

## The Golgi-targeting sequence of the peripheral membrane protein p230

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### SUMMARY

Vesicle transport requires the recruitment of cytosolic proteins to specific membrane compartments. We have previously characterised a brefeldin A-sensitive trans-Golgi network-localised protein (p230) that is associated with a population of non-clathrin-coated vesicles. p230 recycles between the cytosol and the cytoplasmic face of buds/vesicles of trans-Golgi network membranes in a G protein-regulated manner. Identifying the mechanism responsible for Golgi targeting of p230 is important for the elucidation of its function. By transfection of COS cells with deletion mutants of p230 we here demonstrate that the C-terminal domain is necessary for targeting to the Golgi. Furthermore, the C-terminal 98 amino acid domain of p230 attached to the green fluorescent protein (GFP-p230-C98aa) was efficiently Golgi-localised in transfected COS cells. Deletion mutants of GFP-p230-C98aa together with

alanine scanning mutagenesis identified a minimum stretch of 42 amino acids that is essential for Golgi targeting, suggesting that the conformation of the domain is critical for efficient targeting. In COS cells expressing high levels of GFP-p230-C98aa fusion protein, endogenous p230 was no longer associated with Golgi membranes, suggesting that the GFP fusion protein and endogenous p230 may compete for the same membrane target structures. The Golgi binding of GFP-p230-C98aa is brefeldin A-sensitive and is regulated by G proteins. These studies have identified a minimal sequence responsible for specific targeting of p230 to the Golgi apparatus, which displays similar membrane binding characteristics to wild-type p230.

Key words: Trans-Golgi network, Golgi localisation, p230

### INTRODUCTION

Transport through the secretory pathway is mediated by vesicles shuttling between donor and recipient membrane compartments (Rothman, 1994). Vesicle formation requires the association of cytosolic proteins with the membrane, including coat proteins and small GTPases together with their associated activators and effectors. A number of different vesicle coats have been defined, such as the classical lattice-like clathrin coat and two distinct non-clathrin coats, namely COPI and COPII coats, involved in the transport of cargo between the ER and Golgi (Salama and Schekman, 1995; Schekman and Orci, 1996).

The trans-Golgi network (TGN) represents a key sorting compartment of the secretory pathway. From the TGN, proteins are sorted for constitutive transport to the late endosomes or the cell surface, or for granules of the regulated secretory pathway. In polarised cells, two distinct vesicle populations are formed in the TGN to transport proteins selectively to the apical or the basolateral membrane (Wandinger-Ness et al., 1990). Recent data indicate that fibroblasts also have at least two distinct pathways from the TGN to the cell surface (Müsch et al., 1996; Yoshimori et al., 1996). In addition, there are almost certainly retrograde transport pathways from the TGN (Lord and Roberts, 1998). Distinct populations of transport vesicles are required for each pathway, so the biogenesis of vesicles at this site is highly

complex. Clathrin-coated vesicles are involved in the transport step to endosomes; however, the nature of the coats on transport vesicles targeted elsewhere, for example the cell surface, is poorly defined. A TGN-associated myosin II (p200) is associated with the budding of a subpopulation of transport vesicles (Stow et al., 1998). A number of other proteins, including the GTP binding proteins rab6 (Echard et al., 1988) and dynamin II (Jones et al., 1998), and the recycling protein TGN38/41 (Jones et al., 1993) have been implicated in vesicle transport from the TGN. Further, lipid kinases and phosphoinositides are also considered important in the recruitment and/or activation of proteins essential for vesicle transport from the late Golgi (De Camillo et al., 1996).

We have previously identified a 230 kDa peripheral membrane protein (p230) specifically associated with vesicles budding from the TGN (Kooy et al., 1992; Gleeson et al., 1996). p230 recycles between the cytosol and the buds/vesicles of TGN membranes under the regulation of G proteins. For example, binding is stabilised by GTP $\gamma$ S and AIF4- and is disrupted by brefeldin A. Immunolabelling 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 (Gleeson et al., 1996). The predicted p230 protein is highly hydrophilic, consistent with a peripheral membrane protein, and contains a very high frequency of heptad repeats (85% of the polypeptide), characteristic of  $\alpha$ -helices that form dimeric

coiled-coil structures (Erlich et al., 1996). 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.

The location of p230 specifically to TGN membranes indicates the presence of a specific targeting sequence (Gleeson, 1998). In order to define the mechanism of action of p230, we have identified the sequence of p230 responsible for targeting to Golgi membranes. The studies reported here have identified a minimal 42-amino-acid sequence responsible for specific targeting of p230 to the Golgi apparatus, which displays membrane binding characteristics similar to wild-type p230.

## MATERIALS AND METHODS

### Cell culture

Cell lines were maintained in exponential growth as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, non-essential amino acids (ICN Laboratories),  $5 \times 10^{-5}$  M 2-mercaptoethanol, 100 units/ml penicillin and 0.1% (w/v) streptomycin (complete DMEM). HeLa cells expressing a tetracycline-controlled transactivator (Gossen and Bujard, 1992) were maintained in 0.8 mg/ml G418 (GibcoBRL, Australia).

### Antibodies

Autoimmune sera were obtained from Gribbles Pathology (Melbourne, Australia). Polyclonal antibodies to the glutathione-S-transferase-p230 fusion protein were generated in rabbits and affinity-purified as described (Kooy et al., 1992). The 9E10 mouse monoclonal antibody specific for the myc epitope has been described (Evan et al., 1985). The AD7 mouse monoclonal antibody, which recognises the p200 protein, was described by Narula et al. (1992). Rabbit polyclonal antibodies to  $\beta$ -COP were kindly provided by Dr R. Teasdale (Monash University). Monoclonal antibody 2B6, used as an isotype control, is specific for the gastric H/K ATPase  $\beta$ -subunit (Mori et al., 1989). Conjugates were obtained from either Silenus Laboratories (Melbourne, Australia) or Dako Corp. (Botany, Australia).

