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The Organization of the Endoplasmic Reticulum and the Intermediate Compartment in Cultured Rat Hippocampal Neurons

Jacomine Krijnse-Locker,* Robert G. Parton,* Stephen D. Fuller,† Gareth Griffiths,* and Carlos G. Dotti*‡

*Cell Biology and †Structural Biology Programm, European Molecular Biology Laboratory, D-69012 Heidelberg, Germany

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The boundaries of the organelles of the biosynthetic endomembrane system are still controversial. In this paper we take advantage of the unique architectural organization of neurons to investigate the localization of a spectrum of compartment-specific markers with the goal of defining the location of the rough endoplasmic reticulum (ER), smooth ER, intermediate compartment, and the Golgi complex. Markers of the rough ER (signal sequence receptor), Golgi complex (mannosidase II), and the *trans* Golgi network (TGN38) were essentially restricted to the cell body and the initial segment of one of the cell's dendrites. In contrast the cytochemical reaction product for glucose 6 phosphate, a classical ER marker, in addition to staining ER structures in the cell body also reacted with smooth ER elements that extended into both axons and dendrites. These peripheral smooth ER elements also reacted at the immunofluorescence level for ER marker 3-hydroxy-3-methylglutaryl-coenzyme A reductase, as well as for calnexin and protein disulfide isomerase. We also analyzed the location of rab1, rab2, p58, the KDEL receptor, and β-subunit of coatomer. These intermediate compartment markers were found predominantly in the cell body but also extended to the proximal parts of the dendrites. Collectively, our data argue that the ER of hippocampal neurons consists of functionally and spatially distinct and separated domains, and they stress the power of the hippocampal neuron system for investigations of the organization of the ER by light microscopy.

INTRODUCTION

Many essential features of the transport from the rough endoplasmic reticulum (ER)[1] to the cell surface are now well established. It has thus become clear that the exocytic pathway comprises distinct compartments through which membrane and secretory proteins obligatorily and sequentially pass on their way to the plasma membrane, namely the rough ER, the intermediate compartment (IC), also referred to as the *cis* Golgi network (CGN) or endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Hauri and Schweizer, 1992), and the Golgi complex (*cis*, medial, *trans*, and *trans* Golgi network [TGN]).

The ER consists of distinct domains, smooth and rough, that share direct membrane continuities and are regionally segregated (see Sitia and Meldolesi, 1992, for a review). Thus, the inner and outer nuclear envelope are continuous and are directly connected with the membranes of the rough ER. In cells such as hepatocytes or those of the adrenal cortex, which have

‡ Corresponding author: Cell Biology Program, Meyerhofstrasse 1., D-69012 Heidelberg, Germany.

[1] Abbreviations used: BiP, Ig binding protein; BSA, bovine serum albumin; COP, coatomer; DTT, dithiothreitol; EM, electron microscopy; ER, endoplasmic reticulum; G6Pase, glucose 6 phosphatase; GTPγS, guanosine-5′-O-(3-thiotriphosphate); HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IC, intermediate compartment; IF, immunofluorescence; ManII, mannosidase II; MHV, mouse hepatitis virus; pb, permeabilization buffer; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; SFV, Semliki forest virus; SLO, streptolysin O; SSR, signal sequence receptor; TGN, *trans* Golgi network; VSV-G, vesicular stomatitis virus G protein.

extensive regions of smooth ER, it has been shown that the smooth ER is also connected to the rough ER. In neuronal cells, Lindsey and Ellisman (1985a,b) showed that osmium tetroxide stained smooth membraned structures in the neurites and in the cell body. The latter were continuous on the one hand with the perinuclear rough ER and on the other hand with the *cis*-most cisterna of the Golgi complex. Some of these smooth membranes must comprise the compartment now referred to as the IC. Whether the neuronal IC is restricted to the cell body or is also present deep into the processes is not known.

The IC has often been considered to be a distinct (salvage) compartment (Warren, 1987; Pelham, 1989; Mellman and Simons, 1992) although other data support the notion that the IC is continuous with the rough ER, and thus can be considered a subdomain of the rough ER (Griffiths *et al.*, 1994; Krijnse-Locker *et al.*, 1994).

The recent focus on the IC is largely the result of the discovery of a variety of antigenic markers that are enriched in membrane structures that are functionally downstream of the rough ER but are located before the Golgi complex. These markers include the membrane protein p53/p58 (Schweizer *et al.*, 1988; Saraste *et al.*, 1987) and the small GTPases of the rab family, rab1 and rab2 (Chavrier *et al.*, 1990; Saraste *et al.*, 1995). Complementary studies have shown that the IC also provides the membranes for the assembly of corona and vaccinia viruses, and for the assembly of hepatitis B Ag (Tooze *et al.*, 1984; Sodeik *et al.*, 1993; Huovila *et al.*, 1992; Krijnse-Locker *et al.*, 1994).

In contrast to the rough ER it is difficult at both the immunofluorescence (IF) and electron microscopic levels to describe the boundaries of the IC. Some IF studies on the IC have emphasized the fact that the bulk of the IC is located in close proximity to the typical ribbon-like structure that is seen following labeling with markers of the Golgi complex (Schweizer *et al.*, 1988; Lippincott-Schwartz *et al.*, 1990). Other IF studies, however, have provided evidence that in many cell types, and in a significant fraction of the cell population, a large part of IC markers extends well beyond the area of the Golgi complex into the peripheral cytoplasm (Saraste and Svensson, 1991; Lotti *et al.*, 1992). A striking feature of IF localization of many IC-markers is the heterogeneity in the distribution pattern. Some cells show predominantly perinuclear labeling although adjacent cells show an extended peripheral distribution. Thus, the precise boundaries of the IC are still unclear.

To better define the boundaries of the neuronal IC and of the other organelles involved in the membrane biosynthetic pathway we have now analyzed by immunofluorescence microscopy the distribution of a spectrum of defined antibodies that have been extensively used as markers of the rough ER, smooth ER, IC, Golgi complex, and the TGN in cultured hippocampal neurons. Neurons appeared to be particularly suited for this investigation because the central (cell body) and peripheral (processes) domains are well separated. A major finding was that in these cells the smooth ER markers label the cell body and processes whereas the IC markers localized predominantly to the cell body along with the rough ER, the Golgi complex, and the TGN. In addition, the use of a temperature-sensitive mutant of the Semliki forest virus (SFV), as well as several ER resident proteins involved in folding and oligomerization suggests that the entire ER system might be involved in the processing of newly synthesized membrane proteins.

MATERIALS AND METHODS

Cells

Hippocampal cells were prepared from 18-day rat embryos according to the method of Goslin and Banker (1991). Briefly, after dissection from the brain hemispheres, the hippocampi were dissociated by trypsin and mechanical treatments. Cells were plated onto polylysine–treated glass or plastic coverslips. After allowing attachment for 4–12 h in medium containing fetal calf serum (E-MEM/10% FCS), the coverslips were transferred to dishes containing a monolayer of glial cells in serum-free medium. All experiments were performed in cells kept for 10–21 days in culture.

Antibodies

The following primary antibodies were used: rabbit polyclonal anti-signal sequence receptor (SSR; Vogel *et al.* 1990), dilution 1:25; rabbit polyclonal anti-rab2 (Chavrier *et al.*, 1990), dilution 1:20; rabbit polyclonal anti-rab1A (Saraste *et al.*, 1995), dilution 1:10; mouse monoclonal anti-protein disulfide isomerase (PDI) (1D3) (Vaux *et al.*, 1990), dilution 1:100; rabbit polyclonal anti-TGN (TGN38) (Luzio *et al.*, 1990), dilution 1:50; rabbit polyclonal anti-p58 (Saraste and Svenson, 1991); mouse monoclonal anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) reductase (Pathak *et al.*, 1986), dilution 1:50; mouse monoclonal 9AB4 anti-SFV p62/E2 (Burke *et al.*, 1983), dilution 1:100; rabbit polyclonal anti-mannosidase II (ManII) (Moremen and Touster, 1985), dilution 1:500; rabbit peptide serum anti-EAGE recognizing β-subunit of coatomer (β-COP) (Pepperkok *et al.*, 1993), dilution 1:40; rabbit peptide serum anti-calnexin (Hammond and Helenius, 1994), dilution 1:100; and rabbit peptide serum against the mammalian KDEL receptor (Tang *et al.*, 1993; Griffiths *et al.*, 1994), dilution 1:100. Fluorochrome-conjugated species-specific secondary antibodies were obtained from Dianova (Hamburg, Germany).

Virus Infections and Temperature Shift Experiments

The temperature-sensitive mutant of the Semliki forest virus, SFV ts-1 (Saraste *et al.*, 1980), was used. Fifteen– to twenty-one–day old neurons were infected at a multiplicity of infection of 10 for 1 h at 37°C, after which the cells were incubated for 2 h and 45 min at 39.5°C.

The cells were either fixed with 4% paraformaldehyde or transferred to air-medium (Dotti and Simons, 1990) containing 100 μg/ml cycloheximide and equilibrated at 19.5°C or 15°C in a waterbath. The cells were incubated for 2 h at these temperatures, before fixation with paraformaldehyde.

