p230 is associated with vesicles budding from the *trans*-Golgi network

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SUMMARY

Transport vesicle formation requires the association of cytosolic proteins with the membrane. We have previously described a brefeldin-A sensitive, hydrophilic protein (p230), containing a very high frequency of heptad repeats, found in the cytosol and associated with Golgi membranes. We show here that p230 is localised on the *trans*-Golgi network, by immunogold labeling of HeLa cell cryosections using α2,6 sialyltransferase as a compartment-specific marker. The role of G protein activators on the binding of p230 to Golgi membranes and in vesicle biogenesis has been investigated. Treatment of streptolysin-O permeabilised HeLa cells with either GTPγS or AlF$_4^-$ resulted in accumulation of p230 on Golgi membranes. Furthermore, immunolabeling of isolated Golgi membranes treated with AlF$_4^-$, to induce the accumulation of vesicles, showed that p230 is predominantly localised to the cytoplasmic surface of *trans*-Golgi network-derived budding structures and small coated vesicles. p230-labeled vesicles have a thin (~10 nm) electron dense cytoplasmic coat and could be readily distinguished from clathrin-coated vesicles. Dual immunogold labeling of perforated cells, or of cryosections of treated Golgi membranes, revealed that p230 and the *trans*-Golgi network-associated p200, which we show here to be distinct molecules, appear to be localised on separate populations of vesicles budding from the *trans*-Golgi network. These results strongly suggest the presence of distinct populations of non-clathrin coated vesicles derived from the *trans*-Golgi network. As p230 recycles between the cytosol and buds/vesicles of TGN membranes, a process regulated by G proteins, we propose that p230 is involved in the biogenesis of a specific population of non-clathrin coated vesicles.

Key words: *trans*-Golgi network, p230, Coated vesicle, Golgi

INTRODUCTION

Newly synthesised proteins are transported sequentially from the endoplasmic reticulum (ER) to the Golgi and then to their final destination. The transport of these proteins from the ER-to-Golgi cisternae, between adjacent cisternae within the Golgi stack, and from Golgi cisternae to various destinations is mediated by vesicles. Transport vesicles bud from one compartment and then target and fuse with the next compartment, a complex process mediated by an increasing number of structural and regulatory components (Rothman and Orci, 1992; Pryer et al., 1992; Rothman, 1994; Rothman and Warren, 1994; Bennett, 1995).

Vesicle formation requires the recruitment of cytosolic coat proteins with the membrane, which is considered essential to drive the deformation of membrane during the budding process (Melçancon et al., 1991; Mallabiabarrena and Malhotra, 1995; Schekman and Orci, 1996). Three distinct classes of coat protein complexes have been identified to date. The classical clathrin-coat, and the associated adaptor complex, is involved in receptor mediated endocytosis and in transport from the *trans*-Golgi network (TGN) to endosomes (Mallabiabarrena and Malhotra, 1995). Two distinct non-clathrin coated vesicles, coatomer (COPI) and COPII coated-vesicles, are involved in

anterograde and retrograde transport between the ER and the TGN (Kreis et al., 1995; Mallabiabarrena and Malhotra, 1995; Salama and Schekman, 1995). COPs comprise of a complex set of coat subunits. In addition to a role in vesicle biogenesis, protein coats may also be important in the selective packaging of cargo into distinct transport vesicles. For example, COPI interacts with the dilysine motif of the ER retrieval signal (Letourneur et al., 1994; Pelham, 1994).

Genetic and biochemical studies have shown that both monomeric (Rab and ARF-1/sar1p families) and heterotrimeric G proteins are involved in the process of coat formation and vesicle budding (Rothman, 1994). For example, the binding of β-COP, a subunit of COPI, to membranes is regulated by the monomeric G protein, ADP-ribosylation factor (ARF), and also possibly by heterotrimeric G proteins (Donaldson et al., 1991; de Almeida et al., 1993; Rothman, 1994). Uncoating of Golgi-derived coated vesicles is blocked by treatment with the non-hydrolyzable GTP analogue, GTPγS, an activator of all GTP-binding proteins, or with aluminum fluoride (AlF$_4^-$), a selective activator of heterotrimeric G proteins (Donaldson and Klausner, 1994; Kreis et al., 1995; Robinson and Kreis, 1992). On the other hand, binding of either clathrin or COPI proteins to Golgi membranes is inhibited by the fungal metabolite brefeldin A

(Klausner et al., 1992; Robinson and Kreis, 1992), which acts by blocking a Golgi associated GDP/GTP nucleotide exchange activity required for the activation of ARF (Donaldson et al., 1992; Palmer et al., 1993).

From the TGN, proteins are sorted for constitutive transport to either endosomes or the cell surface, or to granules of the regulated secretory pathway. Clathrin coated vesicles are involved in transport to endosomes. Non-clathrin coats on the TGN have been observed (Ladinsky et al., 1994), however, the nature of their cytoplasmic proteins remain poorly defined. Significantly β-COP appears to be absent from TGN derived vesicles (Griffiths et al., 1995; Oprins et al., 1993; Pepperkok et al., 1993). A TGN-associated phosphoprotein, p200, has recently been shown to be associated with the coat structure of a distinct class of non-clathrin vesicle (Narula and Stow, 1995). In addition, the interaction of a cytosolic p62/rab6 complex with TGN38/41, an integral membrane protein predominantly localised to the TGN, has been implicated in the budding of exocytic vesicles from the TGN (Jones et al., 1993).

Using anti-Golgi autoantibodies from a patient with Sjögren's Syndrome, we have identified p230, a peripheral membrane protein localized to the cytosolic face of the *trans*-Golgi region (Kooy et al., 1992). p230 is reversibly dissociated from Golgi membranes by brefeldin A, indicating the existence of membrane and cytosolic pools of p230. We have cloned the cDNA encoding p230 (Erlich et al., 1996). p230 appears to be identical to Golgin-245 (Fritzler et al., 1995), although Golgin-245 is truncated by 145 residues at the N terminus compared with p230. The predicted protein sequence of p230 is highly hydrophilic and contains a very high frequency of heptad repeats, characteristic of α-helices that form dimeric coiled-coil structures (Erlich et al., 1996). About 85% of the p230 is predicted to form a coiled-coil structure. A number of other proteins in the secretory pathway implicated in vesicle transport are also predicted to contain a high content of coiled coil domains for example, Uso1, p115, SNAPs, rabaptin 5 and SNAREs. Dramatic conformational changes occur in coiled-coil proteins involved in membrane fusion events (Oas and Endow, 1994), and the many discontinuities in the heptad repeats of p230 raises the possibility of dynamic coiled-coil formation. Overall the characteristics of p230 suggest a molecule which may participate in the dynamic process of vesicle biogenesis. The present study was undertaken to investigate this possibility. Here we show that p230 is induced to bind to Golgi membranes by G protein activators, and is localised on coated buds and vesicles derived from the TGN. Thus, p230 is likely to be involved in the biogenesis of a population of non-clathrin coated TGN derived vesicles.