### cDNA constructs

Full-length p230 cDNA was generated from the three overlapping clones isolated previously (Erlich et al., 1996), namely px1,  $\lambda$ z9 and  $\lambda$ z16, in pSVT7 (pSVT7-p230). To include a c-myc epitope on the N terminus of p230, pSVT7-p230 was used as template to amplify a 940 bp product by PCR using the sense primer 5'-TCCCCGCGGACC-ATGGAACAGAACTGATCTCTGAAGAAGACCTGCCGTTCAA-GAAACTGAAGCAA-3', which contains a favourable translational initiation codon followed by the coding sequence for the c-myc epitope (underlined), and the anti-sense primer 5'-CCTGGGTAGTCA-TCTGTT-3'. The resulting product was digested with *SacII* and *SpeI* and exchanged for the *SacII/SpeI* fragment of pSVT7-p230 (pSVT7-myc-p230).

A construct lacking the C-terminal 98 amino acids of p230 was generated using pSVT7-p230 as template to amplify by PCR the p230 nucleotide sequence 6020-6676 and introduce a stop codon at position 6677-6679 using the primers 5'-TTGGTCCAACCCAAATTG-3' and 5'-GGGTTATCTGAACCTTGCTCTTT-3' (stop codon underlined). The 662 bp PCR-product was blunt-end ligated into the *EcoRV*-digested pBluescript KS+, and the *BglIII/SalI* fragment from this construct exchanged with the *BglIII/SalI* fragment of pSVT7-myc-p230.

A construct lacking the N-terminal 129 amino acids of p230 was

generated as follows: px1 clone (Erlich et al., 1996) was used as a template to amplify by PCR the p230 nucleotide sequence 676-1478 (Erlich et al., 1996) and introduce a favourable start codon at position 673-675, using the primers 5'-CCGCTCGAGATGTCAGAC-AGTCTCAACAAA-3' (favourable initiation codon underlined) and 5'-CCTGGGTAGTCATCTGTT-3'. The resulting 814 bp PCR-fragment was filled in with the Klenow fragment of DNA polymerase I and dNTPs and cloned into the *EcoRV* site of pBluescript KS+. After *XbaI/BamHI* digestion, Klenow treatment to generate blunt ends, and religation (thus eliminating the *SpeI* site in the multiple cloning site of pBluescript), the 554 bp *SacII/SpeI* fragment of this construct was subcloned into *SacII/SpeI*-digested pSVT7-p230.

For GFP-p230 gene constructions, pSVT7-p230 was used as a template in a PCR amplification of the p230 nucleotide sequence 6676-7035 (Erlich et al., 1996) with the primers 5'-CCGGAATTCTGA-ACAGATTCACAATTA-3' and 5'-CCGGAATTCTCAAGATGAAG-ATCGGAG-3', which incorporate *EcoRI* sites. The resulting 316 bp fragment was digested with *EcoRI* and cloned into the *EcoRI* site of the green fluorescent protein (GFP) vector, pEGFP-C1 (Clontech, Palo Alto, CA, USA). The resulting hybrid cDNA encodes the 98-amino-acid C terminus of p230 fused with the C terminus of GFP (GFP-p230-C98aa). Further constructs of the C-terminal domain of p230 fused with GFP were generated by PCR amplification of pSVT7-p230 as above using the following primers. Sense primers 5'-CCGGAATTCTTT-GACCATACGGATGTC-3' (C31-98aa), 5'-CCGGAATTCTGGAG-AACCTACCGAATTT-3' (C40-98aa), 5'-CCGGAATTCTGAATTTG-AGTATTTGCGA-3' (C44-98aa), 5'-CCGGAATTCTTATTTGCGAA-AAGTGCTT-3' (C47-98aa), 5'-CCGGAATTCTGTGCTTTTTGAG-TATATG-3' (C51-98aa), 5'-CCGGAATTCTAAGACCATGGGAAAA-GTT-3' (C62-98aa) together with the antisense primer 5'-CCGGAATTCTCAAGATGAAGATCGGAG-3'. For constructs C31-88aa, C31-83aa and C31-78aa, the sense primer 5'-CCG-GAATTTTGTACCATACGGATGTC-3' was used with the anti-sense primers 5'-CCGGAATTCTCAAGCATCTTCTTTT-CACC-3', 5'-CCGGAATTCTCACAAAATTTTCTGAGTCTG-3', 5'-CCGGA-ATTCTCACTGATCATCAGGGAACTT-3', respectively. For constructs C1-67aa and C1-36aa the sense primer 5'-CCGGAATTCT-GAACAGATTCACAATTA-3' was used with the antisense primers 5'-CCGGAATTCTCAAACCTTTTGGCCATGGTC-TT-3' and 5'-CCG-GAATTCTCAGACATCCGTATGGTACAA-3', respectively. For construct C44-88aa the sense primer 5'-CCGGAATTCTGAATTTGAG-TATTTGCGA-3' and antisense primer 5'-CCGGAATTCTCAAG-CATCTTCTTTTACC-3' were used. All GFP-p230 hybrid genes was verified by sequencing using the primer GFP-1, 5'-CGACAAC-CACTACCTGAGC-3', which is complimentary to GFP 137 bp upstream of the *EcoRI* cloning site.

To construct a recombinant gene encoding a GFP-p230 fusion protein containing the alternatively spliced C terminus of p230, the primers 5'-CCGGAATTCTGAACAGATTCACAATTA-3' and 5'-CCGGAATTCTCAAGATGAAGATCGGAG-3' were used to amplify by PCR a 379 bp fragment spanning base pairs 6676-7035 from clone  $\lambda$ z7, which contains the alternatively spliced C-terminal sequence FTSPRSIGF instead of SWLRSSS (Erlich et al., 1996).

### Site-directed mutagenesis

Mutagenesis of the GFP-p230-C98aa construct involving serial 4- or 6-alanine substitutions between residues 41-98 of the C-terminal domain of p230 was carried out using inverse PCR mutagenesis (Clackson et al., 1991). For example, to introduce a 6-alanine substitution block at residues 41-46 of the C-terminal domain of p230, the primers 5'-CGTATGGTACAAATTGCC-3' and 5'-GATGTCTC-ACTCTTTGGAGCAGCTGCAGCAGCTGCATATTTGCGAAAAA-GTCTT-3' (alanine substitution underlined) were used to prime the GFP-p230-C98aa template. The resulting PCR-amplified product was filled in with the Klenow fragment and self-ligated. All constructs with alanine substitutions were verified by sequencing with the GFP-1 primer.