Indirect Immunofluorescence

For immunofluorescence localization of SSR, PDI, HMG-CoA reductase, and calnexin, glass coverslip–grown cells were fixed and permeabilized in cold methanol (10 min, −20°C), rinsed in phosphate-buffered saline (PBS), and sequentially incubated in 10% bovine serum albumin (BSA) in PBS (30 min at room temperature), primary antibody (in 1% BSA/PBS, 1 h at room temperature), and species-specific rhodamine or fluorescein-conjugated secondary antibodies (in 1% BSA/PBS). For localization of the rab2 protein, the protocol described by Chavrier *et al.* (1990) was followed. The ManII, TGN38, and p58 labeling was performed on cells fixed with 4% paraformaldehyde. The cells were dehydrated after fixation with increasing concentrations of ethanol (25, 50, 75, 90, 95, and 100%, 5 min each) and subsequently rehydrated with the same ethanol concentrations but in decreasing concentrations. The antibody incubations were done as described above. For the anti-β-COP labeling, the neurons were permeabilized with streptolysin O (SLO, 3 U/ml; Wellcome Diagnostics, Dartford, UK) in the following way. To prevent unwanted depolymerization of microtubules the cells were incubated for 15 min at 37°C in permeabilization buffer (pb; 60 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)-KOH, pH 6.9, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 10 mM EGTA, 2 mM $MgCl_2$) containing 0.5 μM taxol. Taxol was included in all the subsequent incubation steps. The cells were then put on ice and rinsed once with pb containing 1 mM dithiothreitol (DTT) and incubated for 4 min on ice with SLO in pb and DTT. The cells were then rinsed twice and incubated for 30 min at 37°C in pb/DTT and 50 μM GTPγS before fixation with paraformaldehyde. Under these conditions approximately 80% of the cells appeared to be permeabilized as assessed by trypan blue staining. For the antibody labeling (rab1A and β-COP), fixed cells were treated with 0.125% Triton X-100 for 10 min at room temperature and the labeling was performed as above. Microscopy was performed in an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with appropriate filter set. Tech Pan (400ASA, Kodak) film was utilized for photography.

Electron Microscopy (EM)

Glucose 6 phosphatase (G6Pase). Cells grown on plastic coverslips were fixed for 10 min in glutaraldehyde (2.5%)-paraformaldehyde (2%) in 0.1 M sodium cacodylate buffer (pH 7.4). Cytochemical demonstration of G6Pase was performed as described previously (Griffiths *et al.*, 1983) and processed for Epon embedding (Parton *et al.*, 1992).

Wheat Germ Agglutinin-Horseradish Peroxidase (WGA-HRP). Cultured rat hippocampal neurons were washed with air medium (Dotti and Simons, 1990) and then incubated at 4°C with WGA-HRP (20 μg/ml, Sigma) for 30 min. After washing to remove unbound WGA-HRP the cells were incubated at 37°C for 30 min before embedding in Epon as described previously (Parton *et al.*, 1992). Semi-thick sections (150–200 nm) of selected cells were prepared.

Mouse L cells were infected with mouse hepatitis virus (MHV), permeabilized with SLO, fixed, and prepared for cryosectioning at 5 h post-infection as previously described (Krijnse-Locker *et al.*, 1994). The quantitation of calnexin labeling was done as described by Griffiths (1993). Briefly, this consisted of taking 24 systematically sampled micrographs of L cells (infected with MHV and permeabilized with SLO before fixation) labeled with anti-calnexin and protein A gold (10 nm) at a primary magnification of ×13000. The number of gold particles over the different structures was related to the number of intersections of the membrane with lines of a test lattice. The size of the latter was selected such that no more than 150–200 intersections (total) were obtained for the 24 micrographs (i.e., less than 10 intersections per micrograph for each organelle). A simple formula is used to convert the number of intersections to the membrane length in μm (Griffiths, 1993).

RESULTS

Localization of Golgi and TGN markers

To define the localization of the Golgi complex and the TGN in hippocampal neurons we used antibodies to ManII and TGN38. ManII has been extensively used as a marker of the Golgi stack (Lippincott-Schwartz *et al.*, 1990; Moremen and Robbins, 1991) whereas TGN38 is an integral membrane protein of unknown function predominantly found in the TGN (Luzio *et al.*, 1990). In paraformaldehyde-fixed and ethanol-extracted cells the structures labeled by ManII localized exclusively to the cell body (Figure 1, a and b). No labeling was seen in the axons or dendrites. The TGN38 labeling was also essentially confined to the cell body (Figure 1, c and d), mostly showing a tubulo-vesicular labeling pattern. In many cells, however, one of the dendrites (usually the thickest) also labeled for TGN38 in the initial segment (20–30 μm from the cell body). This result agrees with the recent data of Lowenstein *et al.* (1994). The restricted localization of the TGN to the cell body and the initial segment of one of the dendrites was confirmed by EM of internalized WGA-HRP (Figure 2). HRP-conjugated lectins have been shown to be efficiently transported to one side of the Golgi apparatus of neuronal cells, which almost certainly represents the TGN (Gonatas *et al.*, 1977). These results show that the Golgi complex and the TGN, as defined by these markers, are both localized exclusively to the cell body and the initial dendritic segment, as shown to be the case in neurons in situ (Peters *et al.*, 1991) and in culture (de Camilli *et al.*, 1986; Dotti and Simons, 1990; Lowenstein *et al.*, 1994).

Localization of the Rough ER

The SSR is a protein involved in the translocation of secretory and membrane proteins (Wiedeman *et al.*, 1989; High *et al.*, 1991), and so is, by definition, a rough ER marker. Indeed, at the EM level this protein is clearly restricted to the bona fide rough ER (Vogel *et al.*, 1990). Staining of hippocampal neurons with an antibody against the SSR labeled exclusively the cell body (Figure 3), although in some cells some dendritic labeling was also seen in the initial 10–20 μm.

The cell body–restricted localization of Golgi and rough ER markers indicate that the first and last steps of membrane and secretory protein traffic take place in the cell body of neuronal cells.

Localization of other ER Markers

In neuronal cells, the existence of an intricate and interconnected network of endoplasmic reticulum tubules extending from the rough ER in the cell body into the neurites is well established (Broadwell and Cataldo, 1983, 1984; Lindsey and Ellisman, 1985c; Peters *et al.*, 1991). We investigated the extent of this

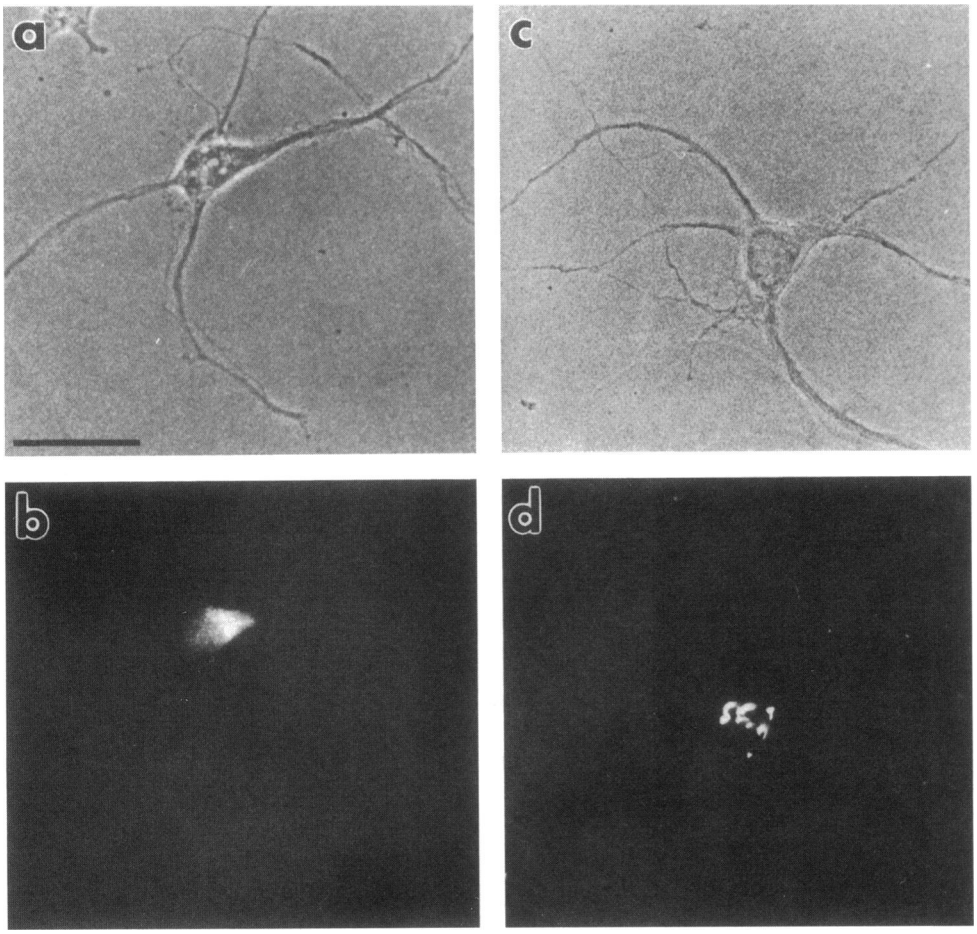

Figure 1. The Golgi apparatus is restricted to the neuronal cell body. Labeling of fully polarized hippocampal neurons with an anti-ManII antibody (b) reveals a distribution of this *cis*-medial Golgi marker, which is restricted to the cell body. Labeling of these cells with TGN38, a specific marker for the trans Golgi network, also reveals only a cell body distribution (d). The phase contrast photographs in panels a and c correspond to the same cells shown in panels b and d. The numerous neurites emerging from these cells are not labeled with either of these Golgi markers. Scale bar, 10 μm.

structure in rat hippocampal neurons using specific cytochemical and immunochemical markers. First we used the well characterized cytochemical marker G6Pase. The reaction product of this enzyme has been used as a morphological marker of the entire ER in a variety of cell types (Farquhar *et al.*, 1974; Griffiths *et al.*, 1983), including neurons (Broadwell and Cataldo, 1983). Electron microscopic analysis of the distribution of G6Pase in hippocampal neurons showed that the entire ER network was stained (Figure 4). Although the most intense reaction was seen in the cell body, including the nuclear envelope and the rough ER, there was also staining of smooth ER elements that extended considerable distances into both the axons and dendrites, including the nerve terminals. In the dendrites, however, the labeling appeared to be more extensive than in the axons.