MATERIALS AND METHODS

Cell culture

HeLa and normal rat kidney (NRK) cells were maintained in exponential growth in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 0.1% (w/v) streptomycin in 10% CO_2/90% air at 37°C. Stable HeLa cells expressing α2,6 sialyltransferase (SialylT) tagged at the carboxy terminus with a VSV-G epitope (Rabouille et al., 1995) were generously supplied by Dr T. Nilsson and were grown in the above medium supplemented with 500 μg/ml G418 (Gibco BRL, Australia).

MDCK cells were maintained as described previously (de Almeida and Stow, 1991) and for experiments grown in DMEM supplemented with 10% FCS as confluent monolayers on Trans-well filters (Costar, Cambridge, MA).

Antibodies

Affinity-purified human autoantibodies to p230 were obtained from a column of recombinant glutathione-S-transferase-p230 fusion protein (Kooy et al., 1992). Polyclonal antibodies to the glutathione-S-transferase-p230 fusion protein were generated in rabbits and affinity-purified as described (Kooy et al., 1992). The AD7 mouse monoclonal antibody, which recognises the p200 protein was described by Narula et al. (1992). Rabbit polyclonal antibodies to β-COP were kindly provided by Dr R. Teasdale (Scripps Institute). The P5D4 mouse monoclonal antibody which recognises the VSV-G epitope was described by Kreis (1986). Conjugates were obtained from either Silenus Laboratories (Melbourne, Australia) or Dako Corp. (Botany, Australia).

Immunofluorescence

Cells, grown either on 12-well glass microscope slides or coverslips, were fixed in 3% paraformaldehyde for 15 minutes, free aldehyde groups quenched in 50 mM NH_4Cl/PBS, and the cells permeabilised with 0.1% Triton X-100 in PBS for 5 minutes. Monolayers were washed with PBS containing 5% FCS (PBS/FCS) for 20 minutes to reduce non-specific binding. Monolayers were then incubated in primary antibody, diluted in PBS/FCS, for 1 hour, washed in PBS, and incubated with fluorescein isothiocyanate (FITC) or tetramethyl-rhodamine isothiocyanate (TRITC)-labelled secondary antibodies for 30 minutes. After washing in PBS, slides were mounted in Mowiol and examined with an Axiophot photomicroscope (Zeiss). In some experiments monolayers were pretreated with 5 μg/ml of brefeldin A (Calbiochem, Australia) (diluted from a stock of 1 mg/ml stock in methanol) for various time periods before processing.

For dual labelling experiments, monolayers were incubated with affinity-purified human anti-p230 antibodies (diluted 1:200 in PBS/FCS) for 1 hour at room temperature, washed and then incubated with FITC-conjugated sheep anti-human immunoglobulin (F(ab′)2 fragment) (Silenus Laboratories, Australia), diluted 1:100 with PBS/FCS. After further washing monolayers were incubated with P5D4 monoclonal antibody for 1 hour at room temperature, washed and incubated with TRITC-conjugated rabbit anti-mouse immunoglobulin (absorbed) (Dako Corp., Botany, Australia), diluted 1:100 with PBS/FCS. After a final wash monolayers were mounted as above. Control incubations demonstrated no cross-reactivity between the anti-immunoglobulin conjugates or between the anti-immunoglobulin conjugates and the irrelevant primary antibodies. Monolayers were examined by confocal microscopy using a Bio-Rad MRC 1000 imaging system.

Permeabilisation experiments

HeLa cells were permeabilised with streptolysin O (SLO), obtained either from Welcome diagnostics (Darford, UK) or Dr S. Bhakdi (Univerity of Mainz, Mainz, Germany). SLO was activated in SLO buffer (25 mM Hepes-KOH, pH 7.4, 115 mM KOAc, 2.5 mM $MgCl_2$) containing 1 mM DTT on ice for 15 minutes immediately prior to use. Monolayers were washed twice with ice-cold PBS and once with ice-cold SLO buffer. SLO, titrated to ensure >95% of cells were permeabilised, was bound to the cells for 10 minutes on ice, the cells washed twice with SLO/DTT buffer and then incubated in the same buffer at 37°C for 30 minutes in the absence or presence of 50 μM GTPγS or AlF_4^- (added as 30 mM NaF and 50 μM $AlCl_3$). After washing, cells were fixed and stained by indirect immunofluorescence, as described above.

Immunoprecipitation

Semi-confluent HeLa cell monolayers were harvested with a cell scraper, washed in PBS, and extracted in 1 ml of 50 mM Tris-HCl, pH

8.0, containing 1% NP-40 10 mM EDTA and protease inhibitor cocktail (Boehringer-Mannheim) supplemented with 1 μg/ml pepstatin, for 30 minutes on ice, and the nuclei removed by centrifugation. Extracts were incubated with purified AD7 antibody (24 μg) for 4 hours with rotation at 4°C and the immune complexes collected by the addition of Protein A-Sepharose (Pharmacia Biotech, Australia). The pellets were washed with buffers as described (Kooy et al., 1992).

In some experiments cells were metabolically labeled with L-[35S]methionine/cysteine (Express [35S]protein labeling mix, Du-Pont-NEN) prior to immunoprecipitation, as described (Kooy et al., 1992).

Immunoblotting

Total cellular proteins or immunoprecipitates were dissolved in reducing SDS electrophoresis sample buffer and separated by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose and immunoblotting was carried out by the method previously described (Kooy et al., 1992) using 5% non-fat milk in PBS containing 0.05% Tween-20 as the blocking solution.

Immunoelectron microscopy

Subconfluent monolayers of HeLa cells were washed twice in PBS, harvested by treatment with 25 μg/ml proteinase K and fixed with 0.1% glutaraldehyde/4% formaldehyde in 250 mM Hepes, pH 7.3, processed for cryosectioning, and sectioned as described (Griffiths, 1993). Thawed sections were labeled with affinity-purified human or rabbit anti-p230 antibody (7.5 μg/ml) followed by Protein A-gold (Dept Cell Biology, Utrecht School of Medicine, Utrecht), as described (Griffiths, 1993). Antibodies and conjugates were diluted in 5% FSC or 1% BSA in PBS. For double labeling of p230 and SialylT, a sequential method was employed; after the first antibody-Protein A-gold pair sections were blocked by treatment with 1% glutaraldehyde fixation step followed by PBS/glycine to quench free aldehyde groups.

In some experiments, Golgi membranes (2.9 mg protein) and cytosol (5.2 mg protein), prepared from rat liver by the method described by Jones et al. (1993), were incubated with AlF_4^- (added as 30 mM NaF + 50 μM $AlCl_3$) at 37°C for 30 minutes to induce budding of Golgi vesicles. Golgi membranes were recovered by centrifugation at 100,000 *g* for 1 hour at 4°C, washed in PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C, overnight. The pellet was washed in PBS, set in 10% gelatin and frozen in liquid nitrogen. Cryosections were double labeled for p230 and p200 by the procedure described by Narula and Stow (1995).