To construct GFP and GFP-p230 genes under the control of a tetracycline-responsive promoter, the 0.8 kb and 1.1 kb coding regions of the GFP and GFP-p230-C98aa cDNAs were excised from the pEGFP-C1 plasmids using the restriction enzymes *NheI/XbaI* and subcloned into the *XbaI* site of the vector pUHD 10-3 (Gossen and Bujard, 1992).

### Transfections

COS cells were transiently transfected using FuGene 6 transfection reagent (Boehringer Mannheim, USA) according to the manufacturer's instructions. Immunofluorescence of transfected cells was performed 48 hours after transfection.

HeLa cells expressing a tetracycline-controlled transactivator (Gossen and Bujard, 1992) were stably transfected with pUHD 10-3 constructs in the presence of 100 ng/ml doxycycline (Sigma), using the calcium phosphate precipitate method (Wigler et al., 1979). HeLa cells were co-transfected with pREP7 (Invitrogen), which contains the hygromycin resistance gene, and transfectants selected in the presence of 250 µg/ml hygromycin (Boehringer Mannheim). Stable transfectants were cloned by limiting dilution and expression of the constructs was induced by withdrawal of doxycycline.

### Immunofluorescence

Cells, grown either on 12-well glass microscope slides or coverslips, were fixed in 4% paraformaldehyde for 15 minutes, free aldehyde groups quenched in 50 mM NH<sub>4</sub>Cl/PBS, and the cells permeabilised with 0.1% Triton X-100 in PBS for 4 minutes. Alternatively, cells were fixed in methanol at -20°C for 5 minutes. Monolayers were incubated 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 tetramethylrhodamine isothiocyanate (TRITC) or CY3-labelled secondary antibodies for 30 minutes. In some experiments monolayers were pretreated with 5 µg/ml of brefeldin A (Calbiochem, Australia) (diluted from 1 mg/ml stock in methanol) for various time periods before processing. After washing in PBS, monolayers were mounted in mowiol and examined by confocal microscopy using a Bio-Rad MRC-1024 imaging system.

### Immunoblotting

Immunoblotting was carried out as previously described (Gleeson et al., 1996).

### Immunoprecipitation

COS cells, transiently transfected with both myc-p230 and GFP constructs, and stable HeLa cell clones expressing GFP constructs, were labelled for 4 hours with 0.25 mCi [L-<sup>35</sup>S]methionine/cysteine (TRAN<sup>35</sup>S-LABEL™, ICN) as described (Kooy et al., 1992). Cells were extracted for 1 hour on ice in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate and Complete™ Protease Inhibitors (Boehringer Mannheim) (lysis buffer). Lysates were incubated with 2 µg anti-GFP monoclonal antibody (Boehringer Mannheim), 2 µg 9E10 monoclonal antibody or 2 µg 2B6 monoclonal antibody, a control antibody specific for the gastric H/K ATPase β-subunit (Mori et al., 1989), for 1 hour at 4°C with gentle rotation. 50 µl of 20% suspension of Protein G-Sepharose (Pharmacia) was added to 500 µl lysate-antibody mixture and incubated at 4°C, with rotation for 30 minutes. The Protein G-Sepharose beads were pelleted at 5,000 rpm (2,000 g) for 20 seconds and washed firstly in 1 ml lysis buffer and then in 1 ml 50 mM Tris-HCl, pH 7.5, containing 0.25 M NaCl, 0.1% NP-40 and 0.05% deoxycholate. Pellets were spun for a further 20 seconds at 14,000 rpm (16,000 g) to remove last traces of supernatant and resuspended in reducing sample buffer and analysed by SDS-PAGE. Gels were treated with Amplify (Amersham) dried and fluorographed at -70°C using Fuji X-ray film.

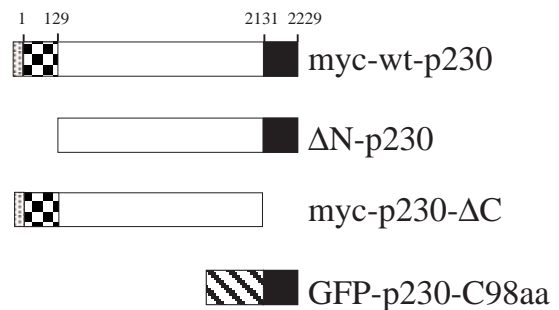
### Permeabilisation experiments

HeLa cells were permeabilised with streptolysin O (SLO), obtained either from Wellcome diagnostics (Darford, UK) or from Dr S. Bhakdi (University of Mainz, Mainz, Germany). SLO was activated in SLO buffer (25 mM Hepes-KOH, pH 7.4, 115 mM potassium acetate, 2.5 mM MgCl<sub>2</sub>) 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. After washing, cells were fixed and stained by indirect immunofluorescence, as described above.

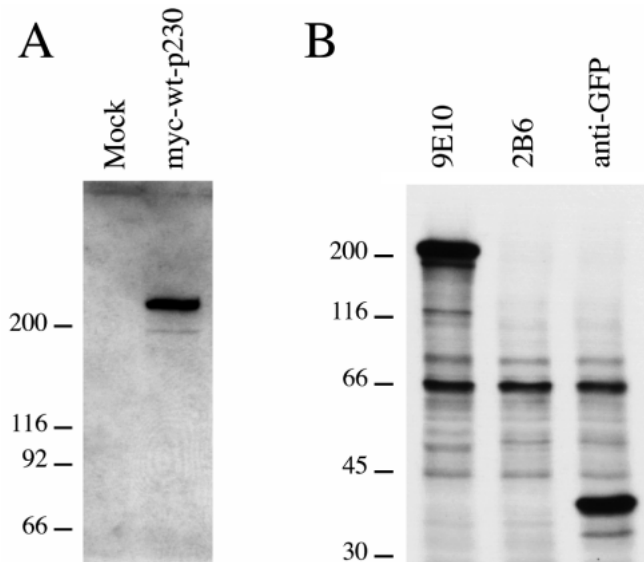
## RESULTS

### The C-terminal domain of p230 is required for Golgi targeting

p230 is a peripheral membrane protein that is localised on the cytoplasmic side of TGN membranes, as well as a subpopulation of non-clathrin-coated vesicles budding from the TGN (Gleeson et al., 1996). As p230 recycles between the cytosol and the membranes of the TGN, there must be a mechanism to target this peripheral membrane protein specifically to TGN membranes. The majority (approx. 85%) of p230 is predicted to form an α-helical coil-coiled structure, notable exceptions being the 120- and 80-amino acid amino and carboxy termini (Erlich et al., 1996) (Fig. 1). To examine the role of these three domains in Golgi targeting, a full-length p230 cDNA was generated from partial cDNA clones, and sequences encoding the myc epitope were fused at the 5' end of the open reading frame (Fig. 1). Expression of this construct in transfected COS cells resulted in a product of approximately 230 kDa, as detected by immunoblotting with the myc-specific monoclonal antibody 9E10 (Fig. 2A), confirming the synthesis of a full-length p230. Furthermore, the myc-tagged p230 was localised to the Golgi region of transfected COS cells (Fig. 3), therefore, the epitope-tagged construct is correctly localised. At very high levels of expression myc-p230 was also detected in the cytoplasm, indicating that recruitment of p230 to Golgi membranes can be saturated (not shown). N- and C-terminal



