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Immunocytochemical localization of β-COP to the ER-Golgi boundary and the TGN

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SUMMARY

Recent data strongly suggest that the coatomer (COP) complex is involved in membrane transport between the ER and Golgi complex. This vesicular coat has been implicated in ER to Golgi, in intra Golgi as well as in Golgi to ER traffic. In this study we present a detailed immunocytochemical analysis of the distribution of β-COP in different tissue culture cells. Our results extend previous studies by showing, using electron microscopy, that β-COP accumulates on vesicular profiles and buds in the intermediate compartment (IC) under conditions that block ER to Golgi transport (15°C). Importantly, under these conditions β-COP co-localizes on these structures with a passenger protein, the membrane glycoprotein of vesicular stomatis virus (ts-O45-G). Furthermore, quantitative immunofluorescence microscopy of cells with ts-045-G accumulated in the ER, IC and *trans*-Golgi network, shifted briefly to the permissive temperature, showed that β-COP was associated with many of the putative transport intermediates containing the viral glycoprotein which is in transit between the ER/IC and the *cis*-Golgi. The simplest interpretation of these data is that COP-coated vesicles are involved in anterograde transport of ts-045-G from the IC to the Golgi complex. Since many putative COP vesicles lacked the G protein following release of the 15°C block this pool could be involved in retrograde transport. We also show that β-COP is present on the membranes of the *trans*-Golgi network. However, in contrast to the ER-Golgi boundary, we could find no convincing evidence that this pool of β-COP is associated with buds or *trans*-Golgi network-derived transport vesicles.

Key words: coatomer, intermediate compartment, ER, Golgi, vesicular stomatitis virus

INTRODUCTION

There is now convincing evidence that coated vesicles are involved in vesicular transport from the endoplasmic reticulum (ER), or intermediate compartment (IC), to the Golgi complex in eukaryotic cells (Palade, 1975; Pepperkok et al., 1993; Balch, 1990; Oprins et al., 1993) and in yeast (Schekman, 1992; Pryer et al., 1992; Hosobuchi et al., 1992; Duden et al., 1994). The available data also argue strongly that clathrin and its adapter proteins are not involved in this process, but that the coat of these vesicles consists of coatomer, the multi subunit COP-complex (Waters et al., 1991; Duden et al., 1991; Oprins et al., 1993). COP has been shown to function in vesicular traffic within the Golgi stack (Rothman and Orci, 1992; Orci et al., 1993) and in transport between the ER/IC (see below) and the Golgi complex (Pepperkok et al., 1993; Peter et al., 1993). The 105 kDa gene product of SEC21, which is essential for ER to Golgi transport in yeast, has been shown to be a subunit of yeast coatomer (Hosobuchi et al., 1992) and subsequently two other members of yeast COP were shown to be required for ER to Golgi transport in this organism (Duden et al., 1994).

More recent data from Barlowe et al. (1994) have shown that, in addition to the conventional COP complex (also referred to as COP I), a second type of coat complex (COP II) mediates the formation of a functionally different class of coated vesicles involved in ER to Golgi transport in yeast. This novel protein complex has no relationship to coatomer and predominantly consists of the gene products of Sar1, Sec23 complex and Sec13 complex (Barlowe et al., 1994). A recent report indicates that COP and COP II form distinct buds from the nuclear membrane in yeast, suggesting that two different types of vesicles may be involved in ER to Golgi transport (Schekman et al., 1994). Two of the subunits of COP II, Sec 23p and Sec 13p, have recently been localised to structures in the transitional element region of the exocrine pancreatic cells close to, but distinct from, the domains where COP buds (Oprins et al., 1993; Shaywitz et al., 1995). COP II will not be discussed further in this paper.

Evidence that coatomer coated vesicles are functioning in ER/IC to Golgi traffic in mammalian cells was provided by in vivo studies with microinjected antibodies against one of the subunits, β-COP, which inhibited transport between these compartments (Pepperkok et al., 1993). This result is consistent with EM results that have immunolocalized β-COP to the so-called transitional elements at the exit sites of the ER (Oprins et al., 1993; Krijnse Locker et al., 1994; Pind et al., 1994). Further evidence for the involvement of COP vesicles

in transport from the ER to the Golgi also came from our recent analysis of the budding compartment of the coronavirus, mouse hepatitis virus (MHV) (Krijnse Locker et al., 1994). This study showed that the compartment where the MHV assembles (Tooze et al., 1988) is the IC, as defined by other markers (p58, rab1 and rab2; see also Griffiths et al., 1994), and that this compartment was both structurally and functionally continuous with the rough ER (RER). Our view from this, and related studies (Griffiths et al., 1994; Sodeik et al., 1993) is that the IC is a distinct domain of the ER from where COP vesicles form which are destined for the Golgi complex. Accordingly, when mouse L cells infected with MHV and permeabilized with SLO were treated with GTPγS, a treatment known to block vesicular transport in vitro, a significant fraction of the IC was covered with COP buds. That the IC may be a domain of the ER is also consistent with the finding that a number of KDEL proteins, such as protein disulfide isomerase (PDI) (Oprins et al., 1993; Hobman et al., 1992; Krijnse Locker et al., 1994; Banfield et al., 1994), the calcium binding proteins CaBP1, CaBP2 and CaBP3 (calreticulin) (Griffiths et al., 1994), a novel HDEL protein ERC-55 (Weis et al., 1994), as well as the spanning membrane chaperone calnexin (Krijnse Locker et al., 1995) are enriched in both the RER and the IC.

The temperature sensitive transmembrane glycoprotein mutant (ts-O45-G) of vesicular stomatitis virus (VSV) has been extensively used as a model protein to characterize the transport of a newly synthesized membrane protein from the ER to the plasma membrane. ts-O45-G is unable to oligomerize into trimers at the non-permissive temperatures, 39°-40°C, and the protein remains in large aggregates in pre-Golgi structures with its N-linked oligosaccharides in the high mannose form (Bergmann et al., 1981; Kreis and Lodish, 1986; Doms et al., 1987; Balch et al., 1989; Hammond and Helenius, 1994). When cells are shifted to the permissive temperature, 31°C, ts-045-G trimerizes and is rapidly transported through the Golgi complex, thereby acquiring the late Golgi sugars on its oligosaccharides, and subsequently to the plasma membrane (Bergmann et al., 1981).

Two additional temperature blocks can be used to follow transport of ts-O45-G to the cell surface. At 15°C it accumulates in the IC (Saraste and Kuismanen, 1984) and at 20°C in the *trans*-Golgi network (TGN) (Matlin and Simons, 1983; Griffiths et al., 1985, 1989). Thus, when the temperature is shifted from 39.5°C to 15°C ts-O45-G protein trimerizes and accumulates in the IC between the ER and the Golgi; at this temperature however, the protein does not enter the Golgi complex and its N-linked oligosaccharides remain in the endo-H-sensitive form that have six or more mannose residues (Balch et al., 1989; Doms et al., 1987; Schweizer et al., 1990; Duden et al., 1991). When the temperature is shifted from 39.5°C to 18-20°C (referred to here as the 20°C block) ts-O45-G is transported through the Golgi stack and accumulates in, but does not leave, the TGN (Griffiths et al., 1985, 1989). Both the 15°C and the 20°C blocks can be reversed simply by shifting the temperature to the permissive temperature, 31°C.

In our recent study microinjected antibodies against β-COP, but not control antibodies, blocked transport of ts-O45-G in infected cells between the IC and the Golgi, but no effect could be detected on transport of the viral glycoprotein from the TGN to the cell surface (Pepperkok et al., 1993). While these data argued strongly that COP is involved in transport into the Golgi complex they also suggested that the COP coat proteins might not be involved in the constitutive transport step from the TGN to the plasma membrane. The latter point is not at first glance easy to reconcile with two other lines of evidence. First, at the immunofluorescence level the localization pattern of β-COP overlaps significantly with the VSV glycoprotein at 20°C in Vero cells (Duden et al., 1991). Second, a small but significant amount of the Golgi-associated β-COP labelling was detected on the TGN of rat pancreatic acinar cells (Oprins et al., 1993).

In the present study we have made a detailed immunocytochemical characterization of COP-coated buds/vesicles using the ts-O45-G system. We provide three significant observations: first, that under conditions where the formation of free COP-coated vesicles is arrested (15°C, or GTPγS) ts-O45-G can be co-localized with COP buds/vesicular profiles, thus providing further evidence in favour of this class of vesicle in forward transport from the IC to the Golgi complex. Second, that most of the ts-045-G is transported in COP-coated transport intermediates between the IC and Golgi complex, but not the TGN and cell surface. Finally, we show that β-COP is detected in significant amounts on the membrane of the TGN but, in contrast to the IC, these COP molecules do not appear to be associated with buds or vesicles.

MATERIALS AND METHODS

Cell culture and immunofluorescence microscopy

Vero cells (African green monkey kidney cells, ATCC CCL81) were maintained and infected with ts-O45 VSV (Indiana serotype) (Kreis and Lodish, 1986) and temperature blocks done as described earlier (Duden et al., 1991; Griffiths et al., 1985). Immunofluorescence was performed as described (Pepperkok et al., 1993) using the following antibodies: monoclonal antibody P5D4 for ts-O45-G (Kreis, 1986), α-EAGE (Duden et al., 1991) and maD (Pepperkok et al., 1993) for β-COP, and a rabbit polyclonal antibody against β′-COP (R. Pepperkok and T. E. Kreis, unpublished). Images of immuno-stained cells were recorded on a Zeiss inverted fluorescence microscope (Axiovert 135TV) equipped with a cooled CCD camera (Photometrics CH250, 1317X1035 pixels, Tucson, AZ, USA), controlled by a Macintosh Power PC 7100. Images were further processed with the software package IPLab spectrum V2-5.6 (Signals Analytics Corp., Vienna, VA, USA) before printing on Tmax 100 film, using a slidewriter IS200 (Focus Graphics, Forster City, CA, USA). The details of the quantification of the immunofluorescence data is given in the legend to Fig. 4.

Electron microscopy

The preparation of cells for cryo sections and immunogold labelling was done as described by Griffiths (1993). Double and triple labelling was carried out by the sequential labelling procedure of Slot et al. (1991). The rabbit affinity-purified rab1A antibody was provided by Dr Bruno Goud (see Saraste et al., 1995). The anti-p53 was provided by Dr Hans-Peter Hauri (Schweizer et al., 1988) and the anti-p58 by Dr Jaakko Saraste (Saraste et al., 1987). The anti-PDI antibody was a gift of Dr Steve Fuller while the anti-γ-adaptin was provided by Dr Ernst Ungewickel. For labelling the ts-045-G we used either a rabbit polyclonal antibody against the luminal domain (Griffiths et al., 1985; a gift from Dr Kai Simons), or the P5D4 anti-G tail mouse monoclonal or a rabbit antibody (P4) against the P5D4 epitope (Kreis, 1986). When the mouse antibody was used a sandwich, rabbit anti-mouse polyclonal antibody was used before the Protein A-gold step. The preparation of L cells for SLO permeabilization as well as the treatment with GTPγS (50 μM) was as described earlier (Krijnse

Locker et al., 1994). For details of the SA:48 cells, which express α2,6 sialyltransferase tagged on its luminal domain with the P5D4 epitope, see Rabouille et al., 1995; Griffiths et al. (1994). For quantifying the COP-labelling the number of gold particles was related to the number of intersections of the membranes of interest with the lines of a test grid (Griffiths, 1993).

