7 protein and the endogenous rab2 occurred in different pools of vesicles with no obvious overlap (Fig. 4C). Calreticulin and calnexin showed, on the other hand, both extensive overlaps of their immunopositivities with VP7 both in cell bodies and processes (Fig. 4D). However, the two endogenous proteins were expressed much more abundantly within the dendritic tree than VP7. Therefore, the overlap in immunostaining occurred as granules among stretches of dendrites with immunopositivity for calreticulin or calnexin only. Double-labeling with the rotavirus NSP4 protein and the endogenous BiP,

PDI, and KDEL-peptide sequence showed that the immunopositivity of the viral protein overlapped with that of the endogenous proteins within soma and to the first dendritic branching site.

DISCUSSION

The two ER-retained proteins BiP and PDI are soluble and serve the function of chaperones in protein folding [11]. Although originally described as restricted to the rough ER, both proteins seem to be located in the lumen of the entire ER and have, therefore, been considered to be general markers for the ER [41]. As shown previously and in the present study, both proteins were present in neurons. Villa et al. [52,53] reported that BiP immunoreactivity extended from the cell bodies to the dendritic spines of rat Purkinje cells *in vivo* and at the ultrastructural level the molecule was detected even in axons within the cerebellar cortex. In cultured hippocampal neurons, Krijnse-Locker et al. [22] also observed that some BiP and PDI-immunopositive granules occurred in thin axon-like structures, while the receptor for the