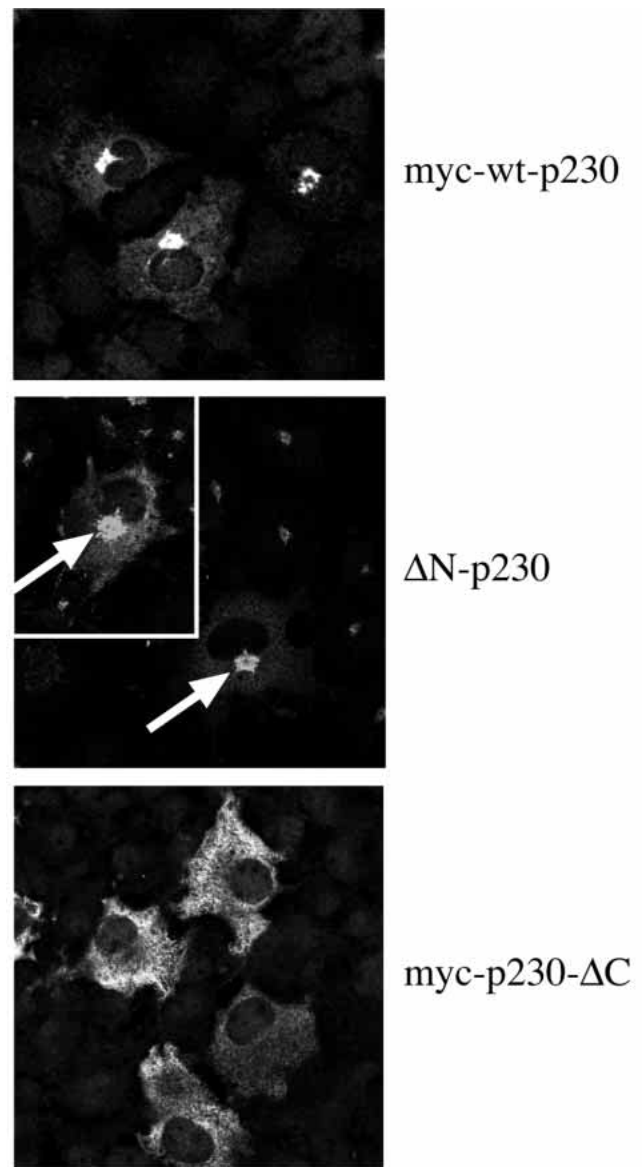
**Fig. 1.** Structure of p230 mutants and GFP fusion protein. Full-length p230 contains an extensive coiled-coil domain (open rectangle) and non-coiled-coil domains at the N terminus (checked) and C terminus (solid). An N-terminal c-myc epitope in wild-type and C-terminal deletion constructs is indicated (stippled). The C-terminal domain of p230 (solid) was expressed fused to GFP (hatched).



**Fig. 2.** Immunoblotting and immunoprecipitation of p230 products from transfected cells. Total cell lysates were prepared from COS cells 48 hours after transfection with pSVT7-myc-p230 (myc-wt-p230) or mock transfected (Mock) and separated on a reducing SDS/5% polyacrylamide gel. After transfer to nitrocellulose, the membranes were blocked and immunoblotted with 9E10 monoclonal antibody using a chemiluminescence detection system as described in Materials and Methods. (B) COS cells were double transfected with expression vectors encoding myc-p230 and GFP-p230-C98aa and metabolically labeled with [<sup>35</sup>S]methionine/cysteine for 4 hours. Labeled cell extracts were immunoprecipitated with either monoclonal antibody 9E10, mouse monoclonal antibodies to GFP, or with the irrelevant monoclonal antibody 2B6, as described in Materials and methods. Immunoprecipitates were dissolved in SDS reducing buffer, proteins separated on a 7.5% polyacrylamide gel and the resulting fluorograph from a 20-hour exposure is shown. The positions of molecular mass markers are shown.

deletion mutants of p230 were constructed as illustrated in Fig. 1. The localisation of the deletion mutants was examined in transfected COS cells using 9E10 monoclonal antibody for the C-terminal deletion and rabbit anti-p230 antibodies for the N-terminal deletion. Although the anti-p230 antibodies recognise both human and simian p230, transfected COS cells expressing the p230 N-terminal deletion mutant could be readily distinguished from non-transfected cells by the dramatically higher level of p230 product in transfected cells (Fig. 3). The N-terminal p230 deletion mutant was localised to the Golgi region of transfected COS cells, indicating that the Golgi-localization motif of p230 does not reside in the N terminus. On the other hand, the C-terminal p230 deletion mutant accumulated in the cytoplasm of transfected COS cells and there was no apparent immunofluorescence associated with Golgi membranes (Fig. 3). Thus deletion of the C-terminal 98 amino acids of p230 abrogates localisation of p230 to the Golgi.