We next analyzed by immunofluorescence microscopy whether the extensive ER revealed by the G6Pase technique was shared by a smooth ER protein of established function. For this we used an antibody against HMG-CoA reductase, a key enzyme in the biosynthesis of cholesterol. In nonneuronal cells, it appears that the bulk of the cellular pool of HMG-CoA is localized to the smooth ER, although it can also be detected in the rough ER (Anderson *et al.*, 1983; Bergman and Fusco, 1991). In agreement with these data we found that HMG-CoA reductase was present in the cell body and in both the axons and dendrites (Figure 5).

Newly Synthesized Viral Membrane Proteins

The above observations showed significant amounts of two smooth ER markers, G6Pase and HMG-CoA

Figure 2 (facing page). Localization of internalized WGA-HRP in hippocampal neurons. Hippocampal neurons were incubated with WGA-HRP at 4°C and were then warmed to 37°C for 30 min to label the TGN. After detection of the HRP reaction product, the cells were embedded in Epon. Semi-thick sections were viewed without further contrasting. Labeling is evident within cisternal and reticular structures (arrowheads) on one side of the Golgi complex (G) both in the cell body close to the nucleus (N, lower panel) and in the proximal segment of one dendrite (upper panel). Putative clathrin coats are evident on buds of the TGN (e.g., double arrowheads in inset). Bars, 100 nm.

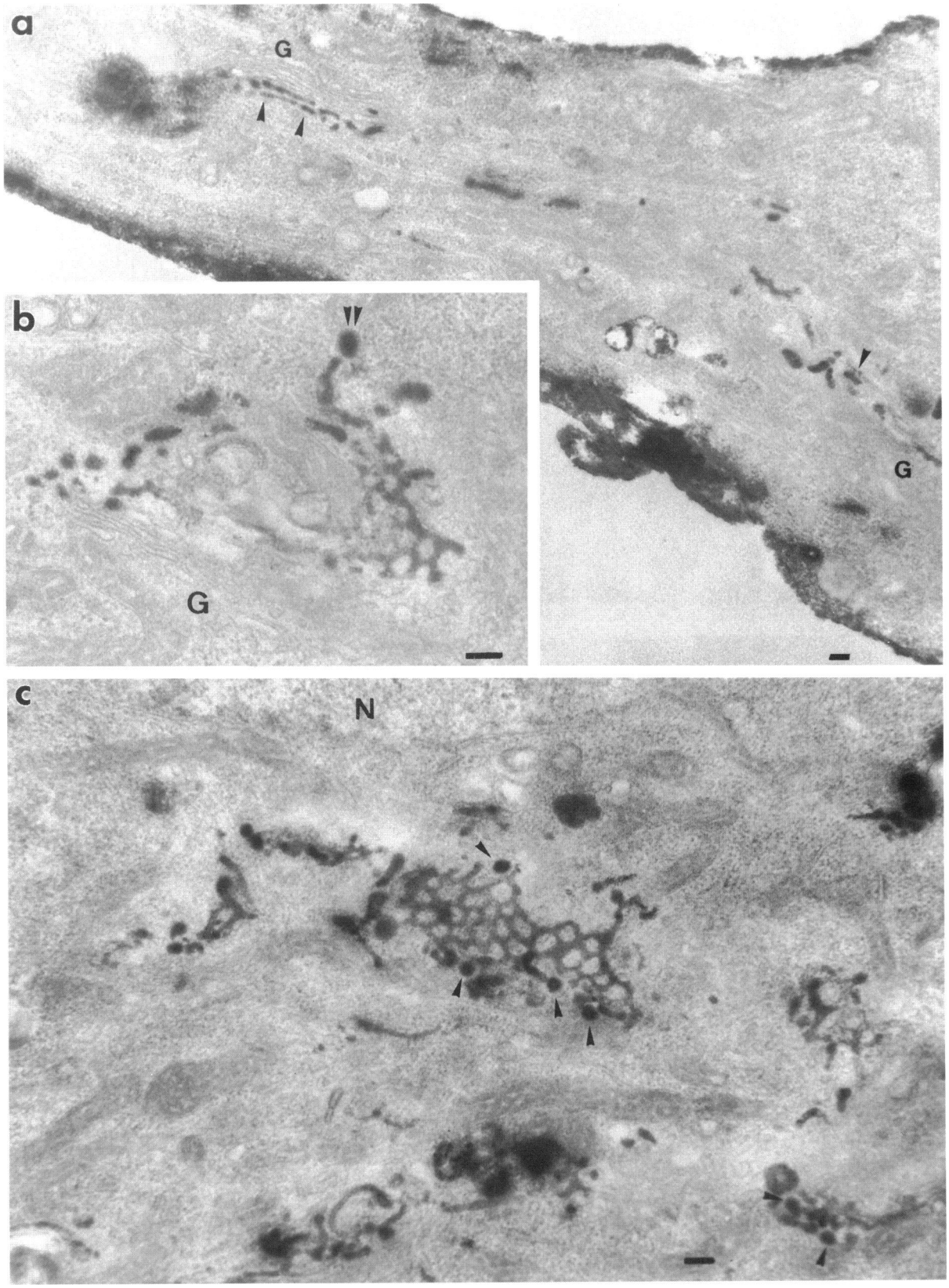

Figure 2.

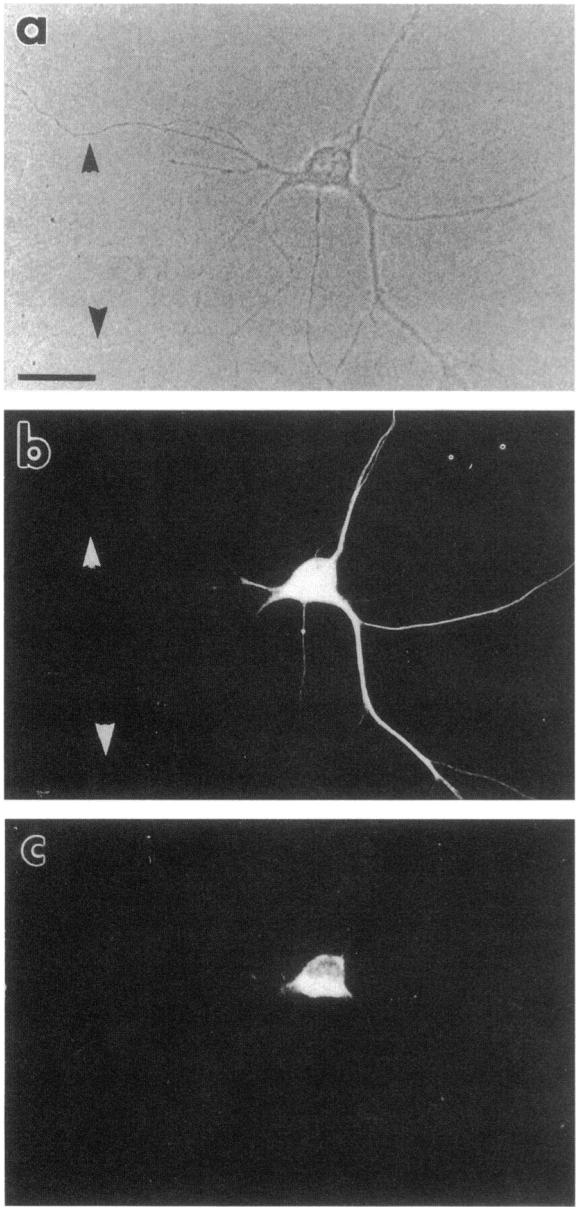

Figure 3. Immunofluorescence localization of the signal sequence receptor in fully polarized hippocampal cells. Hippocampal cells were fixed in methanol and double stained with an anti-MAP2 (b) and anti-SSR (c) antibodies. The SSR labeling is restricted to the cell body whereas dendrites (as revealed by the MAP 2 staining in panel b) and axons (evident in the phase contrast photograph in panel a, arrowheads) are unlabeled. Note that, except for the region of the nucleus, the cell body is entirely and strongly labeled, reflecting a widespread distribution of the neuronal rough ER in the cell body. Scale bar, 10 μm.

reductase, in the axonal and dendritic processes. We asked whether these peripheral elements, which are localized far from both the proximal site of synthesis (rough ER) and the downstream Golgi complex, would be involved in processing newly synthesized proteins en route to the Golgi or whether they represent specialized domains of the ER that are functionally segregated from the cell body. For this we analyzed the localization of newly synthesized SFV viral membrane proteins.

SFV has been extensively used as a model system to study the structure and function of the biosynthetic pathway. The two spanning membrane proteins E1 and p62 (which is cleaved to E2 and E3 in the late Golgi) are normally efficiently transported to the plasma membrane. In a well characterized temperature-sensitive mutant of SFV, ts-1, these proteins are blocked in the ER at the restrictive temperature of 39°C (Saraste *et al.*, 1980; Saraste and Kuismanen, 1984). When neurons were infected with ts-1 for 3.75 h and kept at the restrictive temperature, the monoclonal antibody 9AB4 (Burke *et al.*, 1983), recognizing the p62/E2, gave a labeling that localized preferentially to the cell body and to the proximal segments of dendrites (Figure 6, a and b). It should be noted, however, that the labeling extended greater distances into the processes in some cells. In these cells the SSR labeling (see above) was restricted to the cell body (unpublished results). These results suggest that if transport from the rough ER is impaired the accumulated viral glycoproteins can move into the interconnected smooth ER tubules that extend into the processes.

We next followed the transport of SFV-E2 by incubating for an additional 2 h at either 15°C or 19.5°C in the presence of cycloheximide to accumulate the protein in the IC or the TGN, respectively. At 15°C the immunofluorescence pattern appeared as fine dots essentially restricted to the cell body (Figure 6, c and d). In some cells, however, some residual labeling was seen in the processes although this was less than that seen at 39°C. At 19.5°C the labeling appeared more tubular-vesicular and was mainly confined to the cell body (Figure 6, e and f).