Labeled cryosections were embedded in methylcellulose/uranyl acetate and viewed in either a Zeiss or JEOL transmission electron microscope.

MDCK cell monolayers on filters were perforated by the 'filter stripping' method described by Wandinger-Ness et al. (1990). Perforated cells were incubated with cytosol and AlF_4^-, followed by fixation in 4% paraformaldehyde. Fixed cells were incubated sequentially with primary antibodies and Protein A-gold, and then processed and embedded in Spurr's resin for sectioning.

RESULTS

p230 is localised to the membranes of the TGN

Our previous studies indicated that p230 was localised to the *trans*-Golgi region of human HeLa cells but precise localisation in the stack was not revealed. To more accurately define p230 localisation, we have carried out immunolabeling studies using a HeLa cell transfected with VSV-G epitope tagged human α2,6 sialyltransferase (SialylT) (Rabouille et al., 1995). Immunofluorescence confocal microscopy showed p230
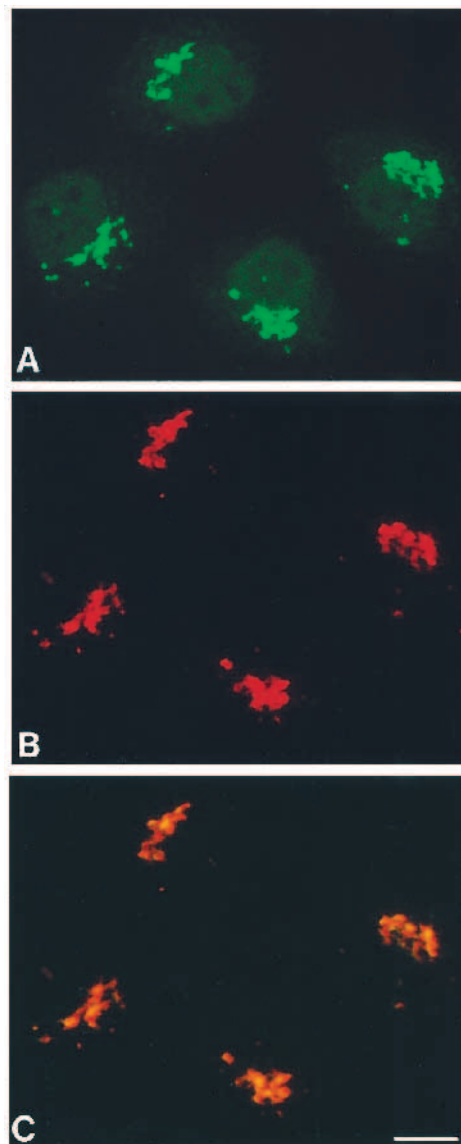

Fig. 1. Co-localisation of SialylT and p230 in SialylT transfected HeLa cells using confocal immunofluorescence microscopy. VSV-G epitope-tagged SialylT transfected HeLa cells were fixed and permeabilised and incubated with affinity-purified human anti-p230 antibody (A) and mouse monoclonal antibody P5D4 to VSV-G epitope (B), as decribed in Materials and Methods. Bound antibody was detected with FITC-labeled anti-human Ig (F(ab′)2 fragment and TRITC-labeled rabbit anti-mouse Ig. (C) Superimposed images of A and B reveals regions of SialylT and p230 co-localisation. Note the lack of green fluorescence emission in C, indicating p230 is included within the region of SialylT localisation. Control incubations demonstrated no cross-reactivity between the anti-immunoglobulin conjugates or between the anti-immunoglobulin conjugates and the irrelevant primary antibodies. Bar, 10 μm.

localised to the Golgi region of permeabilised, SialylT transfected HeLa cells (Fig. 1A). Dual labeling of p230 and SialylT was carried out using affinity-purified human anti-p230 antibodies and monoclonal antibody P5D4, specific for the VSV-G epitope. p230 co-localised extensively with SialylT (Fig. 1B and C), indicating that p230 may be localised specifically to the TGN.
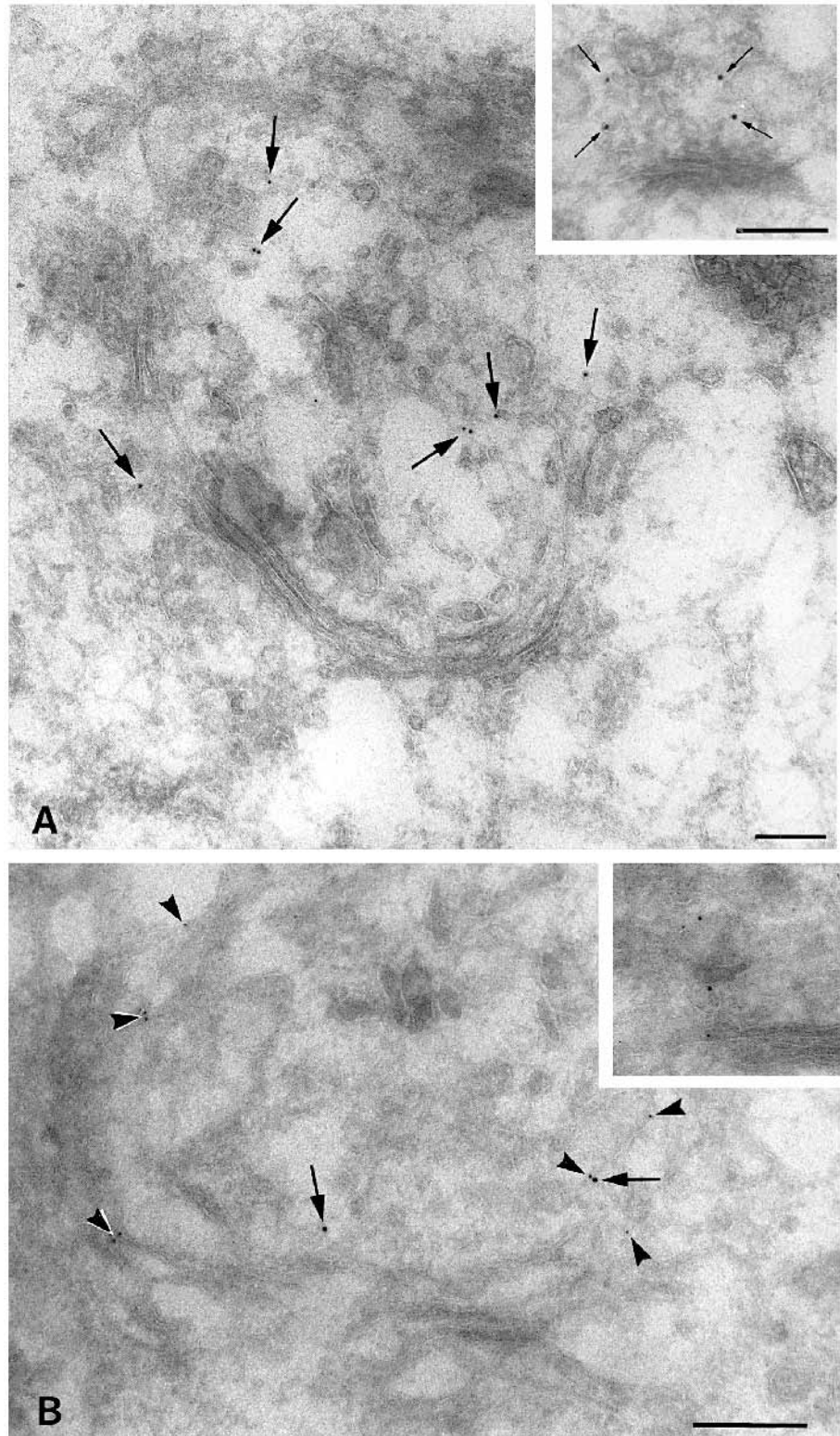
Fig. 2. Immunoelectron microsopic localisation of p230 in SialylT-HeLa cells. (A) Cryosections of SialylT transfected HeLa cells were labeled with either human and/or rabbit (inset) affinity-purified antibodies to p230 (10 nm gold; arrows). Note that the gold particles are located on membranes extending from one side of the Golgi stack. (B) Double labeling of cryosections for p230 (10 nm gold; arrows) and the TGN marker, SialylT (5 nm gold; arrowheads). p230 is localised to the same membrane network as SialylT. Inset (same magnification as main field) shows additional example of co-localisation of p230 and SialyT. Bars, 200 nm.