For the GTPγS experiments using mouse L cells, the cells were infected with ts-O45 for 1 hour at 37°C followed by 2 hours at 39.5°C (Griffiths et al., 1985). One set of these cells were shifted to 31°C by adding pre-equilibrated medium in an incubator and left for 3 minutes at 31°C before incubating for 10 minutes on ice with SLO (1 U/ml; Wellcome Diagnostics, Dartford, UK). Following 2 rinses with ice-cold permeabilization buffer the cells were incubated in the presence of this buffer containing 50 μM GTPγS for 30 minutes at 31°C (see Krijnse Locker et al., 1994). A second set of cells were incubated for 2 hours at 19°C in the presence of 100 μg/ml cycloheximide. These were then permeabilized as above with SLO either in the presence or absence of GTPγS at room temperature for 30 minutes before fixation.

RESULTS

Evidence for COP-containing vesicular intermediates in IC to Golgi but not in TGN to plasma membrane transport

We have shown previously that ts-O45-G accumulated at either 15°C in the IC (Saraste and Kuismanen, 1984) or at 20°C in the TGN (Matlin and Simons, 1983; Griffiths et al., 1985) significantly co-localized with β-COP by double immunofluorescence microscopy (Duden et al., 1991). We therefore reasoned that under these conditions ts-O45-G might accumulate in β-COP-containing buds/vesicles that were unable either to form or to fuse with the *cis*-Golgi (15°C), or to leave the TGN (20°C). If so, shifting these temperature-arrested cells to the permissive temperature, 31°C, should result in a transient association of ts-O45-G with β-COP in discrete transport intermediate structures that might be resolved by light microscopy. We therefore accumulated ts-O45-G protein in the ER at 39.5°C. One set of cells was immediately shifted to 31°C for 4 minutes before fixation. The other two sets were shifted to either 15°C or 20°C for 2.5 hours before raising the temperature to 31°C for 4 minutes before fixation. For comparison, cells were also kept at 39.5°C, 15°C or 20°C, conditions in which ts-O45-G accumulates in the ER, IC or TGN, respectively.

As shown previously, at 39.5°C ts-O45-G localizes to structures that extend throughout the cytoplasm. In contrast, at 15°C this protein has a more restricted perinuclear localization as well as being scattered in discrete spots; under this condition ts-O45-G co-localizes extensively with the well characterized IC marker p53 both at the immunofluorescence and EM levels (results not shown; see Bonatti et al., 1989; Schweizer et al., 1990; Duden et al., 1991; Lotti et al., 1992). At 20°C the overall pattern is similar to 15°C in that the structures labelled with both markers (ts-O45-G and β-COP) are found in perinuclear structures which are often, however, more extended. These extended Golgi associated structures do not significantly overlap with IC markers (not shown; see Duden et al., 1991). At the EM level ts-O45-G accumulates at 20°C in *trans*-Golgi cisternae and in an extended TGN (Griffiths et al., 1985, 1989).

Upon shifting cells with ts-O45-G accumulated in the ER, IC and TGN, respectively, to the permissive temperature, numerous discrete spots scattered throughout the cytoplasm could be observed. Double immunofluorescence labelling for the viral glycoprotein and β-COP revealed some overlap when transport intermediates formed at the ER (39.5°C to 31°C, Fig. 1), little or none at the TGN (20°C to 31°C, Fig. 2), but many of the IC-derived COP-coated vesicle structures also contained ts-O45-G. (15°C to 31°C, Fig. 3). These experiments were further analysed quantitatively (Fig. 4). The total number of discrete COP coated vesicles seen per cell significantly increased when cells with ts-O45-G accumulated in the IC or TGN were shifted to permissive temperature; under all other conditions a similar number of β-COP positive spots was observed. A similar result was obtained when ts-O45-G positive vesicular structures were counted, but the number of spots was reduced to about half relative to those labelling for COP. Strikingly, virtually all of the IC-, but not the TGN-derived transport intermediates containing viral glycoprotein were also labelled for β-COP, suggesting that transport vesicles from the IC to the Golgi complex are COP-coated. Interestingly, however, half of these IC-derived COP coated vesicles did not contain detectable amounts of ts-O45-G. These vesicular structures may contain other cargo and are perhaps recycling between the Golgi complex and the IC (see Letourneur et al., 1994).

Our data suggest that, following the release of both the 39.5°C and 15°C blocks ts-O45-G moves rapidly into discrete structures, presumably including transport vesicles, that contain β-COP. In contrast, after releasing the 20°C block the ts-O45-G is transported from the TGN into structures that either immediately lose their COP coats or that are devoid of COP coats from the beginning. While the above results were obtained using the rabbit antibodies against the EAGE peptide of β-COP, the same results were also seen with the mouse monoclonal antibody (maD) against β-COP, as well as an antibody against another coatomer subunit, β′-COP (not shown).

Co-localization of cargo and coat proteins by immunoelectron microscopy

For the immunoelectron microscopic analysis we were predominantly interested in comparing the distribution of ts-O45-G not only with the distribution of β-COP but also with respect to known compartment markers for the three different temperature blocks, 39.5°, 20° and 15°C. The data from these kinetic blocks were also complemented by the use of the non-hydrolysable analog of GTP, GTPγS, to block vesicular membrane transport in vitro. Since this compound does not enter intact cells we used permeabilized mouse L cells as in our previous analysis of the assembly compartment of MHV (Krijnse Locker et al., 1994).

Transport from the ER

When thawed cryo sections of ts-O45 VSV-infected Vero cells at 39.5°C were labelled with antibodies against either the lumenal or cytoplasmic domains of ts-O45-G, the G protein was found in high concentration in the RER, while the Golgi stack was free of label (not shown; see also Bergman et al., 1981). However, in this initial screening we were surprised that ts-O45-G labelling extended very close to the Golgi stack, and often a tubulo-cisternal element on one side of the stack had specific label associated with it. It now seems clear from the recent literature that much of this labelling is found in the

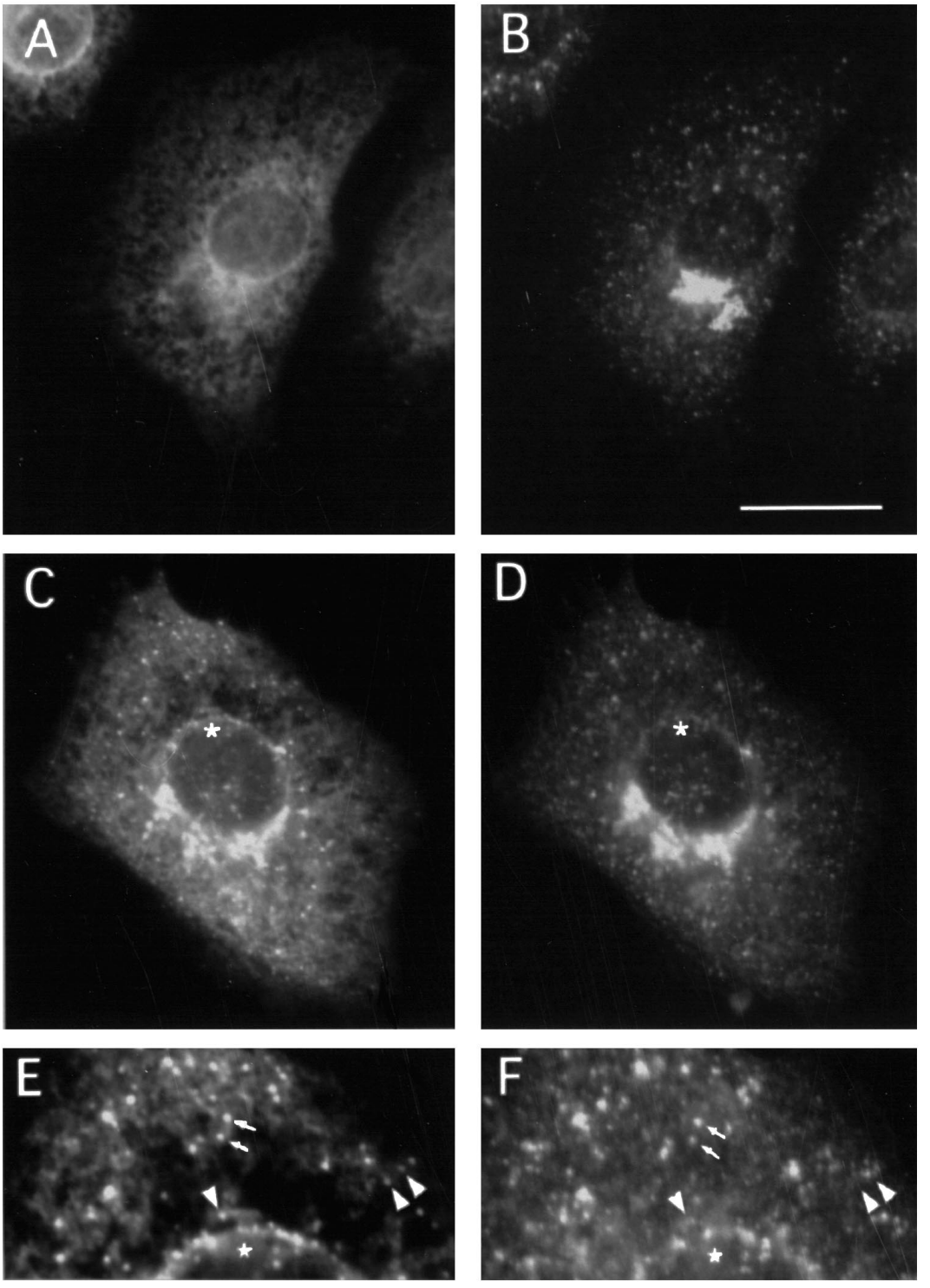

Fig. 1. Movement of ts-O45-G out of the ER. Vero cells were infected with ts-O45 VSV and ts-O45-G accumulated for 2.5 hours at 39.5°C in the ER (A and B). Cells were subsequently shifted to 31°C for 4 minutes before fixation (C and D). All preparations were double labelled for the viral glycoprotein (using the P5D4 monoclonal antibody) (A,C,E) and for β-COP (using the rabbit anti-EAGE antibody) (B,D,F). At 39.5°C the G protein shows an extensive ER distribution which contrasts with the more perinuclear localization of β-COP. Following the shift to the permissive temperature ts-045-G accumulates in many discrete structures that are distributed throughout the cytoplasm. As is evident at higher magnification (E,F) a fraction of these glycoprotein containing vesicular structures co-localize with COP labelling (arrows). The arrowheads indicate G protein containing structures that are negative for β-COP. Bar, 20 μm for A-D; E and F are 2.5× enlarged relative to C and D (the asterisks indicates identical areas).

smooth ER/IC (Bergman and Fusco, 1991; Schweizer et al., 1990; Hammond and Helenius, 1994). We could confirm this interpretation by showing that, at 39.5°C, a fraction of the G protein co-localizes with p53 (in non-permeabilized Vero cells, not shown), with p58 (in the L cells both in non-permeabilized and in SLO-permeabilized cells, not shown), and with rab1 which has recently been shown to extensively co-localize with p58, as well as the budding compartment of MHV (Griffiths et al., 1994) (Fig. 5B). In these IC elements the G protein also co-localizes with PDI (not shown; see below). At 39.5°C a fraction of the G protein was also found in the vicinity of COP labelled buds/vesicles but the COP structures themselves were generally devoid of G protein label (Fig. 5A).