Collectively, these data suggest that at least a fraction of newly synthesized membrane proteins can move into the processes under situations where they are prevented from leaving the rough ER but that essentially all of this "peripheral" pool can leave these processes when transport to the Golgi complex is allowed to continue.

Localization of PDI and Calnexin

We next determined the distribution of BiP and PDI by immunofluorescence microscopy. BiP and PDI are luminal KDEL-bearing proteins of the ER mainly involved in the folding and assembly of newly translocated proteins (Gething and Sambrook, 1992; Helenius *et al.*, 1992). In nonneuronal cells, these proteins are not restricted to the rough ER but are also enriched in the smooth ER and portions of the IC (Bergman and Fusco, 1991; Oprins *et al.*, 1993; Griffiths *et al.*, 1994;

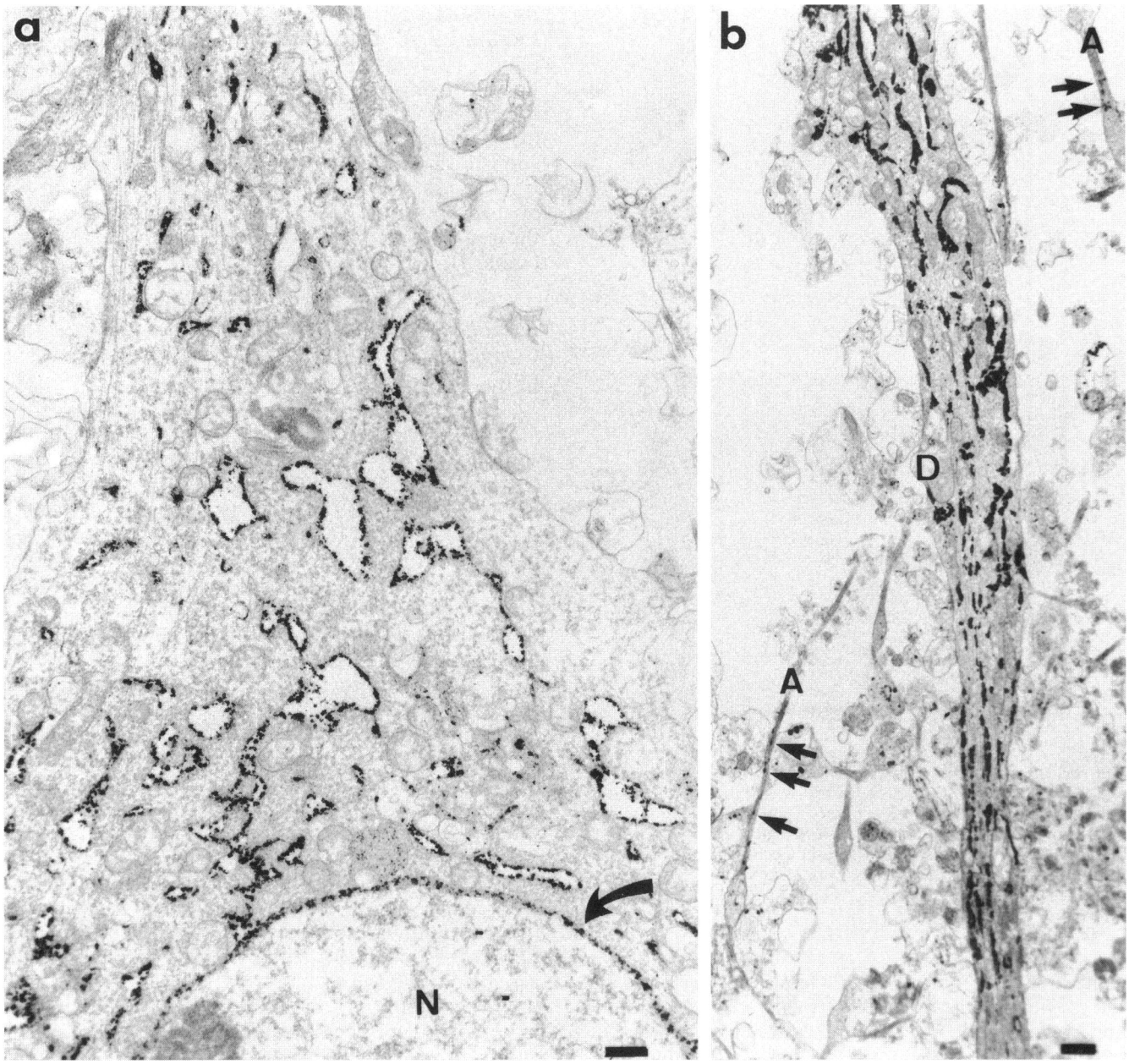

Figure 4. Histochemical localization of glucose-6-phosphatase activity in mature hippocampal neurons. Fixed hippocampal neurons were treated for cytochemical detection of glucose-6-phosphatase and embedded in Epon. Reaction product is evident within the entire ER system including the nuclear envelope (panel a, curved arrow) and cisternal elements that extend into all regions of the dendrites (D; panel b). Low but significant reaction product is also evident within tubular elements within axons (A, small arrows in panel b). Bars, 100 nm.

Hammond and Helenius, 1994; Krijnse-Locker *et al.*, 1994; Weis *et al.*, 1994). In a recent paper by Banfield *et al.* (1994), the presence of PDI was even considered to be a diagnostic marker of the IC. In hippocampal neurons, the labeling for PDI was most intense in the cell body although the signal clearly also extended out to the periphery of axons and dendrites (Figure 7). The labeling for BiP was identical (unpublished results). The presence of BiP and PDI in the neuronal processes may indicate that newly synthesized membrane and secretory proteins may fold and oligomerize at these sites. Alternatively, their location in the processes simply reflects the fact that they may freely diffuse in the lumen of a functionally interconnnected organelle or even perform other functions not related to folding. We therefore used IF to examine the distribution of

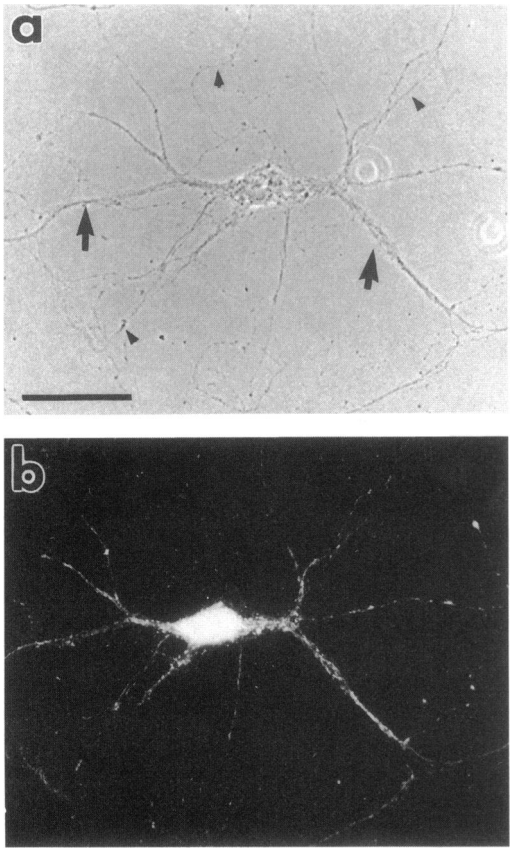

Figure 5. HMG-CoA reductase is present in the cell body, axons, and dendrites of hippocampal neurons in culture. A monoclonal antibody against HMG-CoA reductase was utilized in methanol-fixed cells (b). (a) Phase contrast photograph. Two different types of labeled processes are evident. By morphological criteria they can be distinguished into dendrites (thick and tapering with distance from the cell body, arrows) and axons (thin and of uniform diameter, arrowheads). Note the extensive labeling of essentially all processes. Scale bar, 10 μm.

calnexin, a membrane-bound chaperone that assists in the folding of glycoproteins (reviewed by Hammond and Helenius, 1993) and that localizes to the rough ER by EM (Hochstenbach *et al.*, 1992; Rajagopalan *et al.*, 1994). In hippocampal neurons, calnexin was both in the cell body and extending deep into both the axons and dendrites (Figure 8, a and b). To investigate whether calnexin is also localized beyond the rough ER in other cells, we used EM to analyze its distribution in mouse L cells infected with MHV (Figure 9). The sub-domain of the ER where this virus assembles is enriched for markers of the IC (see below) as well as PDI and other KDEL-bearing proteins (Griffiths *et al.*, 1994; Krijnse-Locker *et al.*, 1994). In preliminary experiments using normal thawed cryosections of MHV-infected cells, we saw sparse calnexin labeling of the ER membranes (unpublished results). However, following permeabilization with SLO before fixation, the protein was clearly not restricted to the rough ER, but localized also to the membranes containing assembling viruses (Figure 9, A–D). The labeling was quantified by using the number of intersections of a test line system with the membrane profiles as an estimate of membrane length (Griffiths, 1993). This analysis showed that the only three organelles that had significant amounts of calnexin were the rough ER, the nuclear envelope, and portions of the IC, defined by the presence of MHV-budded and budding profiles (Table 1).

Localization of Markers of the Intermediate Compartment

In the late 1980's a number of antibodies were raised against novel protein markers that labeled structures at the interface between the rough ER and the Golgi complex (Saraste *et al.*, 1987; Schweizer *et al.*, 1988; Chavrier *et al.*, 1990). Because these membrane elements co-localized with newly synthesized passage proteins (such as the VSV-G protein at 15°C) just before they reached the Golgi complex, they were referred to as the IC or the *cis*-Golgi network. Although the question of whether the IC is a domain of the ER or a structurally distinct compartment is still being debated, it seems clear from ultrastructural studies that this compartment is made up of a heterogeneous network of smooth membranous elements that are in part closely adjacent to the *cis* side of the Golgi complex (Lindsey and Ellisman, 1985a,b; Lotti *et al.*, 1992; Sodeik *et al.*, 1993; Krijnse-Locker *et al.*, 1994).