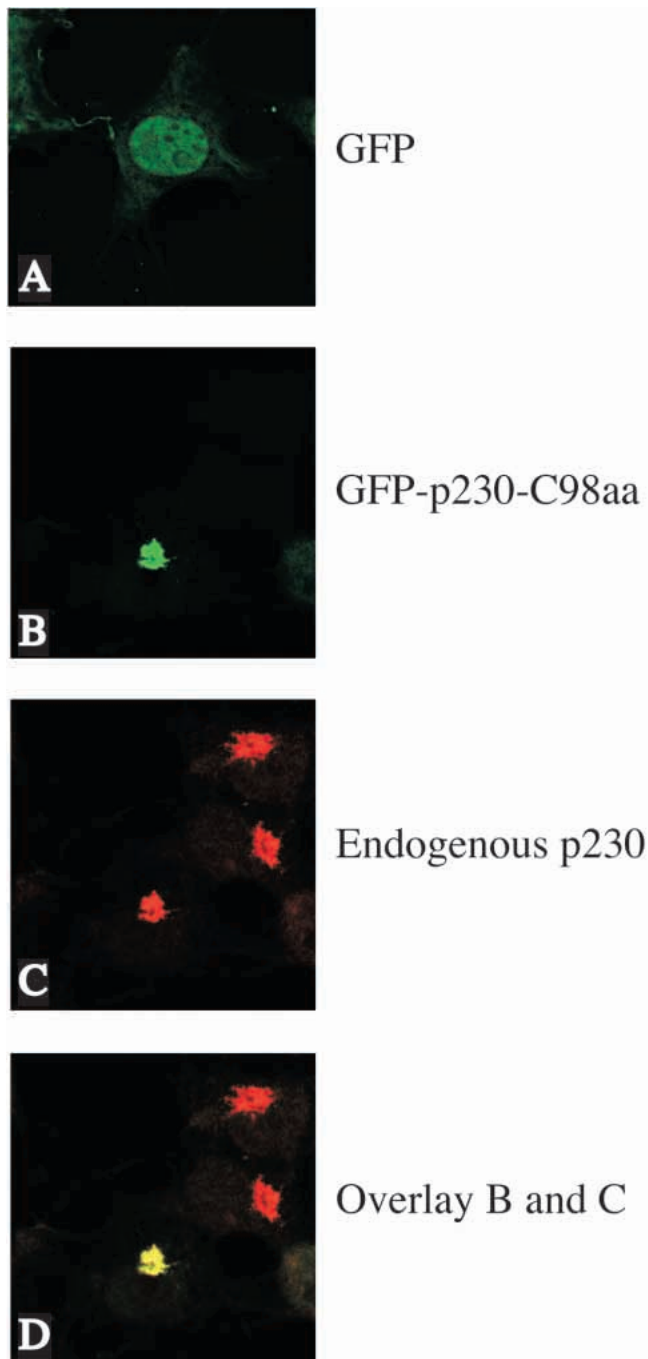
To determine if the C-terminal 98 residues of p230 alone were sufficient for targeting of p230 to Golgi membranes, the p230 C-terminal domain was fused at the carboxy terminus of the green fluorescent protein (GFP) (GFP-p230-C98aa) (Fig. 1). Wild-type GFP was distributed in the cytoplasm and nucleus of transfected COS cells (Fig. 4A), consistent with previous



**Fig. 3.** Deletion of C-terminal domain of p230 abolishes Golgi targeting. COS cells were transfected with pSVT7 constructs encoding myc-wt-p230,  $\Delta$ N-p230 and myc-p230- $\Delta$ C, as indicated, and 48 hours after transfection the cells were fixed and permeabilised and incubated either with 9E10 monoclonal antibody followed by FITC-labeled anti-mouse Ig (myc-wt-p230 and myc-p230- $\Delta$ C) or with affinity-purified rabbit anti-p230 fusion protein antibody followed by CY3-conjugated anti-rabbit Ig ( $\Delta$ N-p230). Confocal immunofluorescence images are shown. The arrows in  $\Delta$ N-p230 indicate staining of transfected COS cells; the neighbouring untransfected COS cells display low levels of endogenous p230.

findings (Liu et al., 1997). In contrast the GFP-p230-C98aa fusion protein was efficiently localised to the perinuclear region of transfected COS cells (Fig. 4B). Endogenous p230 was detected in the GFP expressing cells with a human anti-p230 autoimmune serum; this serum does not recognise the C-terminal domain of p230. Staining of transfected COS cells expressing a low level of GFP-p230-C98aa with the anti-p230 antibodies revealed that GFP-p230-C98aa colocalised with





**Fig. 4.** GFP fusion protein containing the C-terminal 98 amino acids of p230 is efficiently Golgi localised. Confocal immunofluorescence images of wild-type GFP (A) or GFP-p230-C98aa (B-D) transfected COS cells. Cells were fixed 48 hours after transfection, permeabilised and the GFP-p230-C98aa transfected COS cells stained for endogenous p230 using human anti-p230 antibody followed by TRITC-conjugated anti-human Ig. B and C show the same field. Superimposed images of B and C (D) reveal the colocalisation of GFP-p230-C98 and p230 (yellow).

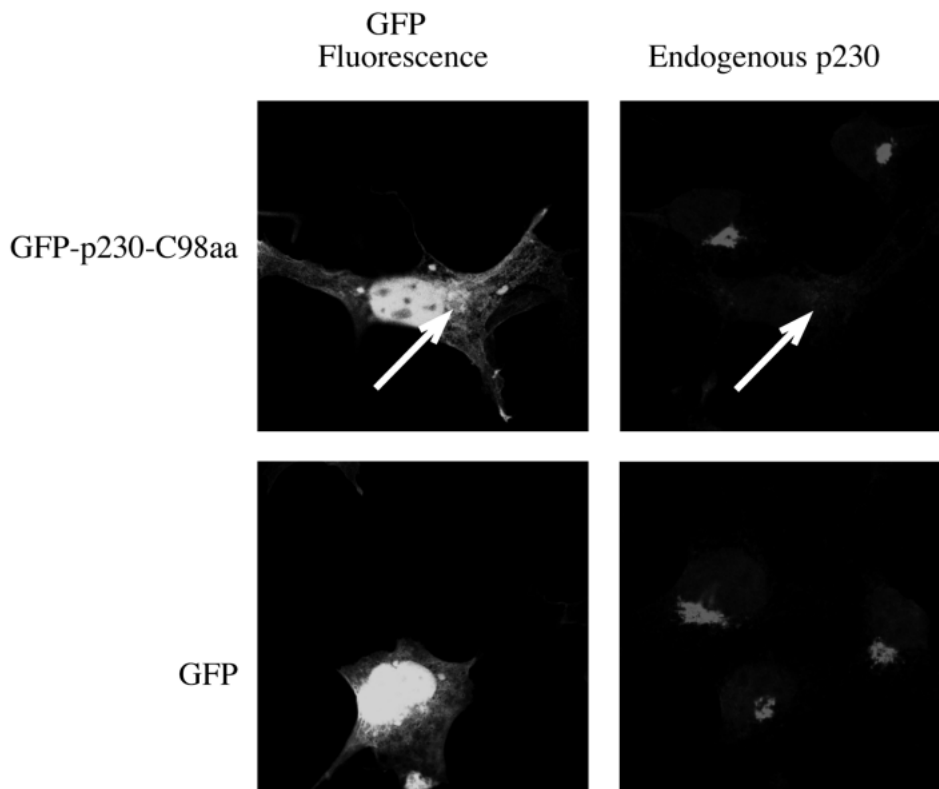
endogenous p230 (Fig. 4B-D). Identical results were also obtained using a rabbit anti-p230 fusion protein antibody, which recognises epitopes within residues 1260-1490 of p230 (Kooy et al., 1992; Erlich et al., 1996).