If the G protein at 39.5°C is present in both the RER and the IC, then upon release of the block by lowering the temperature to the permissive temperature, 31°C, it would be expected that the trimers accumulate at the ER-exit sites (the IC) and are transported in vesicles towards the *cis*-Golgi

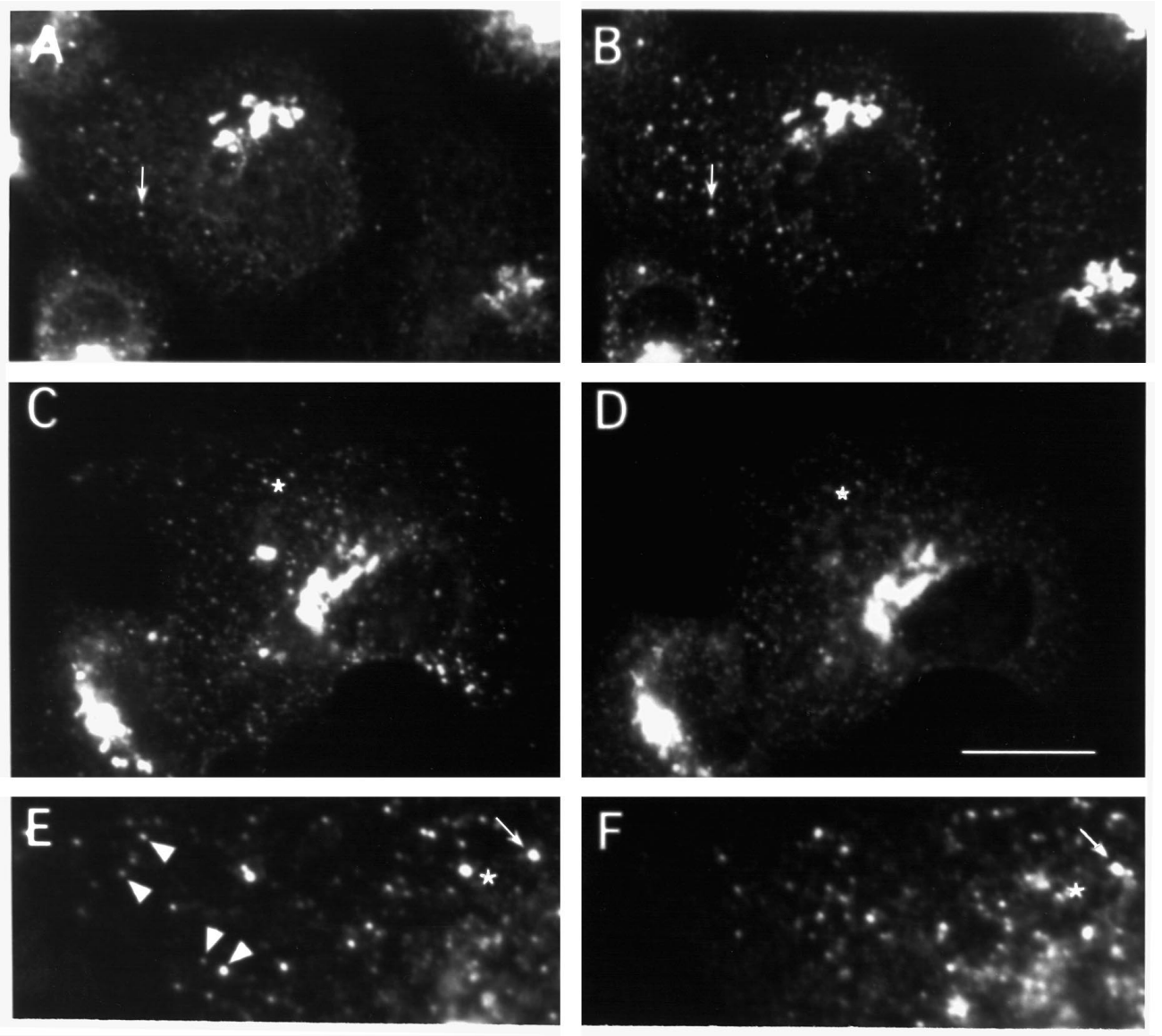

Fig. 2. Transport of ts-O45-G out of the TGN. Vero cells infected with ts-O45 VSV at 39.5°C for 2.5 hours were further incubated for 2.5 hours at 20°C in the presence of cycloheximide to accumulate the ts-O45-G in the TGN. Cells were then fixed (A,B), or transferred to the permissive temperature for 4 minutes before fixation (C-F), and then stained for ts-O45-G (A,C,E) or β-COP (B,D,F). At 20°C ts-O45-G accumulates in the TGN (A), but a few spot like structures (arrows), which are also positive for β-COP (B), are spread over the cytoplasm. These structures probably represent ts-O45-G still in the IC. Upon shift to 31°C the ts-O45-G is transported to the plasma membrane in β-COP-negative vesicular structures (arrowheads in E). Only a few structures are observed which show a co-localization of ts-O45-G and β-COP (arrow in E and F). Close examination of E and F reveal many COP-labelled spots that are devoid of G protein labelling. We assume that at least some of these putative vesicles are derived from the IC. (E and F) A 2.5× magnification of the region marked by the asterisk in C and D. Bar, 20 μm.

where mannosidase 1 is located (which we operationally define as the beginning of the Golgi complex). To try to arrest the transport at the vesicle step the following experiment was undertaken. Mouse L cells were infected with ts-O45 VSV and blocked for 3 hours at 39.5°C. Subsequently, the cells were shifted to 31°C for 3 minutes before being put on ice. At this temperature SLO was added for 10 minutes, the excess washed away and the cells were permeabilized by raising the temperature to 31°C in the presence of 50 μM GTPγS. The cells were incubated under this condition for 30 minutes, then fixed with 1% glutaraldehyde, scraped off the dish, centrifuged and prepared for cryo sectioning. The sections were double-labelled with antibodies against the viral glycoprotein (using either a rabbit antibody against the lumenal domain or P4D5) and β-COP or rab1A.

As shown in Fig. 6, when the cells were permeabilized in the presence of GTPγS a significant number of COP coated buds (and possibly free vesicles) appeared (see also Krijnse Locker et al., 1994). Some of these buds also labelled for rab1A (Fig. 7) in agreement with Pind et al. (1994). Using the cytoplasmic domain specific antibody, we found that much of the Golgi-associated G protein was in these COP/rab1 positive

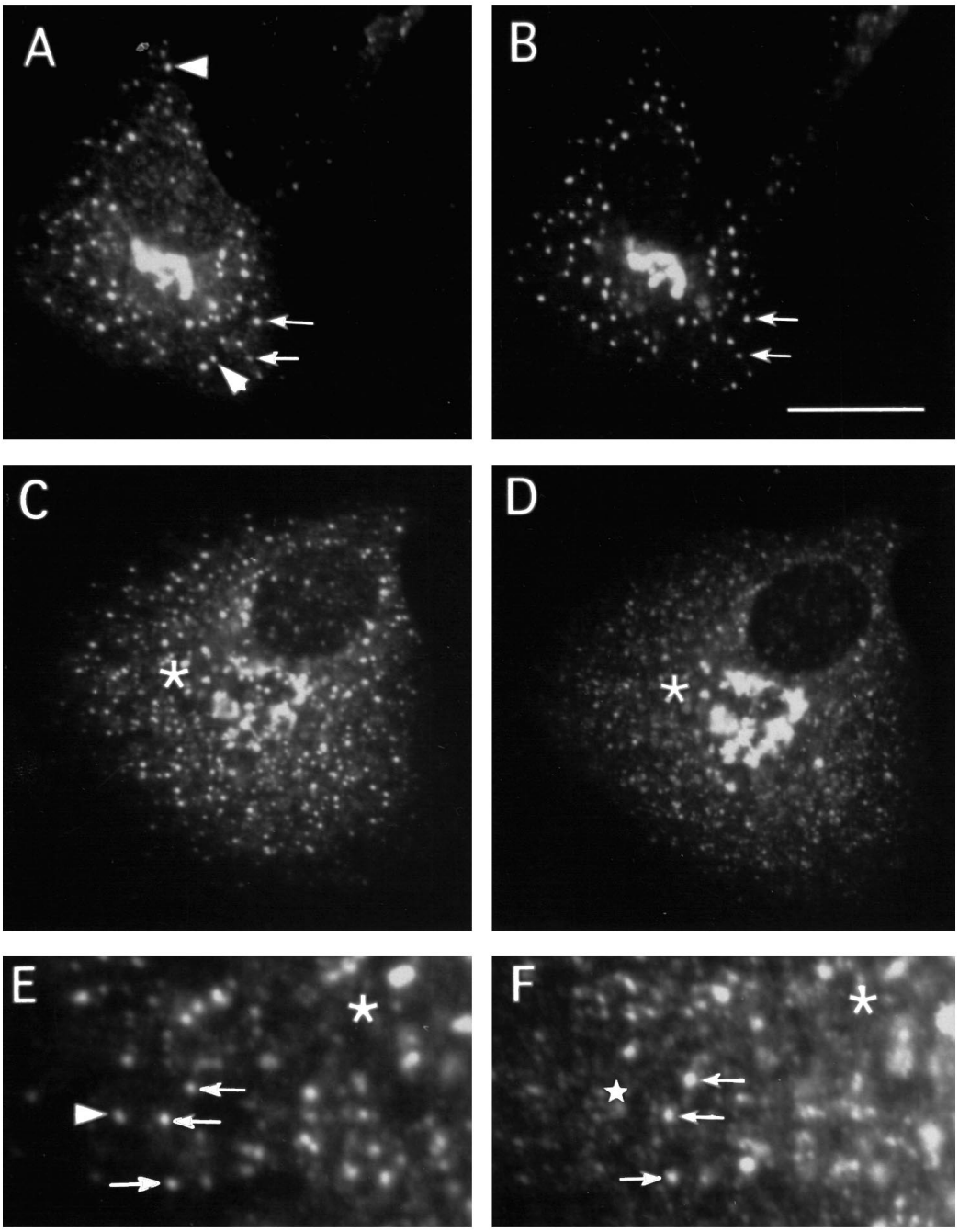

Fig. 3. Transport of ts-O45-G out of the IC in β-COP-coated vesicular carriers. Vero cells were infected with ts-O45 VSV for 2.5 hours at 39.5°C and then shifted to 15°C with cycloheximide for a further 2.5 hours. The cells were then fixed and labelled for G protein (A,C,E) and β-COP (B,D,F). A and B shows the 15°C blocked condition with ts-045-G in the IC whereas C and D (and an area enlarged 2.5× in E and F) shows cells shifted for an additional 4 minutes to 31°C before fixation. At 15°C the distribution of G protein (A) overlaps extensively with that of β-COP (B). The arrows indicate co-localization, whereas the arrowheads indicate some of the few non-COP-coated G protein positive structures. Following the temperature shift (C-F) the two proteins co-distribute into apparently discrete structures that appear to be less intensively labelled but increased in number compared to the 15°C condition. The arrows indicate overlapping structures while the arrowheads point to structures labelled for G that are not labelled for β-COP. The asterisk indicates a region rich in COP-labelled, punctate structures that are devoid of G protein. The star indicates identical areas in C-D and G-F. Bar, 20 μm (A-D).

buds (Figs 6 and 7). The G protein was also detected in extensive IC elements that labelled for rab1 (Fig. 7) as well as p58, though less abundantly (not shown). Following this GTPγS treatment, except for variable labelling of one cisterna, the Golgi stack was essentially free of labelling for the ts-O45-G (Figs 6 and 7).