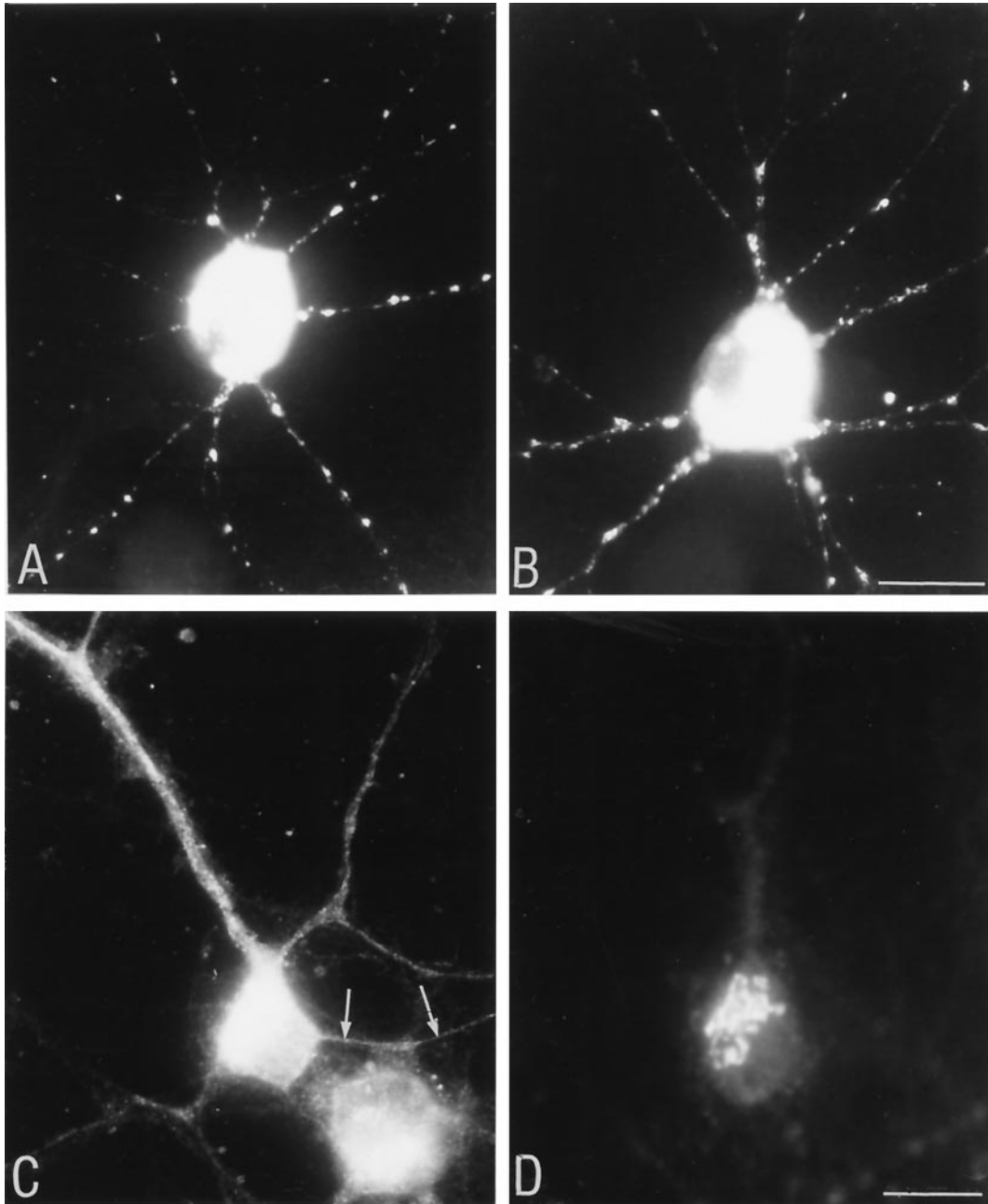


FIG. 3. Immunofluorescence localization of endogenous proteins and rotavirus proteins, VP7 and NSP4, in hippocampal neurons. The VP7 protein extends far into the dendrites (A). NSP4 antibodies label most of the somatodendritic domain (B); while those against calreticulin also label an axon-like process (arrow) (C). Mannosidase II (D) is confined to cisterns in the perikarya. Scale bar = 25 μ m.

KDEL sequence was almost limited to cell bodies. This contrasts the present observation in which these two molecules, as well as the KDEL-peptide sequence, did not seem to enter the axons of the DRG neurons, but extended into dendrites of the hippocampal neurons. However, another soluble luminal ER-protein, calreticulin, appeared in axons of the DRG in spite of the fact that this protein also contains a KDEL sequence. Calreticulin is a major calcium-binding protein in nonmuscle cells [29,30,42]. The protein is galactosylated, which indicates that it must have passed through

the *trans* Golgi cisterns, where galactosylation occurs, before being retrieved back to the ER [34]. It has been postulated that different ER-proteins can escape into different post-ER compartments and that ER-retained proteins can, therefore, be progressively retrieved from a series of positions along the secretory pathways [16,24]. Besides calcium-binding effects, calreticulin also serves the function of a chaperone in the ER, where it acts in concert with the transmembrane ER-protein calnexin in the control of folding of glycoproteins [3,13]. The cytoplasmic tail of calnexin