In COS cells expressing very high levels of GFP-p230-C98aa, the fusion protein was associated not only with the Golgi region but also detected throughout the cytoplasm and the nucleus (Fig. 5). Thus the targeting of GFP-p230-C98aa to the Golgi appears to be saturable. In these high GFP-p230-C98aa-expressing cells, very little of the endogenous p230 was detected in the Golgi region but it was weakly detected throughout the cytoplasm (Fig. 5). From a number of transfection experiments, it appeared that the level of endogenous p230 remaining in the Golgi region was inversely proportional to the level of the GFP fusion protein, suggesting that the GFP fusion protein may prevent endogenous p230 binding to TGN membranes. In cells expressing high levels of GFP-p230-C98aa the localisation of the Golgi markers  $\beta$ -COP (Duden et al., 1991) and p200 (Narula et al., 1992) were similar to untransfected COS cells, indicating that the Golgi structure was intact in these transfected cells (not shown). In contrast, endogenous p230 was not displaced from the Golgi in COS cells expressing equivalent levels of wild-type GFP (as judged by fluorescence intensity; Fig. 5).

To determine if GFP-p230-C98aa can form oligomers with full-length p230, COS cells were transfected with expression vectors encoding both myc-p230 and GFP-p230-C98aa, metabolically labeled and cell extracts immunoprecipitated with antibodies to either the myc epitope or to GFP (Fig. 2B). No association between myc-p230 and GFP-p230-C98aa was detected. Monoclonal antibody 9E10 immunoprecipitated a specific product of 230 kDa as expected, but did not coprecipitate a 40 kDa component, the size of the GFP fusion protein, and vice versa, antibodies to GFP precipitated the 40 kDa GFP fusion protein but did not coprecipitate myc-p230. The additional bands present in the immunoprecipitate are non-specific as they were also observed with the 2B6 control antibody (Fig. 2B). In addition, immunoprecipitations of cell extracts of stable HeLa cells expressing GFP-p230-C98aa with anti-GFP antibodies did not result in the coprecipitation of a 230 kDa component (not shown), again indicating that the GFP-p230 fusion protein does not form complexes with endogenous p230.

#### Identification of a 42-amino-acid sequence necessary and sufficient for Golgi targeting

In order to identify the minimal sequence within the C terminus of p230 that was capable of localising to the Golgi, progressive deletions from the termini of the 98-amino-acid C terminus of p230 were engineered as fusion proteins with GFP (Figs 6A, 7). Progressive deletions from the N terminus of the 98-amino-acid sequence of p230 (Fig. 6A) established that removal of 43 residues (C44-98aa) resulted in a construct that was targeted to the Golgi (Fig. 7), whereas removal of 46 residues (C47-98aa) (Fig. 7) or additional sequences (C51-98aa, C62-98aa) resulted in loss of Golgi localisation. C-terminal deletions were constructed using the C31-98aa construct, since the C31-98aa fusion protein was as efficiently Golgi-localised as the p230-C98aa fusion protein (Figs 6A, 7). Deletion of 10 C-terminal residues from C31-98aa (C31-88aa) had only a marginal effect on Golgi localisation (Figs 6A, 7); however, deletion of 15 or 20 C-terminal residues (C31-83aa, C31-78aa) abrogated Golgi targeting of the GFP fusion protein (Fig. 6A). Deletion of additional C-terminal residues and extending the N terminus (C1-67aa, C1-36aa) did not restore Golgi targeting.

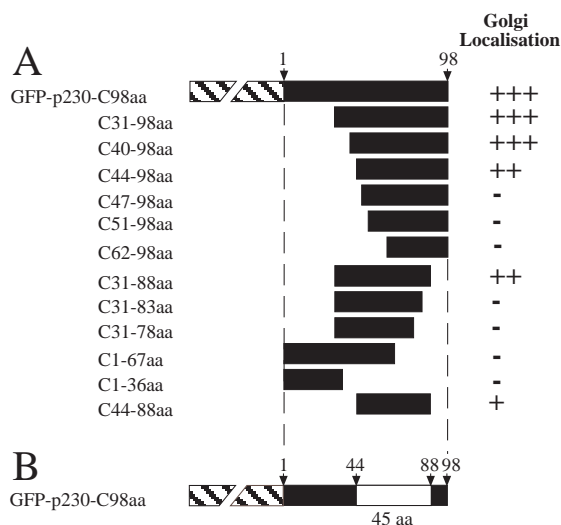


**Fig. 5.** High-level expression of GFP-p230-C98aa fusion protein results in loss of endogenous p230 from Golgi membranes. Confocal immunofluorescence images of transfected COS cells expressing high levels of wild-type GFP or GFP-p230-C98aa. Cells were fixed 48 hours after transfection, permeabilised and stained for endogenous p230 using human anti-p230 antibody followed by TRITC-conjugated anti-human Ig. The same fields are shown for GFP fluorescence and endogenous p230 staining. The arrows in the upper panels indicate the Golgi region of a GFP-p230-C98aa positive cell.