These data are consistent with the notion that, upon release of the 39.5°C block for a short period, combined with permeabilization in the presence of the vesicular transport inhibitor, GTPγS, a significant fraction of the G protein moves into COP/rab1A-containing vesicular buds (and possibly vesicles) but does not enter the central cisternae of the Golgi stack.

Transport from the IC to the Golgi complex

Thawed cryo sections were next prepared of cells in which ts-O45-G was blocked at 15°C. These sections were double-labelled with one of the two antibodies against ts-O45-G and with antibodies against β-COP, PDI or p53. In these preparations there was significant overlap between G protein and p53 by double-labelling (not shown), thus confirming the data of Schweizer et al. (1991). It was also clear that much of the G protein-enriched structures were labelled for rab1 (not shown). A striking accumulation of β-COP reactive buds (or vesicles) was seen in close vicinity to the Golgi complex at 15°C. In these buds there was also significant co-localization with the G protein, using the anti-cytoplasmic domain antibody (Fig. 8).

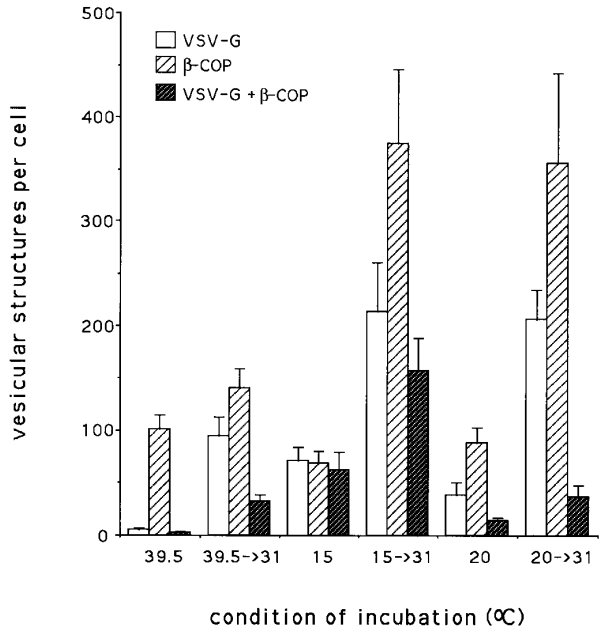

Fig. 4. Quantitative analysis of the number of transport intermediates carrying ts-O45-G and their co-localization with β-COP. Vero cells were infected with ts-O45 VSV and the glycoprotein accumulated in the ER, IC and TGN as described in the legends to Figs 3 and 4. Cells were then shifted for 4 minutes to permissive temperature, fixed and labelled for ts-O45-G and β-COP. Fluorescence images were acquired and digitized with a cooled CCD camera. For each cell analysed a square area (200×200 image pixels, equivalent to 15-30% of the total cell area) was randomly chosen and all the vesicular structures containing coatomer or VSV-G protein counted (circular areas with diameters of ~10 pixels marked for both labels and in the overlay counted positive when >50% overlap observed). The resulting number was then normalized to the whole area the cell occupied, again determined in image pixels. The data represent the mean and standard deviation of the mean of at least 20 cells analysed per condition tested.

We quantified this result by counting the number of gold particles for the G protein cytoplasmic tail epitope labelled with the rabbit antibody that were found in 100 COP buds/vesicular profiles (that had at least one gold particle for β-COP). Under this condition no amplification of the signal is expected (Griffiths, 1993). This analysis showed that on average there was roughly one gold for G-protein per vesicle having at least one gold for β-COP (106 gold for G per 100 buds) as compared with only 8 gold particles per 100 buds using an irrelevant primary antibody. At 15°C anti-rab1 consistently labelled these COP buds (see Pind et al., 1994; Griffiths et al., 1994; not shown).

Protein disulfide isomerase (PDI) is a KDEL-containing protein that, like other KDEL proteins, is enriched in both the RER and the IC (Krijnse Locker et al., 1994; Griffiths et al., 1994; Banfield et al., 1994). This overlap was evident in two ways of visualizing double-labelled sections of the infected cell at 15°C. First, if one searches for all structures in the section that are enriched for G protein these same structures, on average, show a significant labelling for PDI. Second, if one samples only identifiable Golgi stacks the markers co-localize routinely (Fig. 9). When the sections were double-labelled with antibodies against ts-O45-G and PDI a significant overlap of both proteins was seen in membrane structures near the Golgi stacks (Fig. 9). Collectively, these data indicate that, under conditions in which transport into the Golgi complex is blocked the G protein accumulates in the IC and much of the viral membrane protein co-localizes with β-COP-enriched vesicular buds.

Localization of β-COP to the TGN

We next investigated the localization of β-COP in Vero cells infected with ts-O45-VSV and kept at 20°C for 2 hours, a condition which arrests exit from the TGN. In these preparations it was clear that the bulk of ts-O45-G accumulates in tubulo-cisternal elements which we assume to be the TGN based on earlier studies (Griffiths et al., 1985, 1989). These elements were often close to the Golgi stack but their morphology was clearly distinct from the stack (Fig. 10). In general the COP labelling was found scattered over these structures but there was no convincing evidence that this labelling was associated with vesicles or vesicular buds.

Since, at 20°C, G protein also accumulates to a variable extent throughout the Golgi stack (but not the ER), it became clear that an additional marker for the TGN was needed in order to be confident that this organelle specifically labelled for β-COP and, if so, whether this COP was associated with vesicular profiles. Of the possible marker antibodies we tested the most convincing was γ-adaptin. Clathrin buds have been shown to be localized on the TGN (Griffiths et al., 1981, 1985; Orci et al., 1985; Tooze et al., 1988) and immunocytochemical data argue strongly that these buds also contain γ-adaptin (or HA-I) (Klumperman et al., 1993; R. Leborgne et al., unpublished).

When thawed cryo sections of ts-O45-VSV infected Vero cells blocked at 20°C were double-labelled with various combinations for ts-O45-G, γ-adaptin and β-COP, it became clear that all three proteins were found on the same tubulo-cisternal elements (Fig. 11A). As expected, the buds which labelled for γ-adaptin were distinct from those which labelled for β-COP. This could also be confirmed directly in a triple-labelling experiment (Fig. 11B,C). In general, the G protein labelling was excluded from the γ-adaptin buds/vesicles (Fig. 11C).

A careful analysis convinced us, in agreement with Oprins et al. (1993), that the labelling for β-COP was significant over the TGN but we could find no evidence that this fraction of coatomer was associated with vesicular buds. For an alternative approach we also permeabilized ts-O45 VSV infected L cells at 20°C in the presence of GTPγS. Following this treatment there were significant amounts of COP labelling on both sides of the stack, as well as on the rims of the central cisternae. Significantly, the membrane structures where the bulk of the G protein accumulated were positively labelled for β-COP. However, also in these preparations we could not convincingly demonstrate that the β-COP was associated with vesicular buds on the TGN nor that the G protein was preferentially associated with β-COP in any obvious structural element of this organelle (results not shown).

Use of HeLa cells expressing a TGN marker

In order to address more definitively the question of β-COP

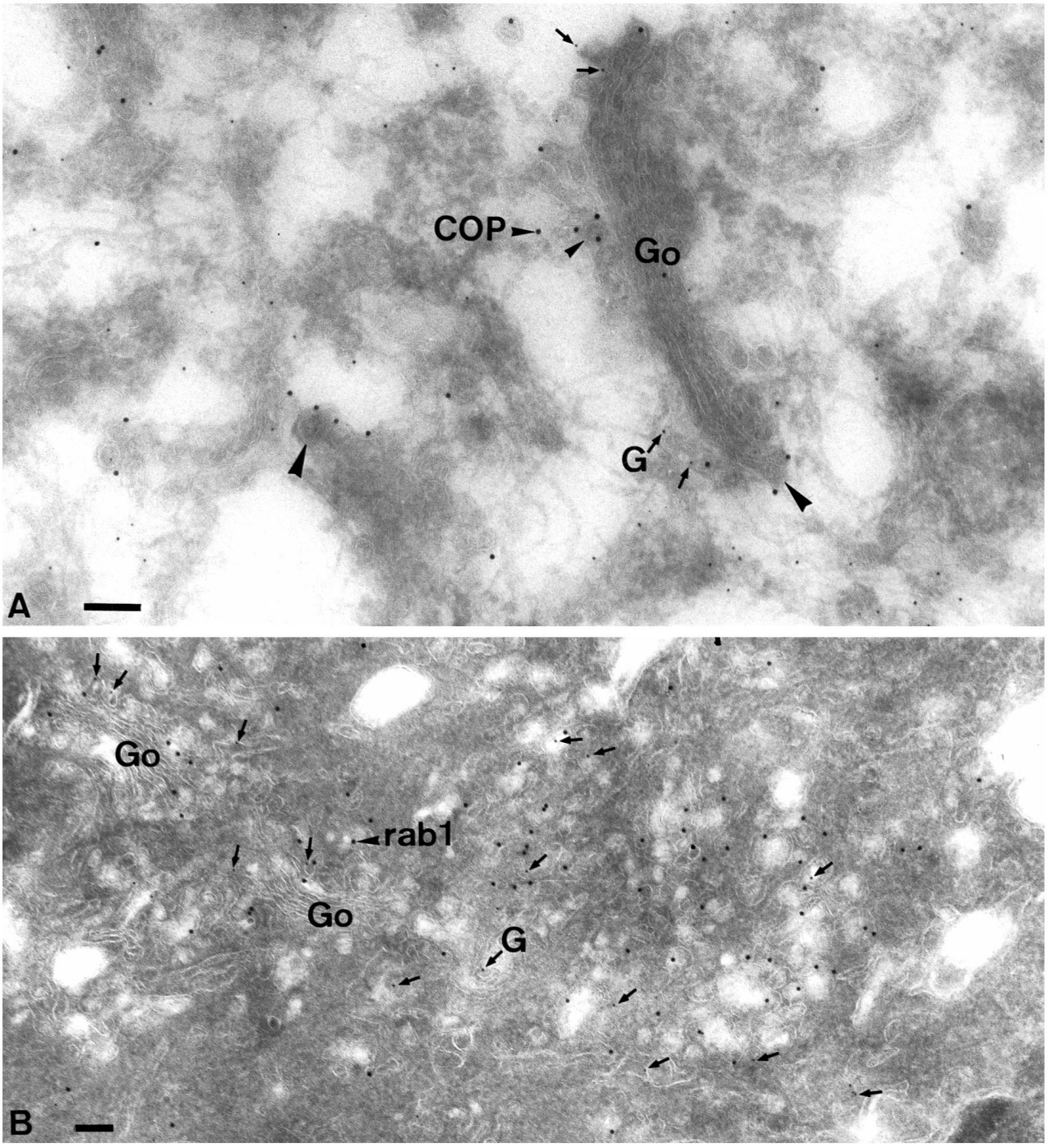

Fig. 5. Double localization of ts-O45-G accumulated in the ER with β-COP or rab1 in vero cells infected with ts-O45 VSV at 39.5°C (4 hours). In both A and B the G protein (G) is labelled with anti-lumenal antibodies and 5 nm gold (arrows). In A the large gold particles labelβ-COP while in B they label rab1. In both micrographs the distribution of G protein extends down to membranes on the side of the Golgi stack (Go). As shown in A the G protein does not label the COP coated buds/vesicles (arrowheads) at 39.5°C. In B note the extensive co-localization of G protein with the IC membrane network labelled by rab1. This co-localization also extends quite close to one side of the Golgi stack. Bars, 100 nm.