For more definitive localisation, immunogold labeling of ultrathin cryosections of SialylT transfected HeLa cells was performed. Gold labeling of p230 was found predominantly associated with membranes of the Golgi apparatus. Significantly, labeling for p230 was observed only on membranes on one side of the Golgi stack (Fig. 2A). Occasionally gold particles were found associated with membrane budding structures in this region (not shown). No labeling was found associated with the centre stack of cisternae, nor with other organelles. To ascertain if the p230 labeled membranes were the TGN, frozen sections of SialylT transfected HeLa cells were labeled for both p230 and SialylT. Gold labeling for

p230 and SialylT was found on the same face of the Golgi apparatus and often colocalised to the same membranes extending from the Golgi stack (Fig. 2B). In addition, SialylT labeling, but not p230 labeling, was also detected on the *trans*-cisternae, consistent with the localisation of endogenous SialylT to the TGN and *trans*-cisternae of the Golgi stack (Roth et al., 1985). These results extend our earlier studies and demonstrate that the membrane-bound form of p230 is localised to the TGN.

p230 is unrelated to p200

p230 has strikingly similar properties to the molecule p200 described by Stow and co-workers (de Almeida et al., 1993; Narula et al., 1992); like p230, p200 is a cytoplasmic protein which binds dynamically to Golgi membranes and is dissociated in the presence of brefeldin A. In addition, p200 is also associated with membranes of the TGN. To investigate the relationship between the two molecules we have carried out comparative immunoblotting and immunoprecipitation experiments of HeLa cell extracts using affinity-purified anti-p230 antibodies and a monoclonal antibody (AD7) to p200 protein. These experiments were performed using HeLa cells as both antibodies show strong reactivity of the human antigens by immunoblotting and immunoprecipitation. In addition to the component of 200 kDa, AD7 also detects a higher molecular mass (~300 kDa) form of p200 (Narula et al., 1992); the ratio of the 200 kDa component to the ~300 kDa component varies between cell types (J. L. Stow, unpublished data). As expected from earlier studies (Narula et al., 1992) the major component detected by AD7 antibody on immunoblots of NRK cells is 200 kDa (Fig. 3A); in contrast immunoblotting of HeLa cell extracts with AD7 monoclonal antibody detected a major component of ~300 kDa (Fig. 3A) and in some experiments a minor component of 200 kDa was also observed. Similar size components were obtained by immunoprecipitation of metabolically labelled NRK and HeLa cell extracts with the AD7 antibody (not shown). The basis for the different molecular mass forms of the AD7 immunoreactive species is not known; nonetheless, anti-p230 antibodies reacted with a component from HeLa cell extracts which was clearly of a different size to the component recognised by AD7 (Fig. 3A).

To determine if there was any cross-reactivity of anti-p230 antibodies with p200, unlabelled HeLa cell extracts were immunoprecipitated with the AD7 antibody and the immunoprecipitate analysed by immunoblotting (Fig. 3B). As expected, AD7 reacted with the ~300 kDa component of the immunoprecipitate, however, no reactivity was observed with the anti-p230 antibodies. In the converse experiment where unlabelled HeLa cell extracts were immunoprecipitated with the anti-p230 antibodies, only p230 was detected by immunoblotting (not shown). Thus, p230 and p200 do not appear to be antigenically related.

Previously, we showed that p230 is dissociated from HeLa Golgi membranes in the presence of brefeldin A (Kooy et al., 1992). As the effect of brefeldin A on Golgi-localised p200 in HeLa cells has not been previously examined, we compared the dissociation rate of p230 and p200 from Golgi membranes in the presence of this drug (Fig. 4). Immunofluorescence staining for p230 and p200 was carried out on HeLa cells treated with brefeldin A for various times. p200
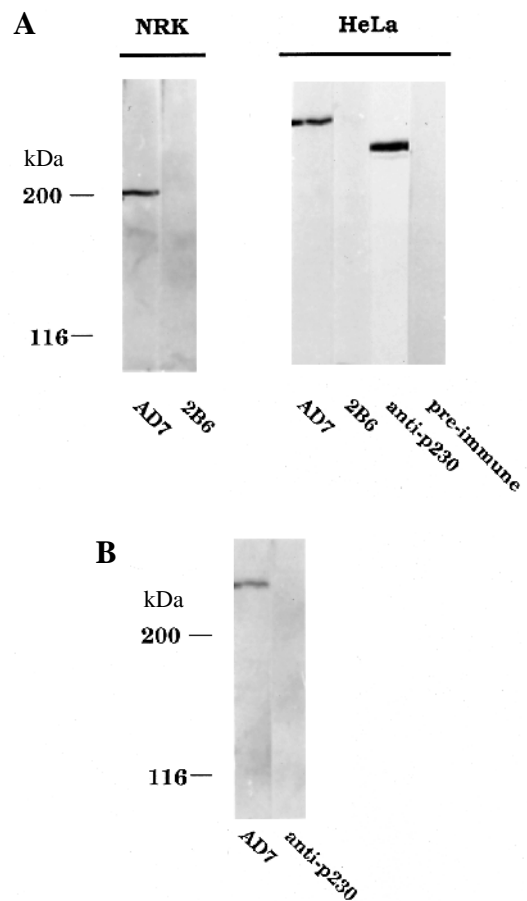