Because our results showed that a part of the smooth ER is well separated from the cell body where the rough ER, the Golgi complex, and the TGN are localized, it seemed logical to ask whether markers of the IC would be restricted to the cell body or, whether, like the smooth ER, they could also extend into the axons and dendrites.

First we took two GTP-binding proteins rab1 (the mammalian homologue of the yeast GTPase YPT1) and rab2, which have been shown to localize to the IC (Chavrier *et al.*, 1990; Griffiths *et al.*, 1994; Pind *et al.*, 1994; Saraste *et al.*, 1995) and to play a role in ER to Golgi transport (Tisdale *et al.*, 1992). Saponin-extracted and paraformaldehyde-fixed neurons labeled with rab2 antibodies showed intense labeling in the cell body (Figure 10). Some labeling was also present in the processes, however, in most cases this was restricted to the first 10–30 μm of one of the dendrites. A similar pattern of labeling was observed with the rab 1 antibody (unpublished results). To increase the amount of rab1 associated to IC membranes, we incubated the cells with GTPγS, which induces the accumulation of pre-Golgi carrier vesicles and vesicular-tubular clusters (Pind *et al.*, 1994). Hippocampal

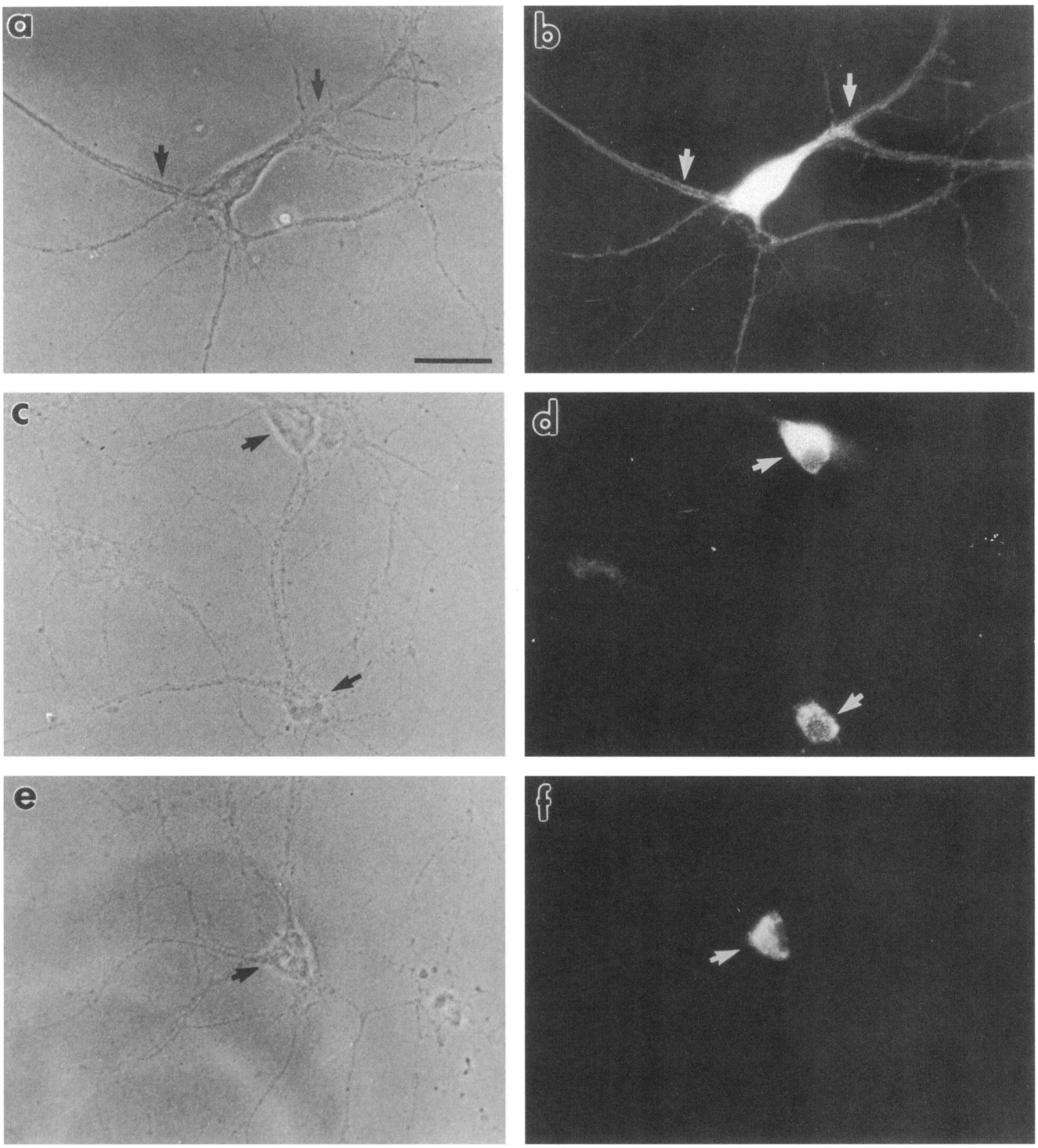

Figure 6. Distribution of the p62/E2 glycoprotein complex of ts1-SFV at nonpermissive and permissive temperatures. Fully mature hippocampal neurons were infected with the temperature-sensitive mutant ts-1 SFV for 1 h at 37°C. The cells were then incubated for 2.75 h at the nonpermissive temperature of 39°C, to arrest newly synthesized proteins in the rough ER. The cells were then either fixed or incubated at 15°C or 19.5°C, to arrest the proteins in the IC or Golgi apparatus, respectively, and fixed; the distribution of the p62/E2 glycoproteins was then analyzed by immunofluorescence. (a–b) Fixation after the 39°C block. Labeling is seen in both the cell body region as well as extending considerable distances into the axons and dendrites. Note also the presence of labeled dots in the peripheral processes (arrows). (c–d) Fixation at the end of the 15°C incubation. Labeling is in the form of dots in the cell body with little, if any, in the processes. (e–f) Fixation at the end of the incubation at 19.5°C. Essentially all of the labeling is found in the cell body. Scale bar, 10 μm.

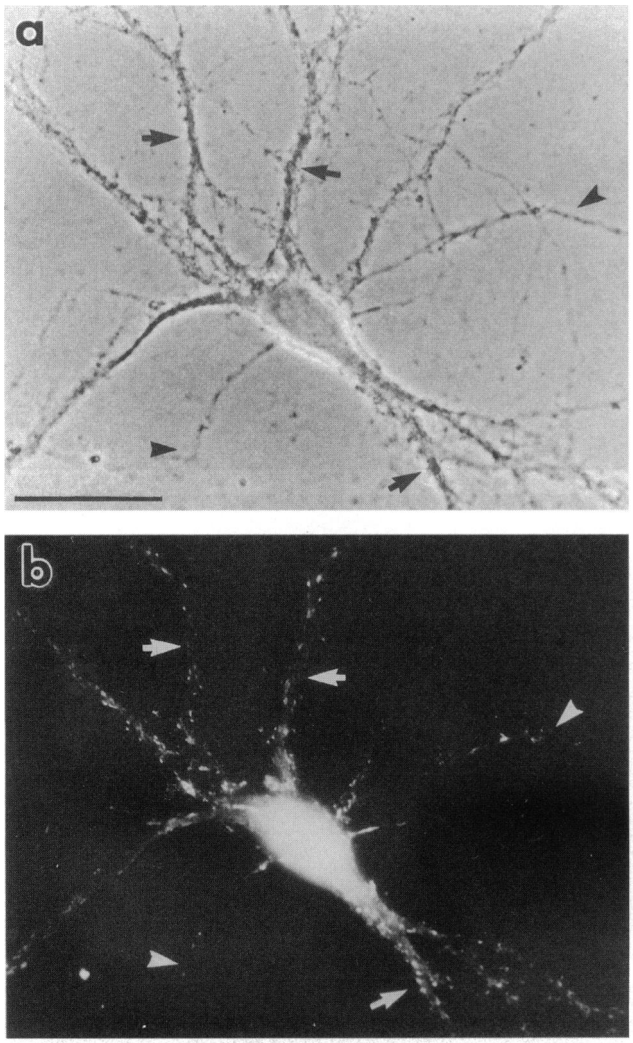

Figure 7. PDI is present in the cell body and processes of polarized hippocampal cells. Hippocampal cells were methanol fixed and labeled with an antibody to PDI (b). Intense labeling is present in the cell body and in numerous neurites. By using morphological criteria to distinguish axons from dendrites, it is clear that the dendrites (the thick processes indicated by arrows in panel a contain most of the PDI whereas in the thin axons (arrowheads in panel a) the labeling is much more sparse. Scale bar, 10 μm.