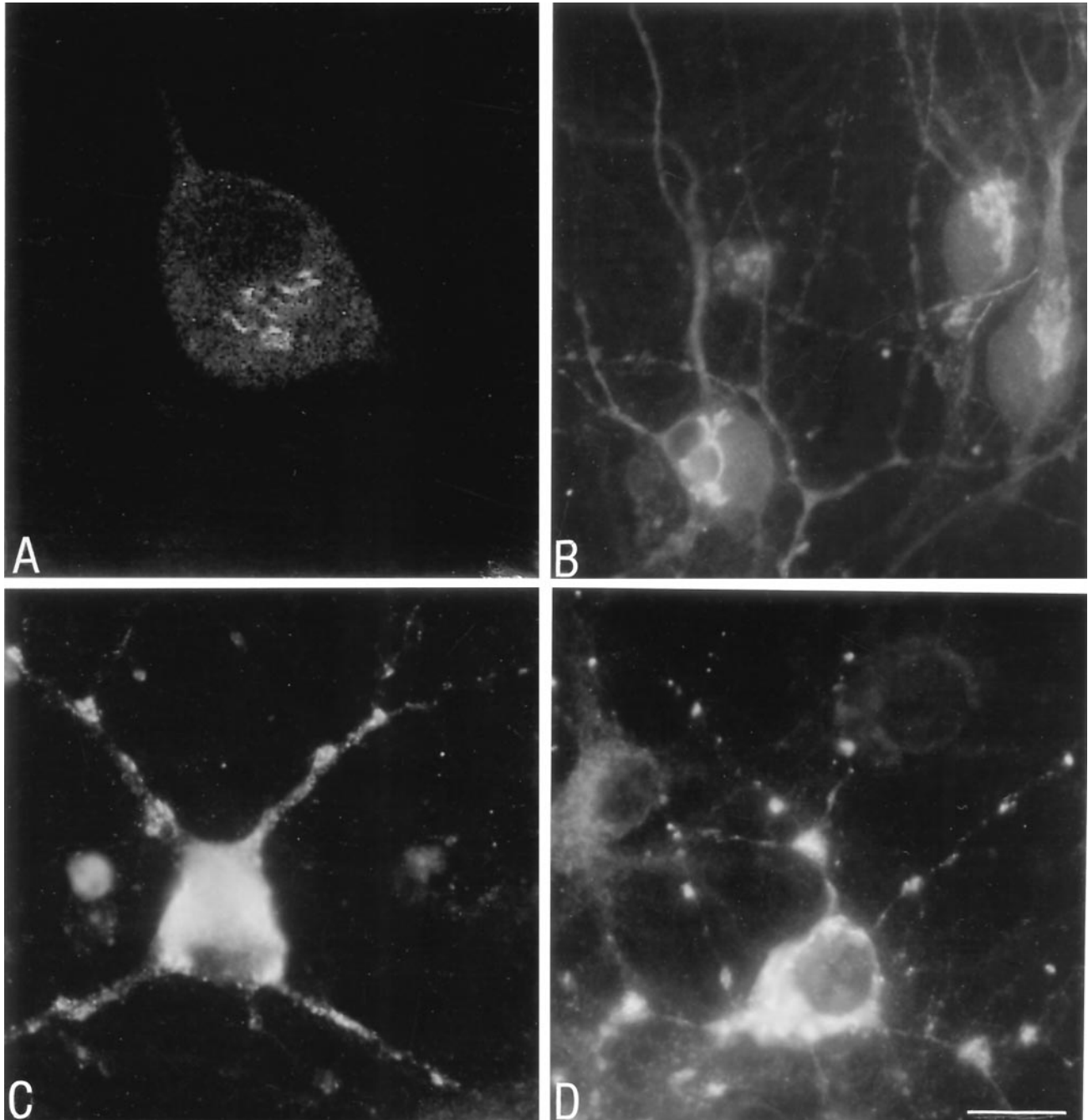


FIG. 4. The DRG neuron immunolabeled with anti-*cis*/medial Golgi-associated mannosidase II antibody showing stacks of cisterns in the perikaryon examined by confocal laser scanning microscopy (A); double-labeling with mannosidase II (strong fluorescence) and MAP2 (weak fluorescence) of hippocampal neurons (B); rotavirus-infected hippocampal neurons showed granules of VP7 and rab2 distinct from each other in cell bodies and dendrites (C); VP7 and calreticulin immunolabeled granules overlapped markedly in cell bodies and dendrites (D). Scale bar = 25 μ m.

carries a RKPRRE sequence that serves as a signal to localize this protein to the ER. In the present study these two chaperones co-localized not only in the somatodendritic domains but also in axons, which corroborates previous observations on the distribution of these proteins in rat and monkey cerebellar neurons [17,53].