Fig. 3. Immunoblotting analysis of cell extracts and unlabeled immunoprecipitations. (A) NRK or HeLa total cell proteins, as indicated, were separated on a 5% polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked and immunblotted with anti-p200 monoclonal antibody AD7, an irrelevant monoclonal antibody 2B6, affinity-purified rabbit anti-p230 antibodies or preimmune rabbit serum, as indicated, using a chemiluminescence detection system. (B) HeLa cells were extracted with 50 mM Tris-HCl/1% NP-40 and extracts immunoprecititated with AD7 antibody and Protein A-Sepharose. The immunoprecipitate was dissolved in SDS reducing buffer, proteins separated on a 5% polyacrylamide gel, as above, and immunoblotted with AD7 and affinity-purified rabbit anti-p230 antibodies, as indicated.

rapidly dissociates from Golgi membranes of HeLa cells in the presence of brefeldin A; by 2 minutes treatment there was a major redistribution of p200 with the disappearance of perinuclear staining and the appearance of cytoplasmic staining, consistent with previous findings reported for p200 in NRK cells (Narula et al., 1992). The rapid rate of dissociation of p200 is similar to the rate of dissociation of β-COP from Golgi membranes in HeLa cells (not shown). In contrast, there was no effect on the perinuclear localisation of p230 after a 5 minute brefeldin A treatment; by 15 minutes treatment, however, there was a reduction in perinuclear staining of p230 and by 60 minutes the typical perinuclear staining pattern had disappeared and only scant vesicle staining remained, consistent with our previous observations
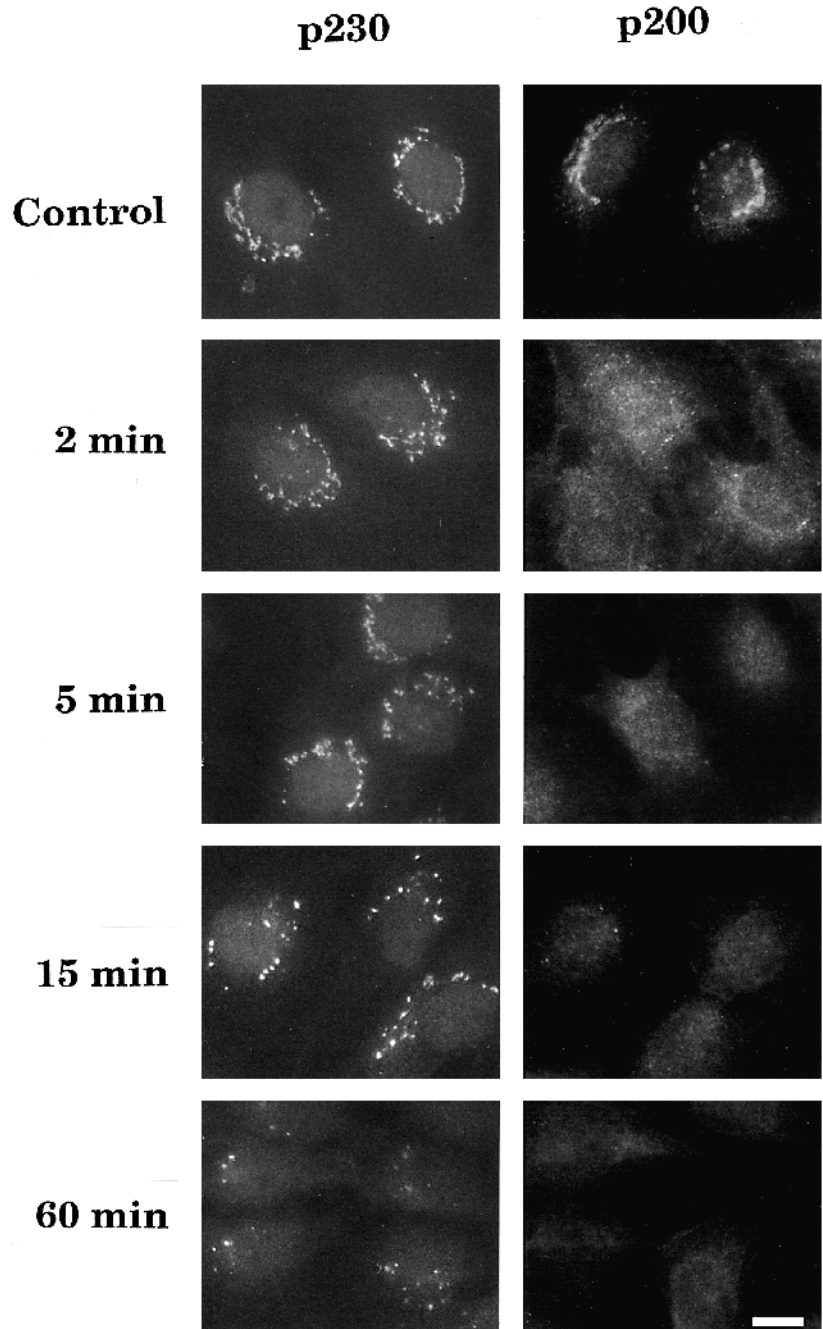
p230   p200

Control

2 min

5 min

15 min

60 min

Fig. 4. Comparison of the rate of dissociation of p230 and p200 from Golgi membranes upon brefeldin A treatment of HeLa cells. HeLa cells were either untreated (control) or incubated for 2, 5, 15 or 60 minutes, as indicated, with 5 µg/ml brefeldin A at 37°C. Cells were fixed with paraformaldehyde, permeabilised with 0.1% Triton X-100, and stained by indirect immunofluorescence using affinity-purified human anti-p230 antibodies (p230) or AD7 monoclonal antibody (p200), as indicated. Bar, 10 µm.

(Kooy et al., 1992). The relatively slow rate of dissociation of p230 from Golgi membranes clearly distinguishes p230 from p200.

Activation of G proteins induces p230 binding to Golgi membranes

The distribution of p230 was examined in HeLa cells treated with GTPγS or AlF_4^- to activate G proteins. GTPγS and AlF_4^- are known to stabilise the membrane association of cytosolic proteins, such as β-COP, ARF, γ-adaptin and p200, and to block vesicle transport from the TGN.

The bacterial toxin streptolysin O (SLO) was used to introduce pores selectively in the plasma membrane. HeLa cells were treated with SLO at 4°C to permeabilise the cell surface membrane, washed, and then incubated in the presence or absence of GTPγS or AlF_4^- for 30 minutes at 37°C. A 30 minute incubation in the absence of G protein activators resulted in very little p230 remaining in the Golgi region (Fig. 5); presumably p230 had dissociated from the membrane during the incubation at 37°C. In contrast, incubation of SLO treated HeLa cells in the presence of GTPγS resulted in intense staining of p230 with affinity-purified anti-p230 antibodies. Similar results were observed on treatment with AlF_4^- (Fig. 5). In contrast, treatment of SLO permeabilised cells with $AlCl_3$ did not show any of the AlF_4^- effects (not shown), demonstrating specificity of AlF_4^- action and indicating a possible

Fig. 5. Effect of GTPγS and AlF₄⁻ on Golgi localisation of p230 in SLO-permeablised HeLa cells. HeLa cells were incubated with SLO at 4°C, unbound SLO removed, and cells permeabilised by incubation at 37°C for 30 minutes in SLO buffer alone (SLO) or in the presence of SLO buffer containing GTPγS (SLO/+GTPγS) or AlF₄⁻ (SLO/+AlFn). Cells were then fixed, permeabilised, and stained for p230 by indirect immunofluorescence as described in Fig. 4. Bar, 10 μm.