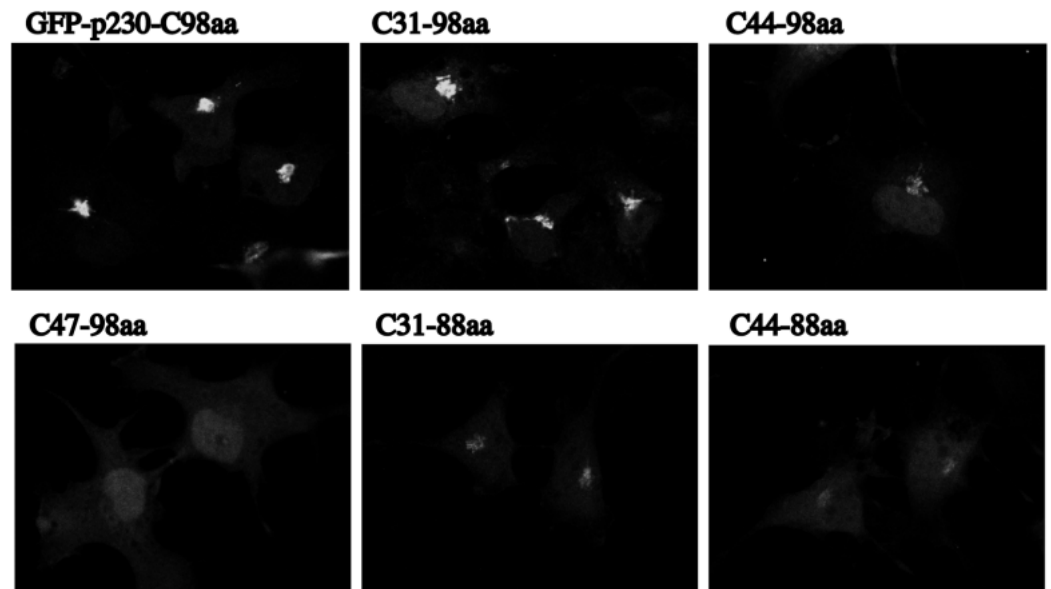
From the results obtained with the GFP-p230-C98aa deletion constructs it is apparent that GFP fusion proteins with either 43 residues deleted from the N terminus or 10 residues deleted from the C terminus of p230-C98aa were capable of being targeted to the Golgi apparatus. These results suggest that a minimum stretch of 45 amino acids is required for Golgi targeting. To directly examine the targeting of the 45-amino-acid domain, a GFP fusion protein was constructed (C44-88aa). C44-88aa was localised to the Golgi of transfected COS cells, albeit less efficiently than either of the fusion proteins C44-98aa or C31-88aa (Figs 6A, 7). Therefore a minimal 45 residue sequence was identified within the 98-amino-acid C terminus of p230 (Fig. 6B), which displayed Golgi targeting; however, it appears that deletion of amino acids flanking the 'minimal sequence' decreases the efficiency of Golgi-targeting.

It is possible that the 45-amino-acid minimum targeting sequence of p230 is required to ensure correct conformation of this C-terminal domain. If so then there may be a short Golgi-targeting signal located within the 45 residue sequence. To further dissect the Golgi targeting sequence, alanine scanning mutagenesis was performed. GFP-p230-C98aa was used to generate the alanine mutations as Golgi localisation of the 98-amino-acid domain was considerably more efficient than for the minimum 45-amino-acid sequence. Blocks of 6-alanine substitutions were engineered across the targeting sequence, as indicated in Fig. 8A. Except for the first three residues of the 45-amino-acid sequence (construct A41-46), which could be substituted without abolishing Golgi localisation, abrogation of Golgi targeting (constructs A47-52 to A77-82), or extremely weak Golgi localisation (construct A83-88) was observed with all other alanine mutants within the 45-amino-acid targeting sequence of GFP-p230-C98aa. On the other hand, GFP-p230-

C98aa fusion proteins with the C-terminal 10 residues substituted with alanines (constructs A89-94 and A95-98) were targeted to the Golgi, consistent with the findings of the



**Fig. 6.** Identification of a 45-amino-acid sequence of p230 sufficient for Golgi localisation. (A) Golgi localisation of deletion mutants of GFP-p230-C98 in transfected COS cells. GFP-p230-C98 constructs were generated with progressive deletions at either the N or C terminus of the 98-amino-acid C-terminal domain of p230, as indicated. 48 hours after transfection, cells were fixed in paraformaldehyde and viewed directly by confocal immunofluorescence. The degree of Golgi localisation of each construct was compared with GFP-p230-C98aa. (B) The location of the minimal 45-amino-acid sequence (open rectangle) within the 98-amino-acid domain of p230 sufficient for targeting a GFP fusion protein to the Golgi.



**Fig. 7.** Localisation of GFP-p230 constructs in transfected COS cells. COS cells were transfected with either GFP-p230-C98aa or deletion mutants of the 98-amino-acid C-terminal domain of p230 as indicated (see Fig. 6A for details of constructs), and 48 hours after transfection cells were fixed and confocal fluorescence images collected.

deletion mutants C31-88aa and C44-88aa (Fig. 6A). The combined deletion and alanine-substitution studies have thus identified a region of 42 residues necessary and sufficient for Golgi-localisation of p230.

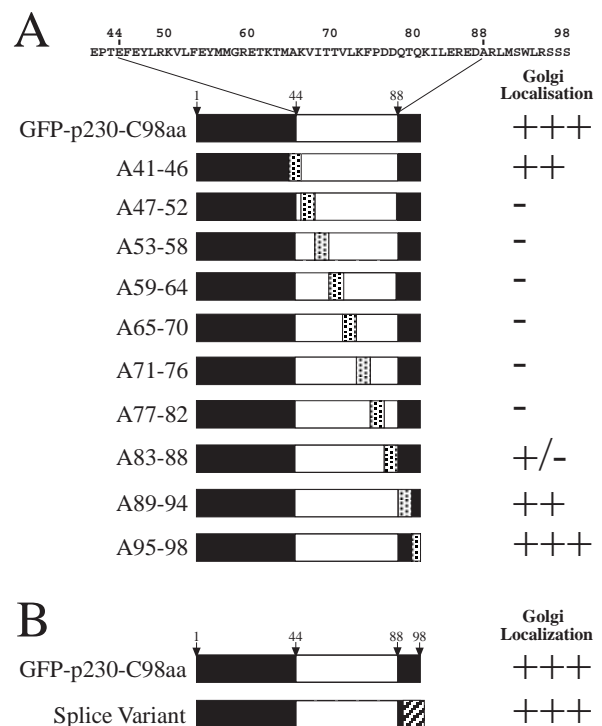
#### A COOH-terminal splice variant of p230 localises to the Golgi