localization in the TGN we used a HeLa cell line that stably expresses the human α2,6 sialyl transferase (ST) tagged with the VSV-G protein cytoplasmic tail epitope P5D4 on its lumenal aspect (Rabouille et al., 1995; Griffiths et al., 1994; a gift from Dr T. Nilsson). This P5D4 containing construct appears to faithfully localize to the TGN (Rabouille et al., 1995; Griffiths et al., 1994; R. Leborgne et al., unpublished). The ST is generally accepted to be a TGN marker (Roth et al., 1985). In order to ensure as much access as possible for the anti-β-COP antibodies to their antigen we used thawed cryosections of SLO-permeabilized cells, both following a normal 37°C or 20°C (2 hours) incubation. As shown in Fig.

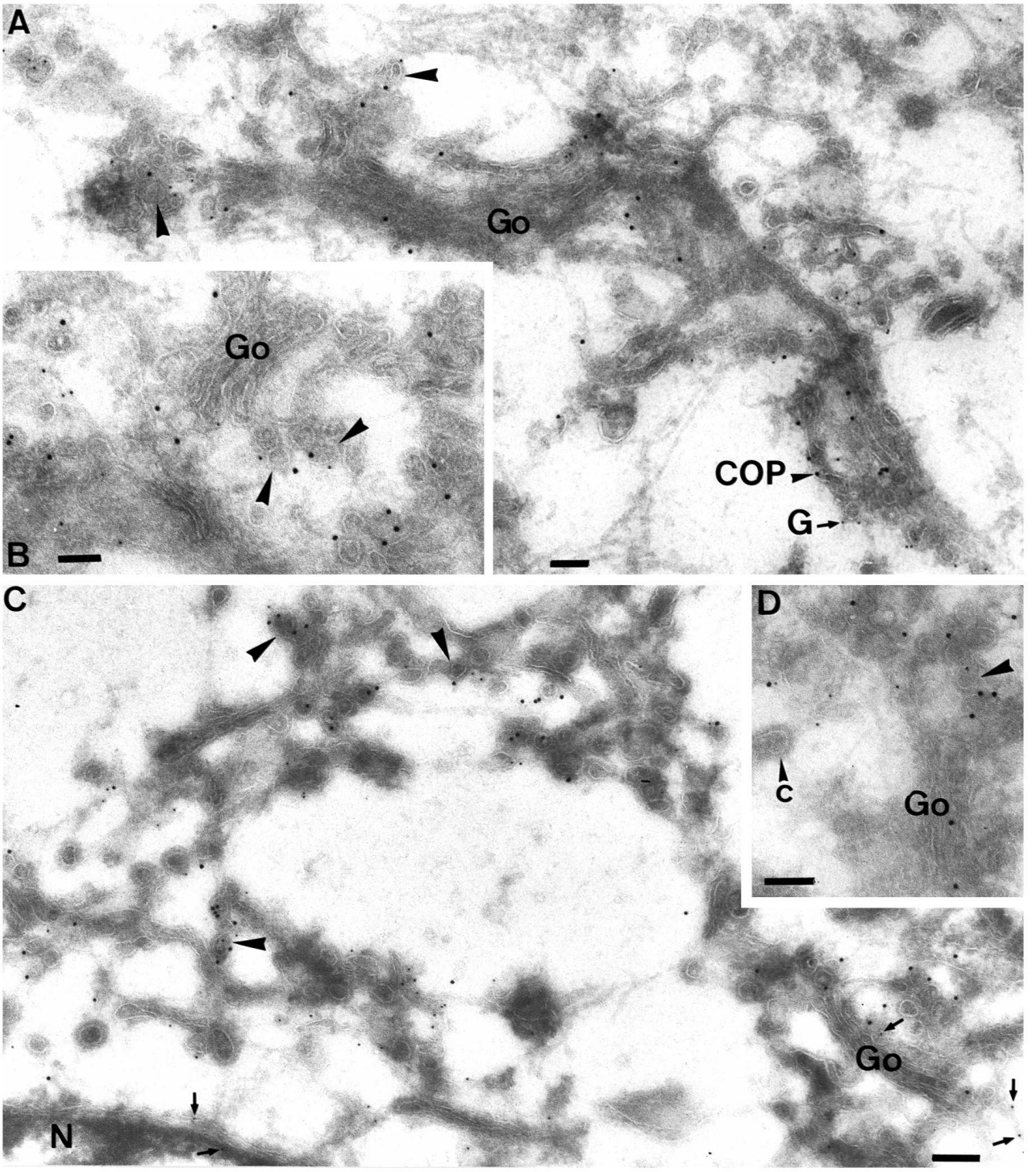

Fig. 6. Double labelling of L cells infected at 39.5°C and shifted for 3 minutes to 31°C before SLO permeabilization in the presence of GTPγS. Following 30 minutes incubation the cells were fixed, prepared for cryo sections and double labelled for G protein (G) (rabbit anti-cytoplasmic domain) using 5 nm gold and β-COP using 10 nm gold. In these figures note the extent of co-localization of G protein in tubular-cisternal elements close to, or within, the Golgi stack (Go) and especially in COP-coated buds (arrowheads in all figures indicate COP buds that appear to label also for G protein). In C the arrows indicate G protein that is on the nuclear envelope membranes as well as on one side of the Golgi stack. In D, c refers to a putative clathrin bud/vesicle that is unlabelled. Bars, 100 nm.

12A and B at 20°C there were significant levels of immunodetectable β-COP on structures identified as the TGN, based on their high concentration of the P5D4-tagged ST molecule. A quantification of this labelling is shown in Table 1. This analysis shows that the density of COP labelling over TGN membranes was over seven-fold higher than the labelling

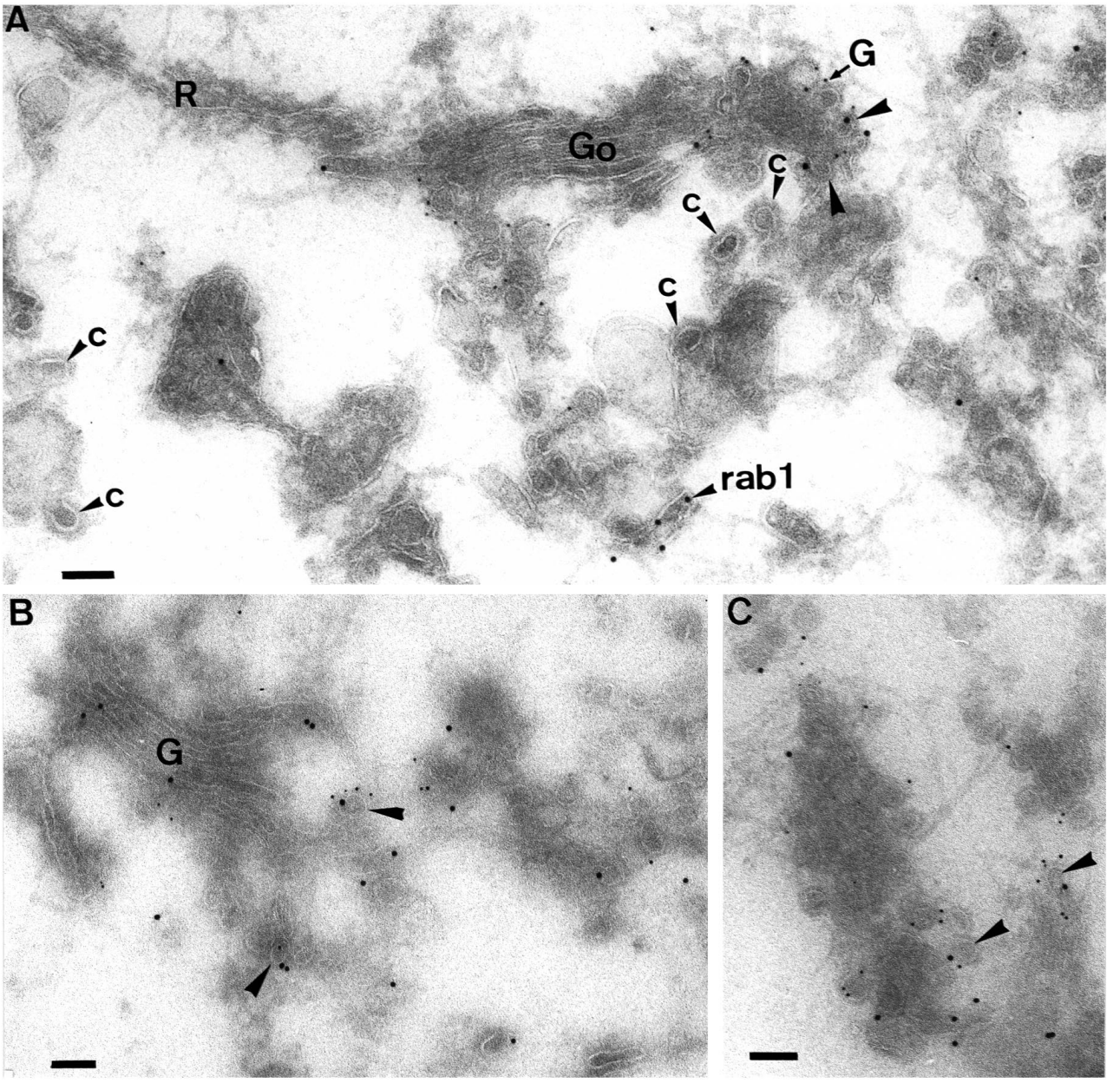

Fig. 7. Double labelling of L cells infected at 39.5°C and shifted for 3 minutes to 31°C before SLO permeabilization and incubation in the presence of GTPγS. The sections were double-labelled for G protein (G) (rabbit anti-cytoplasmic domain, 5 nm gold) and rab1 (10 nm gold). In all figures, note the extensive labelling for both proteins that is localized close to, or on one side of, the Golgi stack (Go; A and B). The arrowheads indicate COP buds that label for both rab1 and G protein; c, putative clathrin buds; R, rough ER. Bars, 100 nm.