involvement of heterotrimeric G proteins in the association of p230 with TGN membranes.

p230 is localised on a distinct population of TGN-derived vesicles after activation of G proteins

To determine if p230 was associated with budding vesicles, immunogold localisation of p230 was carried out on cryosections of isolated rat liver Golgi membranes incubated in vitro with cytosol in the presence of AlF₄⁻ to accumulate budding structures. p230 labeling was found extensively on tubulovesicular structures and small vesicles (~70 nm diameter) associated with Golgi membranes (Fig. 6A-D, arrows). The labeled vesicles had an electron dense coat of about 10 nm thick, readily distinguishable from vesicles with a thicker (>20 nm), clathrin-like coat, which were also found in close proximity (Fig. 6). Occasionally p230 labeling was associated with clathrin-like coated vesicles. p230 labeling was associated with the cytoplasmic surface of the membranes and vesicles, consistent with the expected orientation of p230.

p200 has previously been shown to be associated with a population of coated vesicles which bud from TGN membranes (Narula and Stow, 1995). Cryosections of AlF₄⁻-treated Golgi preparations were double labelled with p230 and p200 antibodies to compare the distribution of both proteins on Golgi derived vesicles (Fig. 6). Vesicles were labeled for either p230 or p200, but rarely both, therefore, p230 and p200 appear to be localised on separate populations of vesicles.

p230 and p200 are also localised on different populations of Golgi-derived vesicles in MDCK cells (Fig. 7). In perforated and AlF₄⁻-treated MDCK cells incubated with the p230 antibody alone, the densely p230 labeled vesicles appeared as discrete clusters, often surrounded by unlabeled vesicles (see Fig. 7A and C). Interestingly some clusters of p230 labeled vesicles also included distinctly tubular elements (Fig. 7D). Dual labeling of perforated cells suggests p230 and p200 labeling on separate clusters of vesicles associated with Golgi stacks (Fig. 7B and E). These data provide further evidence that p230 and p200 decorated vesicles may represent distinct populations.

DISCUSSION

In this study we have investigated whether the peripheral Golgi membrane protein p230 is associated with vesicular transport. Here we have provided compelling evidence for a role of p230 in vesicle biogenesis as: (1) p230 is specifically localised on TGN membranes, denoted by the presence of the resident enzyme SialylT; (2) the binding of p230 to TGN membranes is regulated by monomeric and/or heterotrimeric G proteins; and (3) immunolabelling experiments of isolated membranes
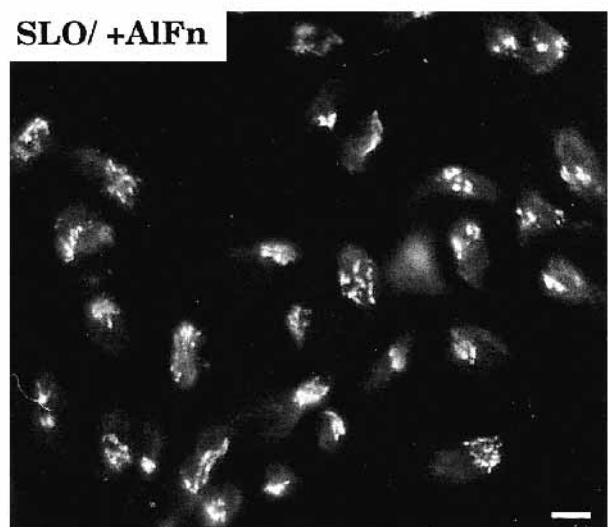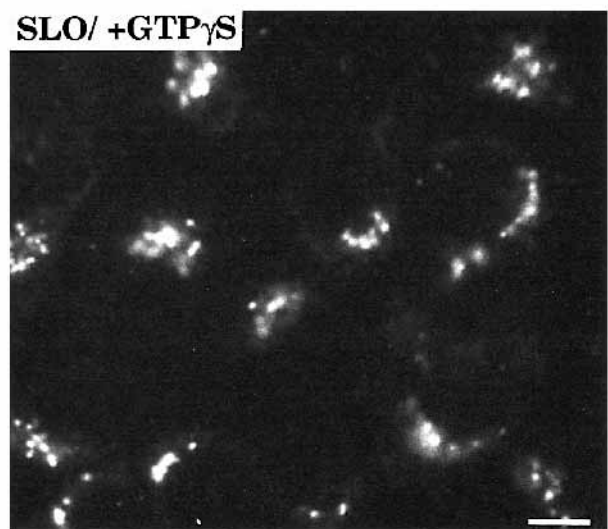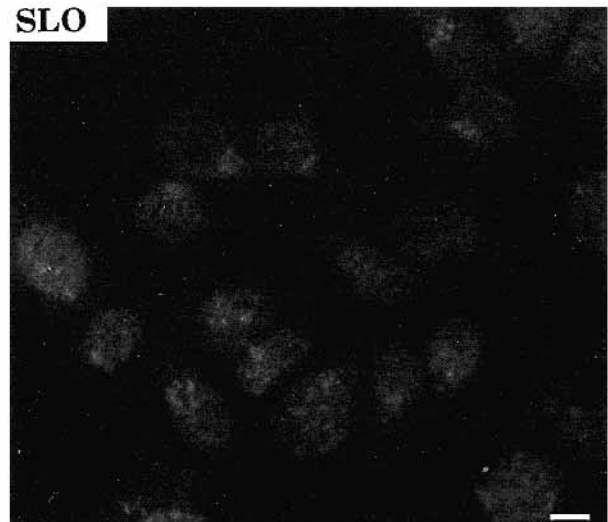
after activation of G proteins demonstrated that p230 is specifically recruited on budding structures and non-clathrin coated vesicles derived from the TGN. Together with our earlier observation that p230 is dissociated from membranes in the
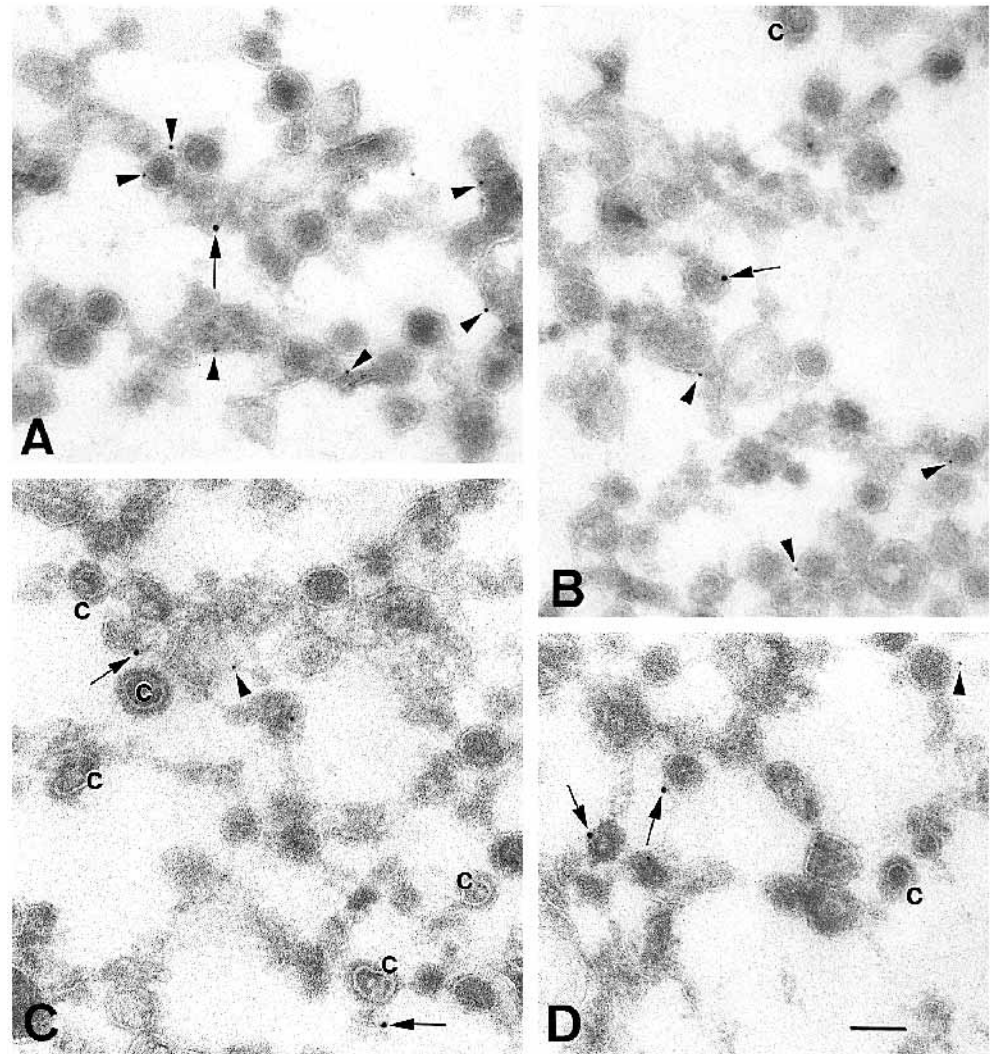
Fig. 6. Immunogold localisation of p230 on vesicles budding from the TGN. Cryosections of isolated Golgi membranes, incubated with AlF$_4^-$ to intiate vesicle budding, were dual labeled with affinity-purified human antibodies to p230 (10 nm gold; arrows) and monoclonal antibody to p200 (5 nm gold; arrowheads). (A-D) Clusters of vesicles with individual vesicles labeled for p230 or p200. p200 was observed on vesicles or tubulovesicular structures (A). An electron dense coat can be seen on p230-labeled vesicles which is distinct from the thicker (>20 nm) coat of clathrin-coated vesicles (c) (in B,C,D). Bar, 100 nm.