The intermediate compartment is an extensive structure of a complex tubulo-vesicular organization that transfers molecules from the ER to the Golgi apparatus [32,33]. Proteins in this

compartment may occupy different subregions [21]. Viral proteins have served as markers for protein transport through the intermediate compartment vesicles and certain viruses can also assemble and mature at this site. For instance, the hepatitis B virus surface antigen co-localizes with rab2 [15] and vesicular stomatitis virus G protein with rab2 and p58 [26,36]. A coronavirus, mouse hepatitis virus, buds into intermediate compartment vesicles containing rab2 and p58, but also into PDI-containing structures [21], and the

vesicles that enwrap and ultimately form double envelopes around intracellular vaccinia virus particles may represent intermediate compartment vesicles [31,43]. In the present study the endogenous intermediate compartment protein ERGIC-53 was confined to the cell bodies and dendrites, while rab2 was found in both dendrites and axons. The latter observation is in contrast to the study on hippocampal neurons by Krijnse-Locker et al. [22], in which rab2 could not be detected in axons. In the DRG axons, the small rab2 vesicles did not colocalize with the VP7 protein, which indicates that also in axons the intermediate compartment vesicles are distinct from the ER. Rab2 belongs to the family of GTP-binding proteins, which use GTP hydrolysis to catalyze the association of recognition molecules in the docking of vesicles and target membranes in a cell [35,46]. Rab2 is considered to be involved in the traffic between ER and *cis* Golgi cisterns and its appearance in axons, in which Golgi apparatus is lacking, is unexpected. In cultured rat embryonic neurons, obtained from tectum and ventral midbrain, injection of purified rab2 protein caused an enhanced adhesion of the cells and an increased outgrowth of dendrite-like neurites. It was suggested that the protein may be involved in the transport and fusion of vesicles with the plasma membrane at the level of the growth cones [2].

In rotavirus-infected neurons the distribution of ER-associated proteins was the same as in controls, indicating that the neurons polarity is maintained during the observation period. The two rotavirus proteins, VP7 and NSP4, which both lack a KDEL sequence, showed marked differences in their distribution. The NSP4 protein had a polarized distribution and appeared in the somatodendritic domain of the neurons, while the VP7 protein also occurred in axons. The NSP4 protein seemed to be distinct in its distribution from the other ER-associated proteins. It did not enter axons but extended into dendrites beyond the level of BiP and PDI. NSP4 is a nonstructural transmembrane rough ER viral protein. It has been suggested to serve as a receptor for subviral particles in the cytosol to mediate their budding through the ER membrane in the process of virus morphogenesis [9]. The motif for retaining the high mannose containing NSP4 to the rough ER has not been identified. In addition to its role as a receptor, NSP4 has been suggested to regulate the intracellular calcium levels of infected cells. This may promote the acquisition of correct protein configuration during virus maturation, but may also be involved in the cytotoxicity [45].

The distribution to both axons and dendrites of VP7 was similar to that of the ER-associated calreticulin and calnexin, but differed from that of rab2. Previously, we have observed that VP7 does not pass through the Golgi apparatus on its way to axons [50]. The mechanisms by which the endo-H sensitive VP7 is retained in the ER remains an interesting question in cell biology since it does not contain a KDEL or RKPRRE sequence and it is not membrane spanning. Although the localization of VP7 and calreticulin to axonal ER-like structures may be similar, their routes to reach this compartment may differ; calreticulin being transferred through the Golgi apparatus to become galactosylated and VP7 being directly transferred. Anatomically, a direct communication between the rough ER of the nerve cell bodies and the smooth ER of the axons has repeatedly been proposed [4,14,25,37] and VP7 may, therefore, be a reporter to study this route of delivery of molecules to axons. Recently, a nonclassical pathway for vesicular transport to the apical cell surface that bypasses the Golgi apparatus has been detected also in epithelial cells by the use of rotavirus [18].

From these studies it appears that the ER in neurons has a heterogeneous composition. Certain of the ER proteins may enter the axons after being transferred through the Golgi apparatus, while others may have a direct communication. Calreticulin in axons may serve as a calcium-binding protein, but, since it appears

in context with calnexin, a role as a chaperone for proteins transported through the axons may also be suggested. By virtue of the relative ease by which viral proteins can be genetically manipulated, they provide useful tools for future studies to dissect the mechanisms of delivery of proteins to the various compartments of the ER.

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