Table 1. Quantification of β-COP labelling on the TGN* using cryosections of SLO-permeabilized cells

Gold per linear micron membrane†		
TGN	Nuclear envelope (inner and outer membranes)	Mitochondria (outer membrane)
1.31 (0.2)	0.14 (0.05)	0.18 (0.12)

*The TGN is defined as structures enriched for the P4D5 epitope. The latter was labelled with 5 nm gold and β-COP with 10 nm gold.
†Determined using intersection counts.
Numbers in parentheses - standard error of means.

over either the nuclear membrane (both inner and outer) or the outer membrane of mitochondria. When double labelling for β-COP and P5D4 was carried out on SA:48 cells that had been permeabilized with SLO in the presence of GTPγS (50 μM) there appeared to be more labelling for β-COP on the TGN (Fig. 12C) than that seen in the absence of GTPγS. However, both in the presence and absence of GTPγS we again could find no convincing evidence of β-COP labelling of vesicles or vesicular buds. Collectively, these data indicate that while low but significant amounts of β-COP are present on the TGN it is evidently not associated with buds or vesicles.

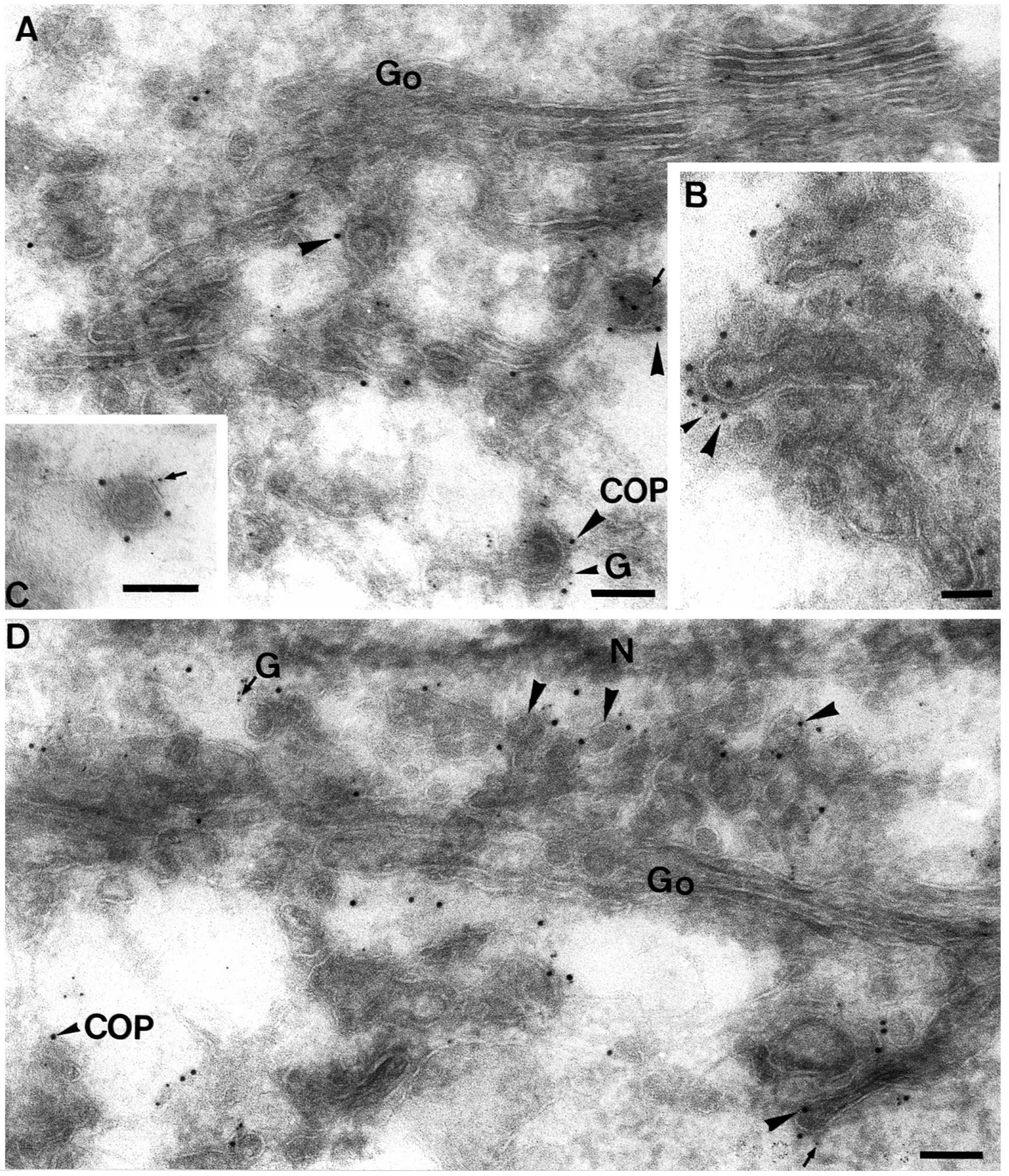

Fig. 8. Co-localization of G protein and β-COP at 15°C. Following the 39.5°C treatment infected vero cells were shifted to 15°C for 2 hours. The sections were double labelled for G protein (P4D5 followed by a sandwich rabbit anti-mouse and 5 nm gold; small arrowhead) and β-COP (10 nm gold, large arrowhead). Note the extensive co-localization of both proteins in structures that are positioned predominantly on one side of the Golgi stack (Go). Note the COP labelled buds that also label for G protein (G) (A-C, large arrowheads in D). Note also that the latter are often positioned close to the nucleus (N). The fact that the G protein labelling in many buds (e.g. see Fig. 7B) is farther away from the membrane than that for COP is due to the extra rabbit anti-mouse antibody layer when using the P5D4 monoclonal. Bars, 100 nm.

DISCUSSION

The main finding of this study is that two well-characterized conditions which block ER to Golgi transport lead to an accumulation of the newly synthesized G cargo membrane protein in β-COP-coated, vesicular buds on the membranes of the IC. The first inhibitory condition, 15°C, was introduced by Saraste and Kuismanen (1984). It has subsequently been shown to be

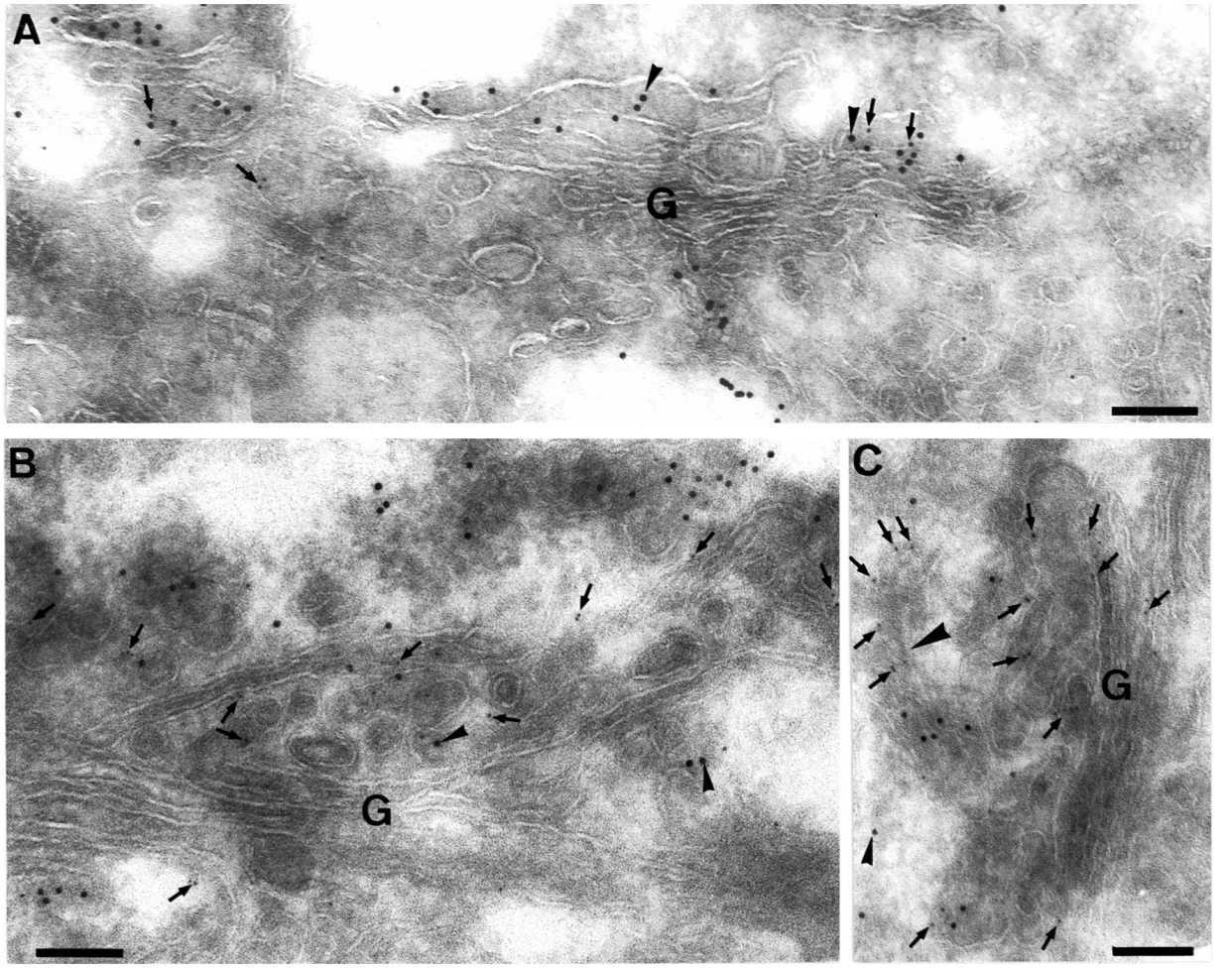

Fig. 9. Co-localization of G protein and PDI at 15°C in tubulo-cisternal elements close to, as well as in one cisterna of, the Golgi stack (A-D). In all figures the G protein (rabbit and anti-G tail in A and luminal domain antibody in B and C) is labelled with the small gold particles (arrows) while the PDI is marked by the large gold particles (arrowheads). Note the variability in the amounts of both proteins at the boundary, or within, the Golgi stack. In the example shown in A there is relatively abundant labelling for PDI and scarce labelling for the G protein. In C the large arrowhead indicates a structure enriched in G but for the most part not labelled for PDI. G, Golgi. Bars, 100 nm.

a general block for all newly synthesized proteins that are destined for the Golgi complex. The best characterized protein in this respect is probably the one used in this study, the glycoprotein of the ts-O45 mutant of VSV. For this protein it has been clearly demonstrated that at 15°C its N-linked oligosaccharides are in the high mannose form (Balch, 1989) and by immunocytochemistry the protein accumulates in the IC (Schweizer et al., 1988). The latter point could be confirmed in the present study using both p53 (Schweizer et al., 1990) and rab1 (Saraste et al., 1994; Griffiths et al., 1994) as markers of the IC, thus confirming earlier reports (Schweizer et al., 1990; Lotti et al., 1992; Saraste et al., 1994).