presence of brefeldin A (Kooy et al., 1992), these data collectively indicate that p230 recycles between the cytosol and buds/vesicles of TGN membranes and is associated with a specific population of TGN derived transport vesicles.

p230 has similar characteristics to another peripheral membrane protein also localised on TGN membranes, namely p200 (de Almeida et al., 1993; Narula et al., 1992; Narula and Stow, 1995). p200 is also associated with budding structures from the TGN and has also been implicated in vesicle formation. However, the results presented here clearly demonstrate non-identity of p230 and p200 as: (1) comparative immunoblotting and immunoprecipitation experiments demonstrated a distinct difference in size between the two components; (2) immunoprecipitation followed by immunoblotting did not reveal any cross-reactivity of anti-p230 and anti-p200; (3) the kinetics of brefeldin A-induced dissociation of p230 from Golgi membranes of HeLa cells differs significantly to that of p200; (4) dual labelling studies with antibodies to p200 and p230 have revealed that budding structures labeled for p230 are not labeled for p200, and vice versa, indicating these two molecules are decorating distinct populations of vesicles; and finally (5) the putative partial nucleotide sequence of p200 (B. Burke, K. S. Matlin and J. L. Stow, unpublished data) is

unrelated to the cDNA sequence p230 (Erlich et al., 1996). Therefore, p230 is a novel cytoplasmic protein which is associated with the formation of transport vesicles from the TGN.

The association of p230 with Golgi membranes is regulated by G proteins. The immunofluorescence data clearly show that p230 accumulates on Golgi membranes of SLO permeablised cells in the presence of either GTPγS or AlF$_4^-$, whereas in the absence of these G protein activators there is no detectable membrane bound p230 after a 30 minute incubation at 37°C. The loss of Golgi staining for p230 in the absence of G protein activators is likely to reflect the movement of p230 from membranes to cytosol and subsequent leakage of soluble p230 from the SLO permeablised cells. GTPγS is an activator for both heterotrimeric and monomeric G proteins whereas AlF$_4^-$ is an activator of heterotrimeric G proteins but not the monomeric G proteins (Kahn, 1991). Therefore, the effect of AlF$_4^-$ on membrane binding of p230 indicates that heterotrimeric G proteins are involved in the recuitment of this cytoplasmic protein to the TGN membranes.

Heterotrimeric G proteins have been implicated in various stages of the endomembrane system, including the regulation of vesicular transport from the late Golgi or TGN (Stow et al., 1991; Leyte et al., 1992; Ohashi and Huttner, 1994; Pimplikar