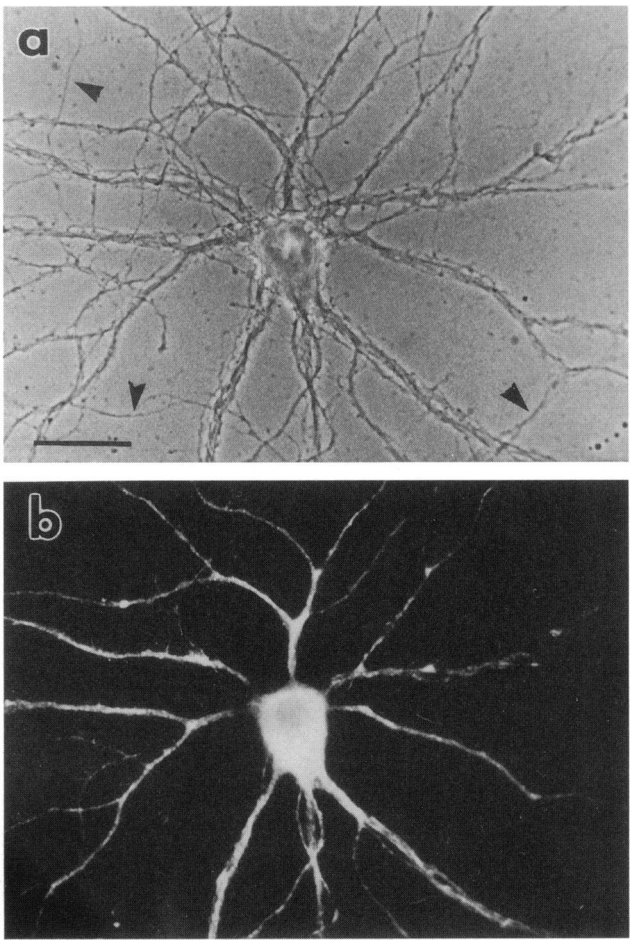

Figure 8. Calnexin is present in the cell body and most but not all processes of mature hippocampal neurons. Methanol-fixed/permeabilized cells reacted with the anti-calnexin antibody show intense labeling in the cell body and in numerous processes, which by morphological criteria, thick and tapering, are dendrites (b). Many of the thin axons evident in the phase contrast photograph (a, arrowheads) are not labeled. Scale bar, 10 μm.

neurons permeabilized with SLO and treated with GTPγS (see MATERIALS AND METHODS) showed strong cell body labeling, although some punctate was also evident in the proximal parts of the processes (Figure 11, a and b).

We next analyzed the distribution of the membrane protein p58, previously localized to the IC (Saraste *et al.*, 1987; Saraste and Svensson, 1991). In nonneuronal cells, this protein shows a perinuclear concentration as well as peripheral punctate staining (Saraste and Svensson, 1991). In paraformaldehyde-fixed and ethanol-extracted neurons, however, intense labeling was predominantly confined to the cell body (Figure 11, c and d).

Figure 9 (facing page). Thawed cryosections of SLO permeabilized L cells that were infected with MHV. The section was labeled with anti-calnexin (cytosolic domain) and protein A gold. These figures show that on the average the membranes of both the rough ER and the IC, defined as the site of MHV assembly, label for calnexin. The budding or budded virions are indicated by small arrowheads in all figures. (B) Labeled membranes (large arrowhead) are continuous with virion-enclosing unlabeled membranes (arrowheads). (C) An extensive area of the rough ER (ER) is shown as well as the nuclear envelope (N; nucleus), which is labeled (arrows). (D) Note the labeling in membranes close to the Golgi stack. The large arrowhead indicates a labeled membranous "vesicle" whose structure is characteristic of one part of the IC, as defined by other IC markers. Note that the main part of the Golgi stack is unlabeled. Bars, 100 nm.

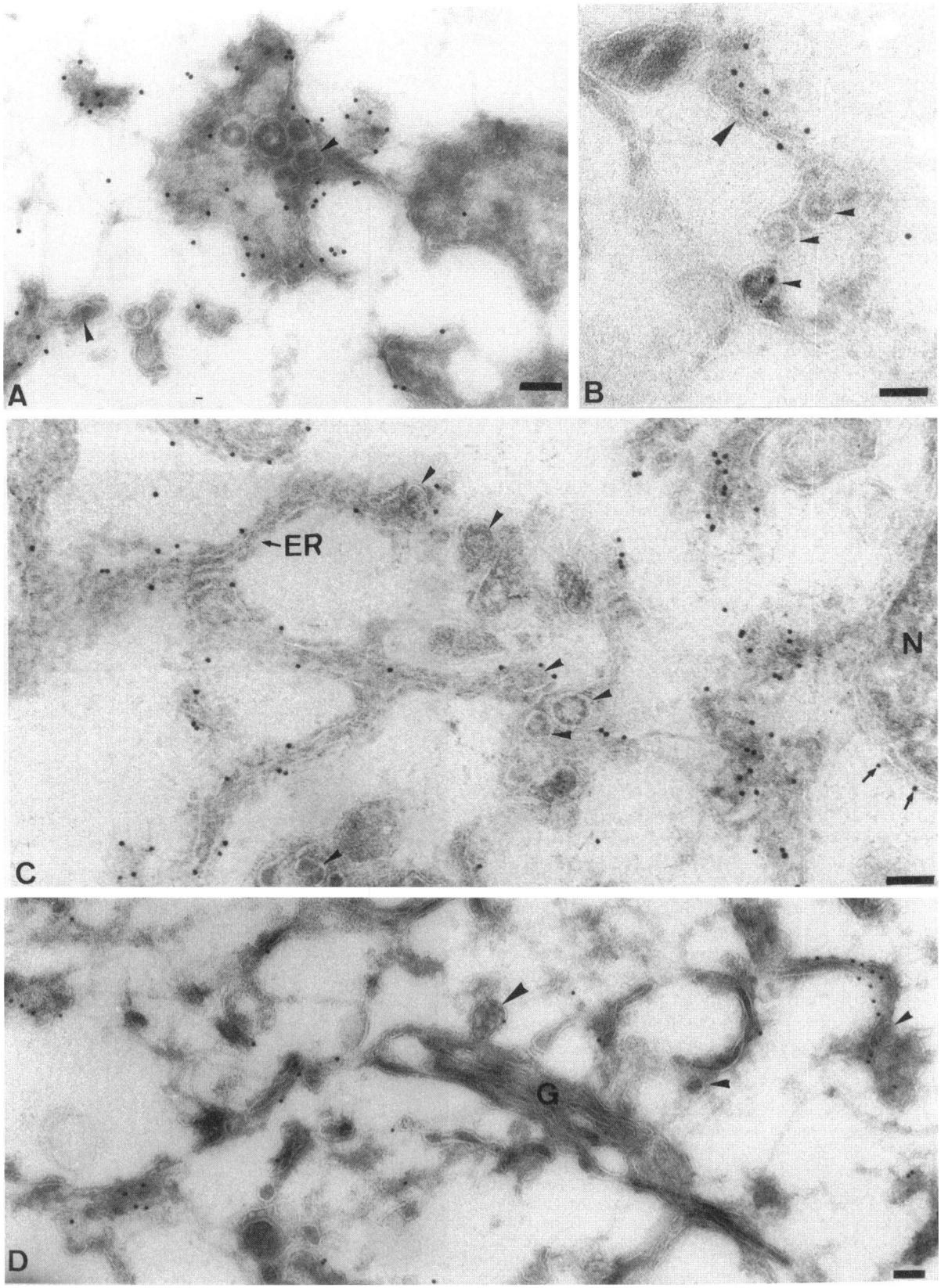

Figure 9.

Table 1. Quantification of calnexin labelling†

	Gold per μm membrane*
Budding compartment (IC)	1.52 (0.24)
Rough ER	3.90 (0.63)
Nuclear envelope	2.07 (0.27)
Mitochondria (outer membrane)	0.45 (0.08)

† From labeled cryosections of SLO-permeabilized MHV-infected L cells.

* Data expressed as mean and standard error of mean.

Finally, we determined the distribution of the KDEL receptor, an integral membrane protein involved in the retrieval of lumenal and type II membrane proteins with the carboxyl-terminal sequence Lys-Asp-Glu-Leu (KDEL) from the Golgi apparatus to the ER (Pelham, 1989). As expected from its known distribution to the IC and Golgi complex of nonneuronal cells (Griffiths *et al.*, 1994), the KDEL receptor was almost exclusively found in the cell body of the hippocampal neurons, although, as with all IC markers, some labeling was also evident in the initial 10–20 μm of one of the cells' dendrites (Figure 12).

β-COP Localizes to the Cell Body As Well As to the Periphery of the Processes

Although initially identified as a coat protein involved in intra-Golgi transport (Rothman and Orci, 1992), at the EM level the bulk of the β subunit of COP is localized to the *cis*-Golgi region, on structures reminiscent of the IC, and which were labeled with antibodies to sec23, a protein involved in ER to Golgi transport in yeast (Duden *et al.*, 1991; Hendricks *et al.*, 1993; Oprins *et al.*, 1993). We therefore analyzed β-COP distribution in permeabilized and nonpermeabilized hippocampal neurons. In nonpermeabilized neurons β-COP labeling was mostly restricted to the cell body (unpublished results). In SLO-permeabilized cells the bulk of the labeling was present in the cell body, although some β-COP–positive structures were also observed in the processes (Figure 13).