The second condition we used which facilitated the accumulation of the G protein in β-COP buds, and possibly vesicles, was to release the 39.5°C block by shifting to the permissive temperature, 31°C for 3 minutes, and then to permeabilize the cells in the presence of GTPγS. The rationale here was that even in this short period a significant amount of the G protein should be able to form trimers and enter transport vesicles. While some of the transport competent G protein might be able to enter the first cisternae of the Golgi stack (which we operationally define as that where the Golgi mannosidase 1 is localized) in the few minutes that the cells were left at 31°C, before permeabilization and the addition of GTPγS, we expected that this would only be a small fraction of the total. Indeed, in these preparations only a small amount of label for G protein was found in the central cisternae of the stack and it was evident that much of the G protein accumulated peripherally in COP buds. This is consistent with our results using the same L cell system infected with MHV (Krijnse Locker et al., 1994). In the latter study the newly synthesized M protein of MHV could acquire an IC-localized modification, *N*-acetyl galactosamine, in the presence of GTPγS (as well as at 15°C in vivo) but not the more distally acquired Golgi modifications. In the present analysis the results with GTPγS following the temperature shift were indistinguishable from the 15°C experiments: a significant fraction of the G protein could be detected in COP buds on the membranes of the IC (see also Pind et al., 1994).

At both 15°C and 39.5°C the results of the EM analysis were complemented by double-labelling immunofluorescence microscopy. When the temperature was shifted to the permis-

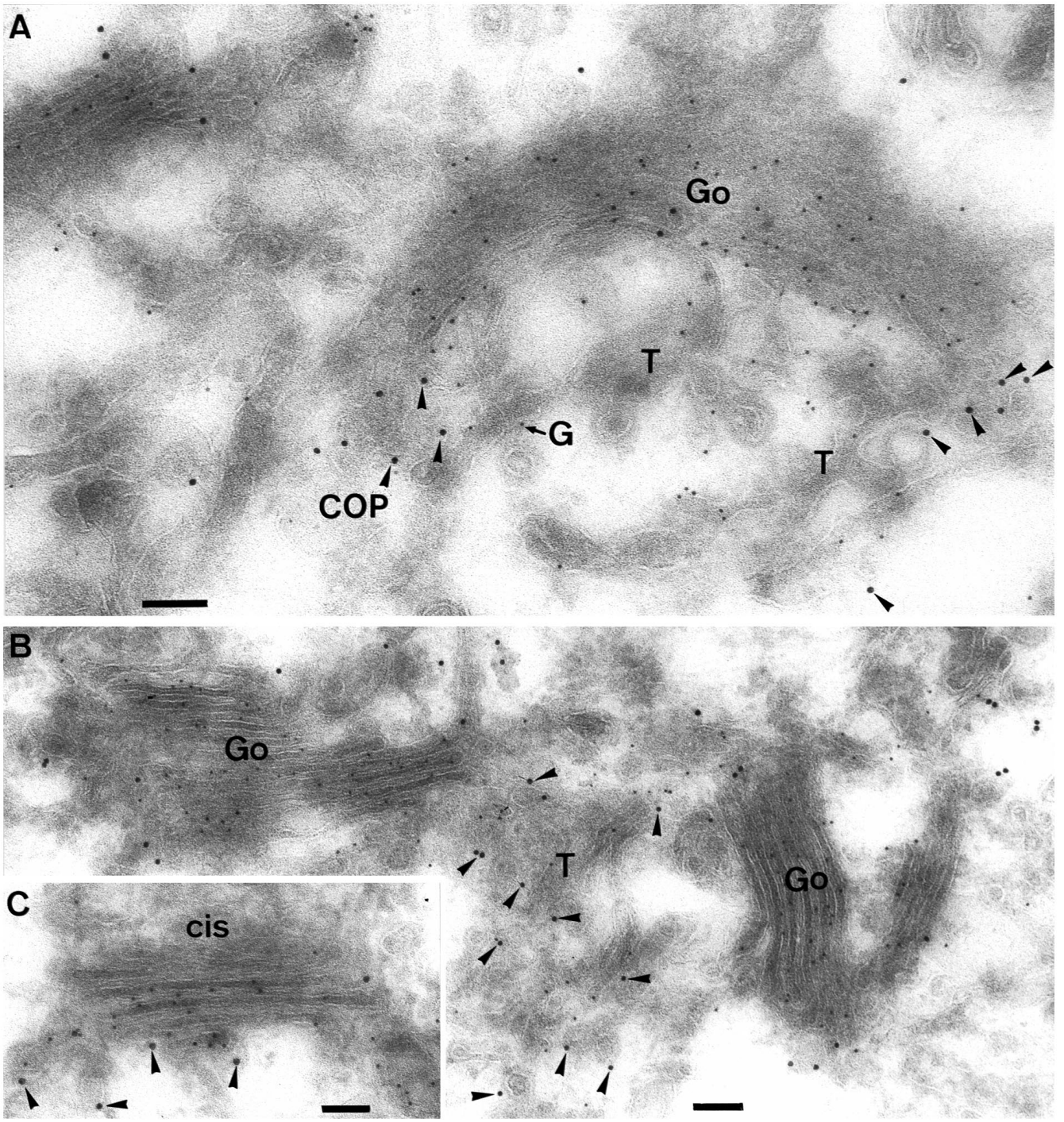

Fig. 10. Co-localization of G protein with COP at 20°C. In all figures the G protein is labelled with 5 nm gold (A and C - anti-luminal domain; B - anti-P5D4 followed by rabbit anti-mouse) while the β-COP is labelled with the 10 nm gold (arrowheads). Note the significant labelling for the β-COP in TGN structures (T) that are either heavily labelled for G protein (B) or that have the distinct morphology of G protein-modified TGN; in many of these tightly packed structures there is less access for G protein antibodies (see Griffiths et al., 1985, 1989). In C the polarity of the stack is evident since the *cis*-side is unlabelled for the G protein; the other side labels for both β-COP and G protein. In A and B there is extensive labelling for G protein (G) throughout the Golgi stack (Go). In C only the *trans*-cisternae are labelled. Bars, 100 nm.

sive temperature for 4 minutes after inhibiting at either 39.5°C or 15°C the G protein co-localized with β-COP in small punctate structures that, within the limits of light microscopic resolution, appeared to be discrete. Upon release of the 15°C block there was an apparent co-localization of G protein with β-COP in punctate structures some of which probably represent transport vesicles. Interestingly, however, a quantitative analysis revealed that, of the total discrete, punctate structures labelled for COP following a few minutes release of the 15°C block, only about half also labelled for G protein. It

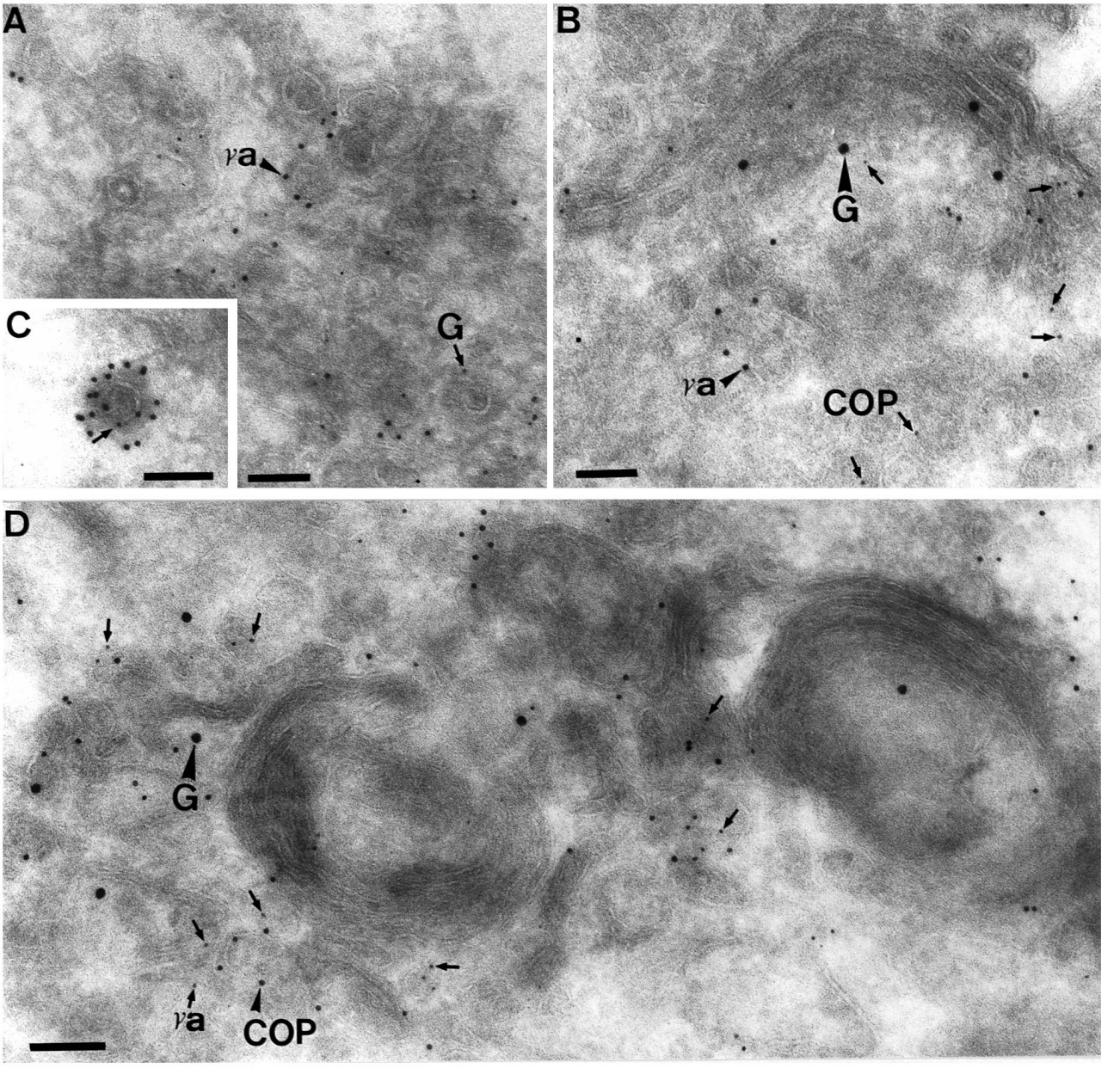

Fig. 11. Double and triple labelling of infected Vero cells at 20°C. In A and C the cells were double labelled for G protein (P5D4, 5 nm gold) and γ-adaptin (10 nm gold; γA). Note that with the exception of a single gold for G in C the γ-adaptin positive membrane buds are negative for the G protein. In B and C the sections were triple labelled on section for G protein (lumenal domain antibody; 15 nm gold), γ-adaptin (10 nm gold) and β-COP (5 nm gold). Note that areas of these sections that label for both G protein and γ-adaptin also appear to label for β-COP. Bars, 100 nm.

is tempting to suggest that this pool of putative COP vesicles might be involved in retrograde, Golgi to IC/ER transport, as implied by the recent data of Letourneur et al. (1994).