Fig. 7. p230 and p200 decorate separate clusters of vesicles budding from the TGN in perforated MDCK cells. MDCK cells were perforated, as described in Materials and Methods, and treated with cytosol and AlF₄⁻ to accumulate Golgi vesicles, fixed, and labeled with antibodies to p230 (10 nm gold) alone (A,C,D) or in combination with antibody to p200 (5 nm gold) (B,E). (A) A Golgi stack surrounded by accumulated vesicles shows p230 labeling along the membrane of the TGN and on a cluster of vesicles apposed to the TGN (arrows). p230 labeled vesicles appear as discrete clusters, often surrounded by unlabeled vesicles (see A and C). Some clusters of p230 labeled vesicles also include distinctly tubular elements (see D). (B,E) Double labeling of Golgi stacks shows clusters of vesicles labeled separately for either p230 (arrows) or p200 (arrowheads). B shows distinct clusters of p230 and p200 labeled vesicles budding off the TGN. Some areas (see E) also show individual vesicles labeled for either p230 or p200 budding off adjacent sites on the Golgi. Bar, 200 nm.
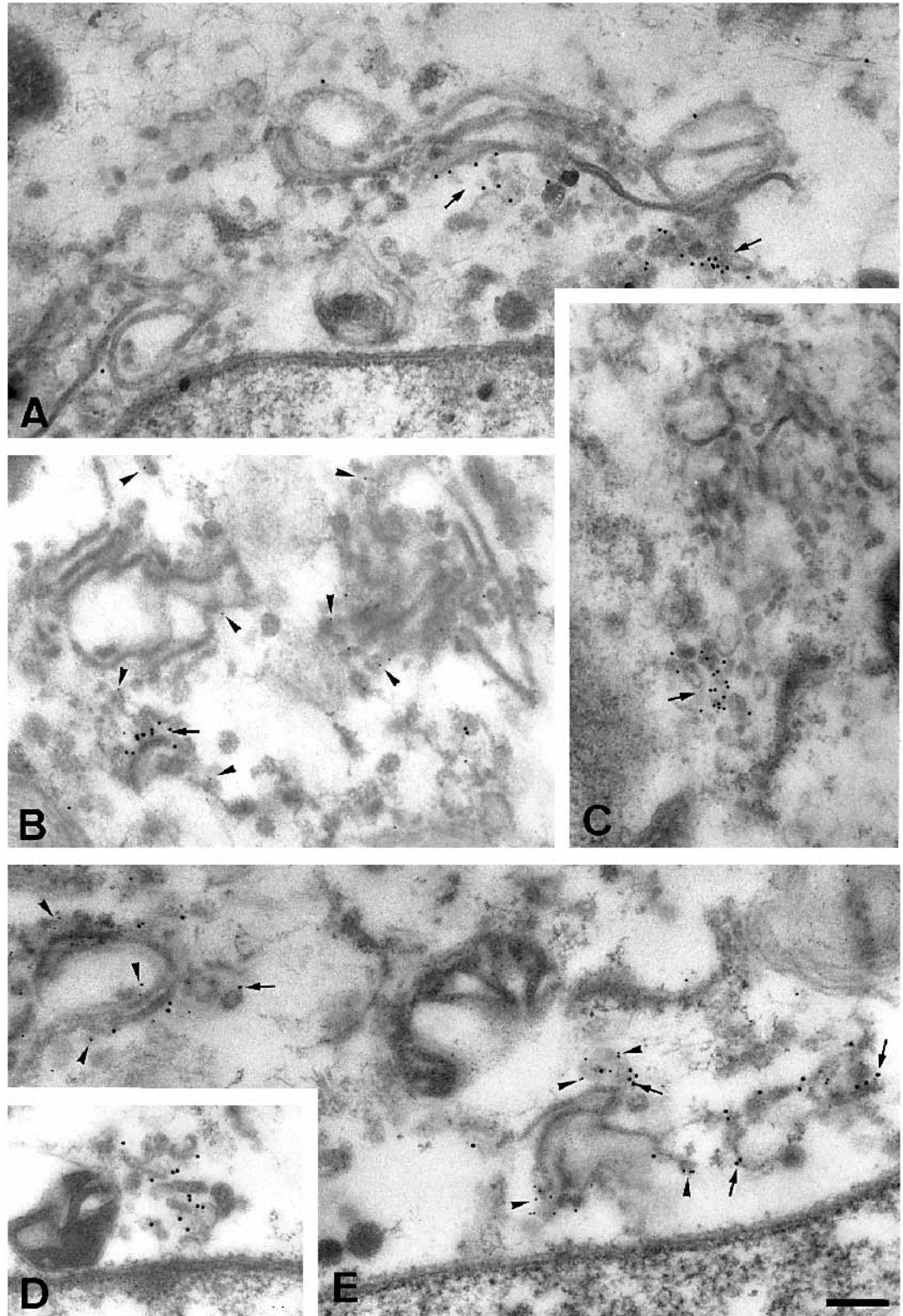
and Simons, 1993). Membrane recruitment of the brefeldin A-sensitive, small GTP-binding protein, ARF, and a number of vesicle coat associated proteins (for example β-COP and γ-adaptin) are also regulated by heterotrimeric G proteins (Donaldson et al., 1991; Robinson and Kreis, 1992). As pre-

treatment of cells with AlF₄⁻ prevents brefeldin A-induced redistribution of β-COP and γ-adaptin from Golgi membranes, heterotrimeric G proteins probably function upstream from monomeric G proteins (Donaldson et al., 1991; Robinson and Kreis, 1992). The sensitivity of p230 to not only AlF₄⁻ but also

brefeldin A, which is known to act on ARF, suggests that both heterotrimeric and monomeric G proteins are involved in p230 membrane association, or that there are additional, as yet undefined, targets of brefeldin A.

Significantly, p230 was found to be predominantly localised to budding structures and coated vesicles of AlF₄⁻-treated Golgi preparations, suggesting that p230 is involved in the dynamic process of vesicle biogenesis. The p230 labeled vesicles have a relatively thin (~10 nm) electron dense cytoplasmic coat which distinguishes them from the thicker (>20 nm) clathrin coated vesicles. p230-labeled vesicles appear to be distinct from p200-labeled vesicles, although both vesicle populations have a cytoplasmic coat of similar thickness. p200-labeled vesicles are devoid of β-COP (Narula and Stow, 1995). It is likely that p230-labeled vesicles also lack coatomer, as firstly, coatomer has been shown to be involved predominantly in ER-to-Golgi transport and intra-Golgi transport (Kreis et al., 1995; Salama and Schekman, 1995) and, secondly, although β-COP has been found in the TGN, there is no evidence that β-COP is associated with buds or vesicles derived from the TGN (Griffiths et al., 1995). Importantly, the differential labeling of vesicles with p230 or p200 indicates that there are a number of distinct populations of vesicles, with electron dense coats of similar thickness, arising from the TGN. Distinct populations of secretory vesicles budding from the TGN have been identified (Saucan and Palade, 1994; Wandinger-Ness et al., 1990). However, at this stage it is unclear how many different populations of carrier vesicles are involved in trafficking membrane and secretory proteins from the TGN. Clearly, it will be important to further characterise the nature of the coats on the p230- and p200 labeled vesicles and to define their cargoes and destinations.

What is the role of p230 in vesicle formation? The cycling of p230 between the cytoplasm and the membranes of budding vesicles, a process regulated by G proteins, is consistent with a role for p230 in the assembly of a vesicle coat structure. One possibility is that p230 represents a novel coat protein of TGN derived vesicles. Indeed, membrane coats distinct from clathrin have been identified by high-voltage EM which bud from tubules continuous with TGN membranes (Ladinsky et al., 1994), and it is possible p230 may form part of the coat on these vesicles. However, the relatively slow dissociation of p230 from Golgi membranes in the presence of brefeldin A contrasts with the rapid dissociation (<2 minutes) of other proteins, such as β-COP (Robinson and Kreis, 1992). This difference in behaviour may suggest that p230 is not a typical coat protein. Alternatively, p230 may not be an integral component of the coat per se but may be involved in other facets of vesicle biogenesis. For example, p230 may play a role in the structural organisation of TGN membranes into long tubules from which vesicles appear to bud (Ladinsky et al., 1994). Another possibility is that p230 may be involved in the sorting of cargo into a specific vesicle population. The extended coiled-coil structure of p230 provides the possibility of dynamic interactions with other components of the machinery for vesicle formation, and this is currently under investigation.

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