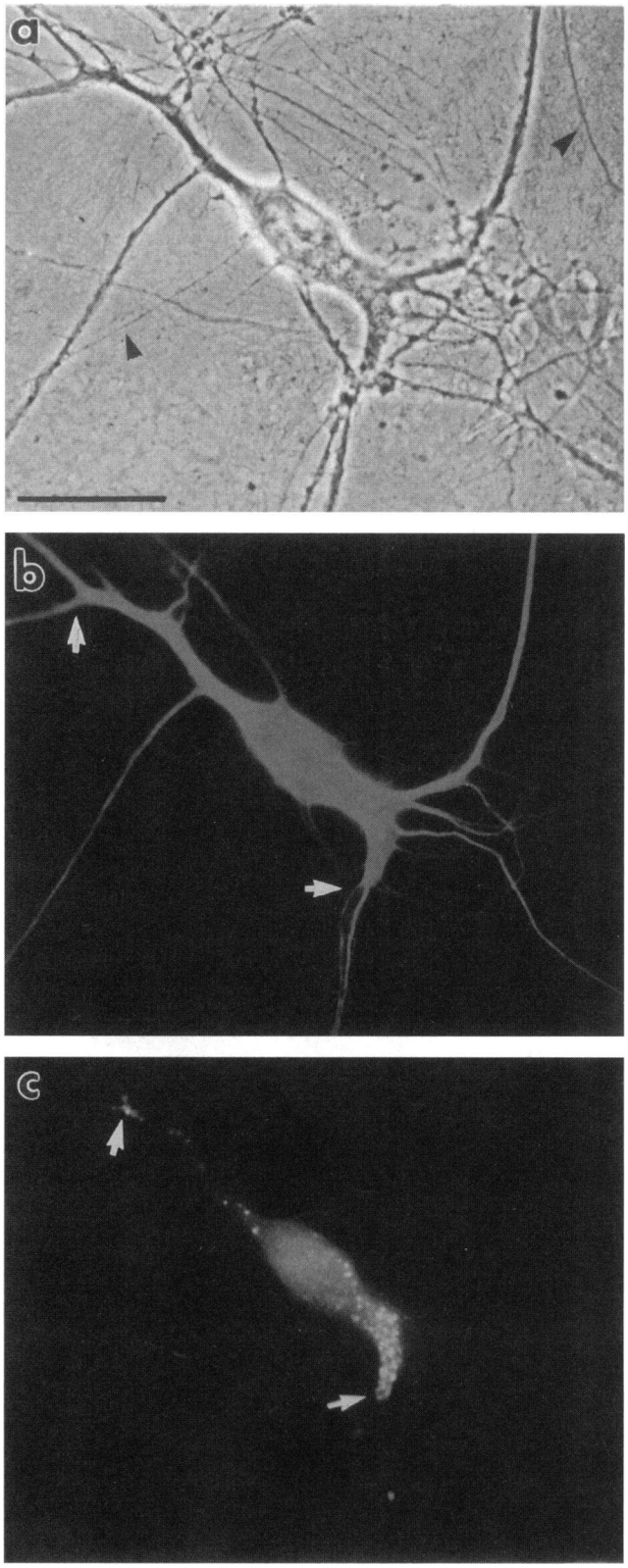

Figure 10.

Figure 10 (facing column). Immunofluorescence localization of rab2 in mature hippocampal neurons in culture. Saponin extracted and paraformaldehyde fixed cells reacted with the anti-Rab2 antibody (c) show that rab2 is found exclusively in the cell body and initial dendritic segments (arrows). The dendrites are recognized in panel b by their positive labeling for MAP2, a dendritic-specific marker. All the axons, not labeled by MAP2 and evident in the phase contrast photograph in panel a (arrowheads), do not contain detectable amounts of rab2. Beyond their proximal parts the dendrites are also negative. Scale, 10 μm.

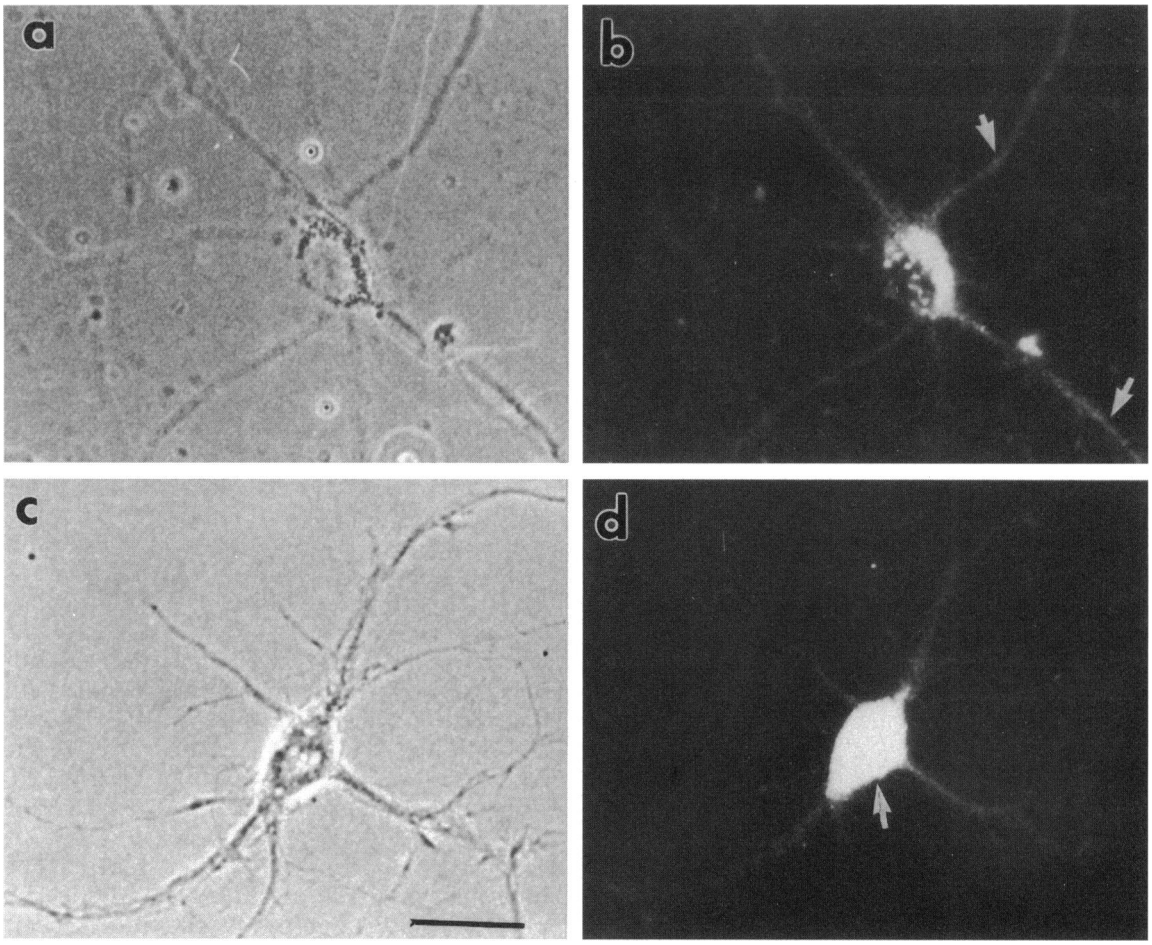

Figure 11. Immunofluorescence distribution of the IC markers rab1A and p58 in mature hippocampal neurons. For the rab1A labeling (a–b), neurons were permeabilized with SLO and treated with GTPγS before fixation (see MATERIALS AND METHODS). The rab1A labeling is mostly confined to a vesicular perinuclear location in the cell body although fine punctate is present in some of the dendrites (arrows in panel b). For the p58 labeling, cells were fixed in paraformaldehyde and extracted in ethanol (c–d). In the phase contrast photograph (c) numerous processes are evident, and compared with the IF-picture in panel d, it is evident from the IF figure (d) that p58 labels only the cell body. Scale bar, 10 μm.

DISCUSSION

Our goal in this study was to determine the spatial limits of the organelles of the biosynthetic pathway, focusing in particular on the IC. For this purpose we used cultured rat hippocampal neurons whose architecture facilitates the distinction between the perinuclear region and the peripheral domains of the cell that make up the axons and dendrites. This specialized cytoarchitecture of neurons enabled us to visualize distinct endomembrane domains that would be difficult to identify in fibroblast-like cells where the rough ER alone occupies a significant fraction of the whole of the cytoplasm. Our results are summarized in Figure 14.

We found that markers of the rough ER (marked by SSR), the *cis*-medial Golgi compartments (enriched for ManII), and the TGN (marked by TGN38) were restricted to the cell body and the initial segment of one dendrite. In contrast, markers of the smooth ER fill the entire neuronal cytoplasm. This latter finding agrees with numerous previous observations of an intricate and extensive neuronal smooth ER (Broadwell and Cataldo, 1983, 1984; Peters *et al.*, 1991). The smooth ER network reacted for the classical cytochemical marker of the whole ER, G6Pase (Teichberg and Holtzman, 1973; Broadwell and Cataldo, 1983). HMG-CoA reductase also localized to the periphery of both axons and dendrites. This protein, a rate-limiting enzyme in cholesterol biosynthesis, is predominantly a smooth ER marker (Anderson *et al.*, 1983; Bergman and Fusco, 1991) and therefore it seems that in neurons, as in other cells, one of the functions of the smooth ER is to synthesize cholesterol for cellular membranes. Collectively, these

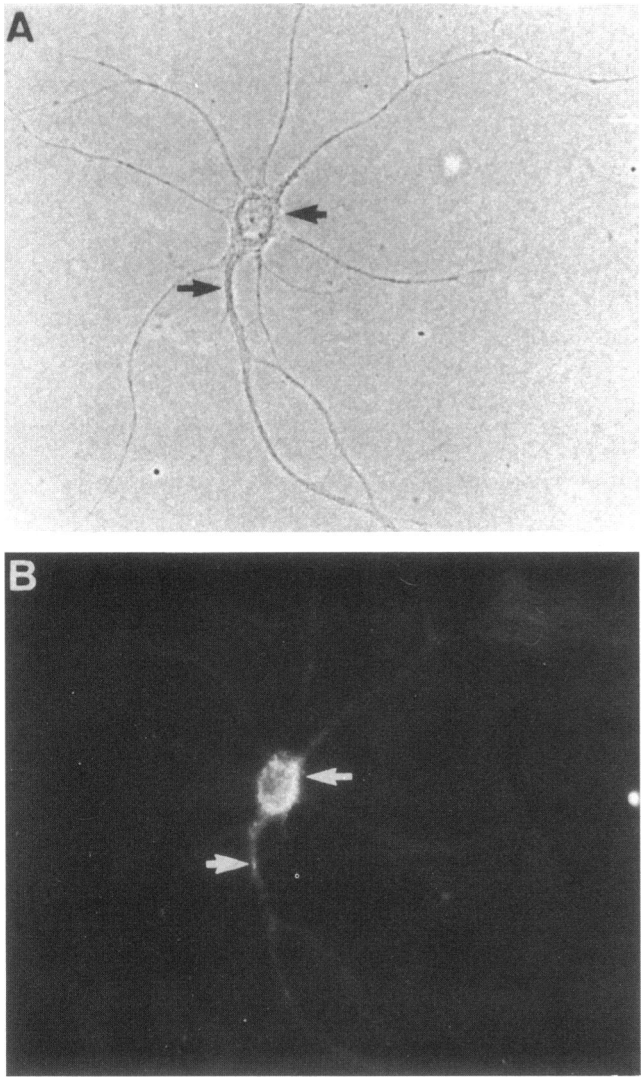

Figure 12. KDEL receptor localization in mature hippocampal neurons. Hippocampal neurons maintained in culture for 14 days were fixed in paraformaldehyde, permeabilized in ethanol, and labeled with the anti-KDEL antibody. In the phase contrast photograph (A) numerous processes are evident. In the corresponding fluorescence photograph (B), it is clear that the KDEL labeling is restricted to the cell body and initial segment of one of the cell dendrites (arrows). Scale bar, 10 μm.

data argue that the smooth ER, as defined by these classical markers, extends considerably beyond the region of the cell body where the rough ER, the Golgi complex, and the TGN are localized.