The simplest interpretation of all our data is that conditions which block transport out of the IC, either 15°C or GTPγS, arrest the G protein destined for the Golgi in a COP bud that is unable to form into a vesicle. In contrast, at 39.5°C, the G protein does not trimerize and cannot enter the COP bud. Whether COP buds and vesicles devoid of G protein can still form normally under this condition remains to be determined. Following release of the 39.5°C block the G protein can enter the COP buds and vesicles. Following the 15°C block, upon raising the temperature to 31°C the arrested COP buds form vesicles carrying the G protein towards the Golgi compartment containing mannosidase 1.

There is now an extensive body of evidence that COP buds and vesicles are also involved in transport across the Golgi stack (see Rothman and Orci, 1992) although the precise transport step(s) remain to be defined. We did not focus directly on these structures in the present study but we would emphasize that some COP coated buds and vesicular profiles were regularly seen on the rims of the central cisternae of the Golgi stack, and this was, as expected, especially pronounced after GTPγS (see Orci et al., 1993). We should point out,

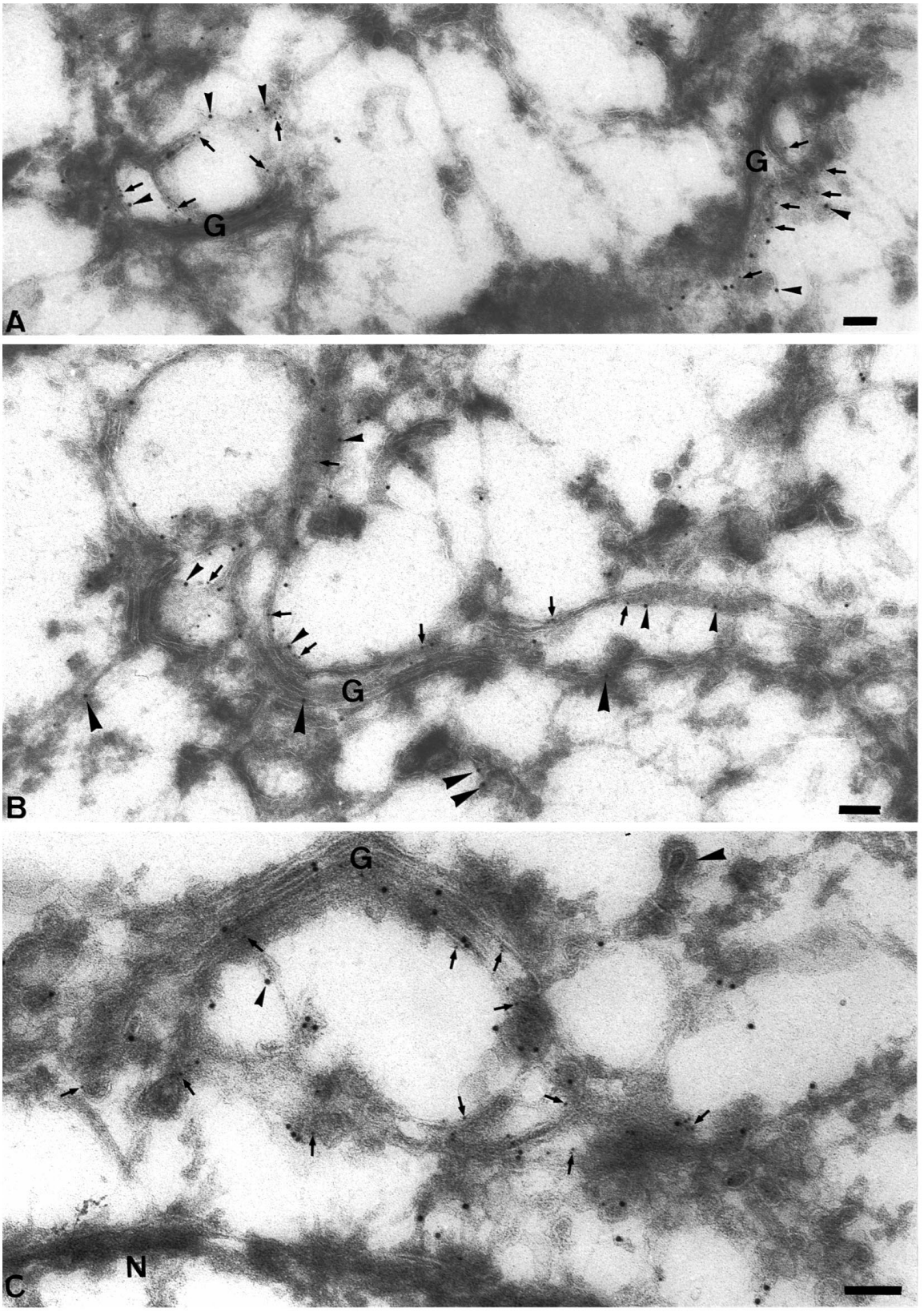

Fig. 12. Cryosections of SA:48 cells (a HeLa stable cell line expressing sialyltransferase - G protein P5D4 epitope) that were permeabilized with SLO before fixation and double-labelled for β-COP (10 nm gold - small arrowheads) and for the G protein tag (using the rabbit anti-cytoplasmic domain; 5 nm gold - arrows). A and B show untreated cells while C is from a cell permeabilized in the presence of 50 μM GTPγS and incubated for 15 minutes prior to fixation. Note the extensive co-localization of β-COP with TGN membranes (identified using G protein) in all figures. The large arrowhead in C shows a putative (unlabelled) clathrin bud. G, Golgi stack; N, nucleus. Bars, 100 nm.

however, that in all our experiments using GTPγS either with in vitro Golgi fractions (Duden et al., 1991) or with permeabilized cells (Krijnse Locker et al., 1994; this study) the great majority of COP-coated structures are buds rather than free-vesicles; this observation differs from the interpretation of Rothman and Orci (1992) who have argued that GTPγS arrests a more distal step following the release of free vesicles.

A significant increase (about threefold) in the number of COP positive vesicular structures was also observed by immunofluorescence when the 20°C temperature block was released after accumulation of cargo in the TGN (see Fig. 4). Since only few β-COP positive buds could be observed by immuno-EM on TGN membranes, we assume that all the discrete, punctate structures labelled for COP by immunofluorescence at 20°C represent COP vesicles originating from the IC, from which vesicular transport is probably, to a large extent, also inhibited at 20°C. We suggest that the COP-positive, but G protein-negative spots seen by immunofluorescence microscopy following release of the 20°C block may correspond to such IC derived COP vesicles that lack labelling for the G protein.

Nevertheless, our immunofluorescence and immuno EM data argue strongly that β-COP is enriched above background over TGN membranes. By both immunofluorescence (Duden et al., 1991; this study) and by immuno EM the labelling for COP overlaps with the structures where the G protein accumulates at 20°C, a condition which is known to inhibit the exit of proteins from the TGN (Griffiths et al., 1985, 1989). Significant COP labelling was also seen over Golgi-associated membranes that were enriched in γ-adaptin and, most convincingly, over the TGN as defined by the sialyltransferase-tagged molecules in the SA:48 cells. However, by immunofluorescence microscopy using cells switched for a few minutes from 20°C to 31°C we were unable to convincingly demonstrate co-localization of the G protein with COP in discrete, vesicular-like structures. This result is also consistent with the failure of microinjected anti-β-COP antibodies to affect transport from the TGN to the plasma membrane when the 20°C block is released by shifting to 31°C (Pepperkok et al., 1993). Thus, while our detailed analysis failed to see a co-localization of β-COP with vesicular buds on the TGN our collective data argue strongly that significant amounts of this protein are localized to the TGN. Moreover, in the quantitative analysis of the rat pancreatic acinar cells by Oprins et al. (1993) a small, but significant amount (≈7%) of the total Golgi-associated β-COP labelling was also seen on this organelle. The significance of this TGN-associated pool of β-COP must now await a more detailed functional characterization.

Finally, the results of this study are consistent with the model we have proposed (Griffiths and Rottier, 1992; Krijnse Locker et al., 1994) whereby the IC is the exit domain of the ER from which COP vesicles form that are destined for the Golgi compartment where mannosidase 1 would be localized. We believe that this model is the simplest one to explain the presence of the G protein at 39.5°C in both the RER and IC. It is supported by the presence of detectable levels of PDI in both compartments: at both 15°C and 39.5°C this protein co-localized extensively at the EM level with the G protein. This result is also consistent with the results of Bergman and Fusco (1991) showing that the G protein accumulates in the extensive smooth ER that accumulates in UT1 cells that are defective in cholesterol biosynthesis. It was not shown in the latter study whether or not known IC markers are enriched in these smooth membranes but the authors presented convincing evidence that they contained significant amounts of BIP. This result would agree with our findings that several KDEL (HDEL) proteins are enriched in both the rough ER and the IC (see Introduction). Nevertheless, we emphasize that the data presented in this paper do not formally rule out the possibility that the IC is not continuous with the RER and that there is one additional vesicular transport step from the ER to the IC.

Our interpretation of the organization of the ER-Golgi boundary differs from other studies that have used the G protein of VSV as a marker in EM morphological studies. First, a study by Lotti et al. (1992) showed convincingly that the tubulo-vesicular clusters where the G protein localizes at 15°C often extend some distance away from the Golgi region (see also Saraste and Svensson, 1991). These elements indeed labelled at the EM level for an IC protein, rab2, but were interpreted by the authors to be discrete elements rather than extensions of the RER. We assume that these structures correspond to some of the peripheral dots that co-label for both G protein and β-COP by immunofluorescence microscopy in this study (Fig. 3). Further studies will be required to establish the structure and functions of these elements. Second, the recent studies by Balch et al. (1994) and Pind et al. (1994) have been interpreted to mean that these so-called tubulo-vesicular clusters represent the formation of an aggregate of vesicular elements where the G protein concentrates and that these structures move en masse towards the Golgi complex. A detailed critique of these latter two papers is provided elsewhere (Griffiths et al., 1995). Moreover, we would also like to point out that in the study by Lotti et al. (1992) the density of a defined pool of G protein in the rab2-positive membrane aggregates decreased progressively with increasing time of transport. This argues strongly against the notion that these membrane structures move in concert with the cargo.

The notion that the IC is functionally continuous with the RER would also be the simplest way to explain the recent biochemical data of Hammond and Helenius (1994) who showed that, at 39.5°C, the misfolded G protein is not restricted to the RER but is also in detectable amounts in the IC. The presence in the IC of proteins such as PDI, BIP and calnexin (Krijnse Locker et al., 1994, 1995; Griffiths et al., 1994) that assist the folding of newly synthesized proteins strongly suggests that the IC, along with the RER, may play an important role in the phenomenon of protein folding referred to as quality control (Helenius et al. 1992; Helenius, 1994; Doms et al., 1993). The position of the IC at the exit of the ER would make it the ideal candidate to control that only properly folded and oligomerized proteins are free to leave for the Golgi complex, a process we argue is mediated by COP vesicles.

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