It has been very difficult from the recent literature to decide whether the classical smooth ER and the more recently identified IC (whose membranes are also devoid of ribosomes) represent the same set of structures, whether they partly overlap, or whether they really represent different domains of a multi-domain ER network. Although our data do not have sufficient resolution to make a definitive statement about the interrelationship between the smooth ER and the IC, none of the IC markers we tested (rab2, rab1, and p58) extended far from the cell body whereas classical ER markers and PDI and calnexin were located deep into the processes, suggesting that they represent distinct functional domains. Hence, the peripherally located smooth ER elements would perform functions like intracellular calcium homeostasis (Rosier and Putney, 1991) and lipid synthesis and/or transport (Pfenninger and Johnson, 1983; Fawcett, 1986; Vance *et al.*, 1991) whereas the IC may represent the exit domain of the RER that would provide the last chance for "quality control" and may form transport vesicles destined for the Golgi complex.

We observed that two ER lumenal proteins, the KDEL-containing soluble protein PDI and calnexin, a membrane-spanning protein, considered to be components of the folding and oligomerization of newly synthesized proteins (Gething and Sambrook, 1992; Helenius *et al.*, 1992; Hammond and Helenius, 1993; Doms *et al.*, 1993) extended deep into the processes of the neurons whereas the KDEL receptor, which is involved in their retrieval from the Golgi back to the rough ER, appeared restricted to the cell body-dendrite initial segment. These results suggest that the peripheral ER may also carry out "house-keeping" functions involved in quality control of protein export. That the peripheral SER may also play some role in membrane protein export was also suggested by the finding that the SFV mutant glycoproteins are localized to the neuronal processes some distance from the cell body, under conditions where they are unable to fold or oligomerize. Moreover, this pool of misfolded viral proteins was able to be chased back into the perinuclear region upon reversal to the permissive temperature. However, at the moment we cannot exclude the possibility that PDI and calnexin may localize to different (sub)domains of the ER network at the ultrastructural level (see Villa *et al.*, 1991). Because certain KDEL-bearing ER lumenal proteins can bind Ca^{2+} with high affinity (Nguyen Van, *et al.*, 1989; Cala *et al.*, 1990), we also cannot exclude the possibility that the PDI is involved in calcium homeostasis in the neuronal processes.

β-COP was found preferentially in the cell body of hippocampal neurons but also in small amounts in the processes. The presence of β-COP in the processes became more pronounced when the cells were permeabilized in the presence of GTPγS, an effect that appears to "block" the COP coats onto buds (and perhaps free vesicles) in the IC as well as throughout the Golgi complex (Orci *et al.*, 1989; Duden *et al.*, 1991; Krijnse-Locker *et al.*, 1994). Our data agrees with many immunofluorescence studies

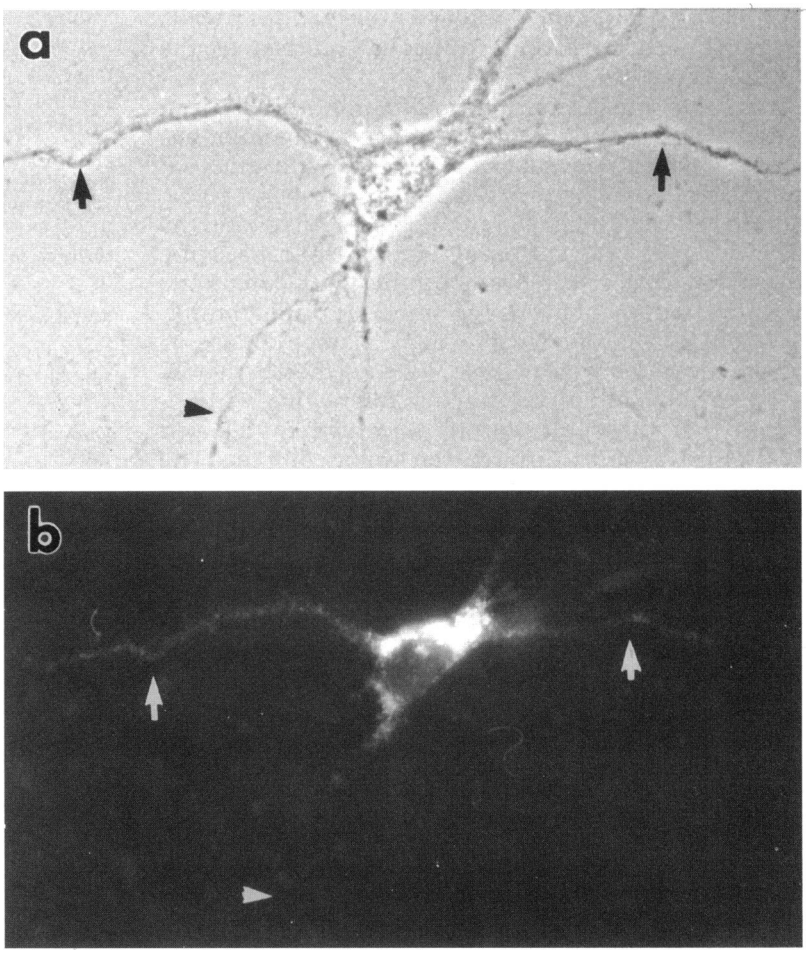

Figure 13. β-COP localization in mature neuronal cells. Hippocampal neurons were permeabilized with SLO and treated for 30 min with GTPγS, before fixation with paraformaldehyde. Panel a shows the phase contrast and panel b shows the IF image. Note that the bulk of the β-COP labeling is in the perinuclear region of the cell body. Some of the processes are also labeled to a variable extent. Scale, 10 μm.

on cultured fibroblastic type cells in which COP-enriched structures often extend quite some distance from the perinuclear site of the Golgi complex (Duden *et al.*, 1991; Tisdale *et al.*, 1992; Pind *et al.*,

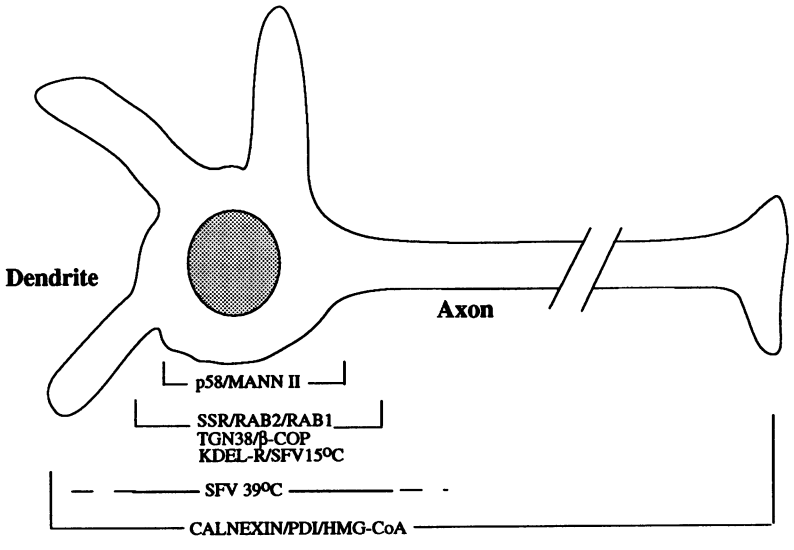

Figure 14. Pattern of distribution of markers specific for the RER, IC, Golgi complex, and TGN in mature hippocampal neurons in culture. Markers for the RER (SSR), the IC (KDEL receptor, b-COP, rab2, rab 1, p-58, and the E2/p62 complex of SFV at 15°C), and the Golgi-TGN complex (ManII and TGN38) are mostly restricted to the cell body and to a minor extent in the initial segment of one of the processes, usually a dendrite. Calnexin, PDI, and HMG-CoA reductase fill the entire neuronal ER. In SFV-infected cells kept for several hours at the restrictive temperature of 39°C, the E2/p62 complex can be found deep into the processes.

1994). The significance of these observations awaits detailed structural and functional studies of the COP protein complex.

What emerges from this study is that the ER is an extensive network of interconnected membranes that comprises a number of domains with different functions (see also Silia and Meldolesi, 1992). Among these domains are the nuclear envelope, the rough ER, and the various smooth membrane structures that label with the smooth ER and IC markers. In hippocampal neurons the smooth ER membranes seemed far more extensive than IC membranes as assessed by our markers, yet were reached by newly synthesized viral glycoproteins. Interestingly, it appears that these glycoproteins return to the cell body for transport through the Golgi complex. The use of the cultured hippocampal neurons facilitated the identification of the limits of the different neuronal ER compartments, perinuclear and peripheral, and it should be a useful experimental system for future immunolocalization studies.

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