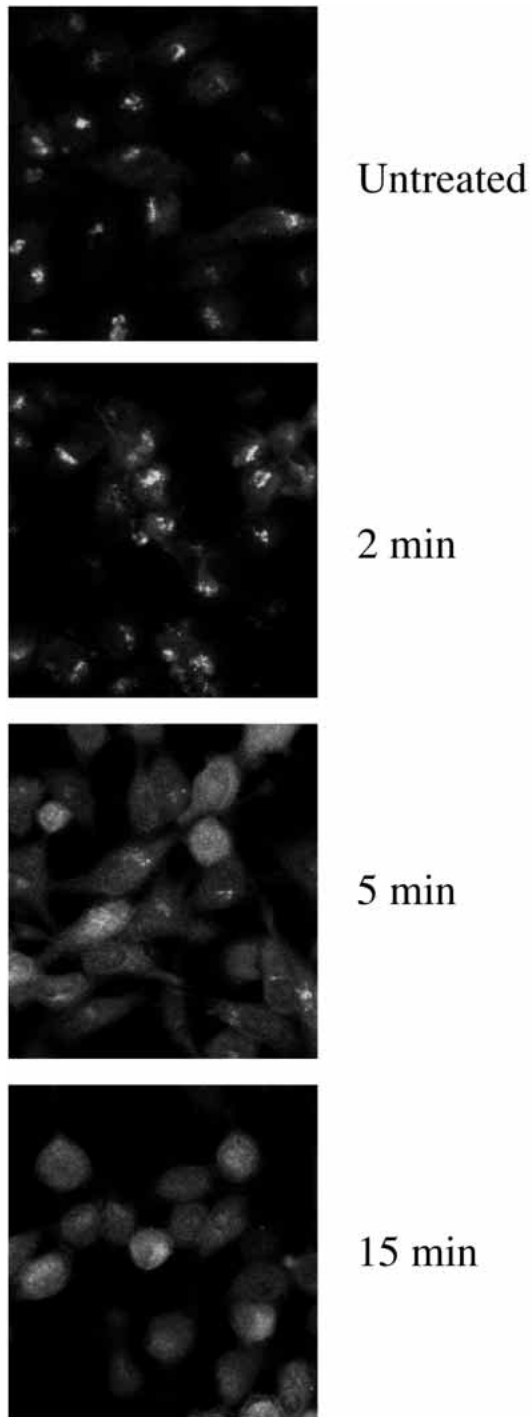
The cloning of p230 (Erlich et al., 1996) revealed a splice variant in which the C-terminal 7 residues SWLRSSS had been replaced with 9 residues of the unrelated sequence, FTSPRSGIF. To assess whether the C-terminal sequence within this splice variant had an influence on intracellular localisation, we examined the localisation of a GFP-p230 fusion protein containing the alternatively spliced sequence. As indicated in Fig. 8B the splice variant was as efficiently Golgi-localised as GFP-p230-C98aa, consistent with the ability to delete the C-terminal 10 amino acids without loss of Golgi targeting. Therefore, these splice variants do not appear to play a role in the localisation of p230.

**Fig. 8.** (A) Alanine scanning mutagenesis of GFP-p230-C98aa. COS cells were transfected with either the GFP-p230-C98aa construct or constructs in which stretches of 6-alanine substitutions (indicated by stippled regions) were introduced sequentially across the Golgi targeting domain of the C-terminal domain of p230. Numbers refer to the residue position within the C-terminal 98 amino acids of p230. The first stretch of alanines (A41-46) was introduced at position -3 with respect to the 45-amino-acid targeting sequence. Constructs A89-94 and A95-98 had six and four alanine substitutions respectively, introduced within the C-terminal 10 amino acids of p230. 48 hours after transfection, cells were fixed in paraformaldehyde and viewed by confocal fluorescence microscopy. The degree of Golgi localisation of each construct was compared with GFP-p230-C98aa. (B) Analysis of the Golgi localisation of a C-terminal splice variant of p230. COS cells were transfected with either GFP-p230-C98aa, which terminates in residues SWLRSSS, or with a GFP-p230 fusion protein splice variant, which contains the alternatively spliced C-terminal sequence FTSPRSGIF (indicated by hatching). 48 hours after transfection, cells were fixed in paraformaldehyde and the localisation of GFP fusion proteins assessed by confocal immunofluorescence as above.

#### Binding of GFP-p230-C98aa to Golgi membranes is regulated by brefeldin A and GTP $\gamma$ S

p230 has been shown to dissociate from Golgi membranes in the presence of brefeldin A (Kooy et al., 1992; Gleeson et al., 1996). To determine if the sensitivity to brefeldin A is mediated via the C terminus of p230 or through other regions of p230, the effect of brefeldin A on binding of GFP-p230-C98aa was investigated. A HeLa cell clone stably expressing the fusion protein GFP-p230-C98aa was generated and treated with brefeldin A for 2, 5 or 15 minutes before fixation and analysis (Fig. 9). There was no effect on the perinuclear localisation of GFP-p230-C98aa after a 2 minute brefeldin A treatment; however, after 5 minutes of treatment some of GFP-p230-C98aa





**Fig. 9.** Binding of GFP-p230-C98 to Golgi membranes is brefeldin A-sensitive. HeLa cells stably expressing GFP-p230-C98 were either untreated or incubated for 2, 5 or 15 minutes, as indicated, with 5  $\mu\text{g/ml}$  brefeldin A at 37°C. Cells were fixed and images collected by confocal fluorescence microscopy.

had dissociated from Golgi membranes and was located in the cytoplasm. After 15 minutes of treatment the fluorescence associated with the Golgi had completely disappeared and GFP-p230-C98aa was detected in the cytoplasm. Staining of the cells with antibodies specific for the endogenous p230 revealed a

similar rate of dissociation from the Golgi in the presence of brefeldin A as GFP-p230-C98aa (not shown), consistent with the behaviour of p230 in untransfected HeLa cells (Gleeson et al., 1996). However, in contrast,  $\beta$ -COP was rapidly dissociated from Golgi membranes of transfected HeLa cells in the presence of brefeldin A; by 2 minutes of treatment there was a major redistribution of  $\beta$ -COP, with the disappearance of perinuclear staining and the appearance of cytoplasmic staining (not shown).

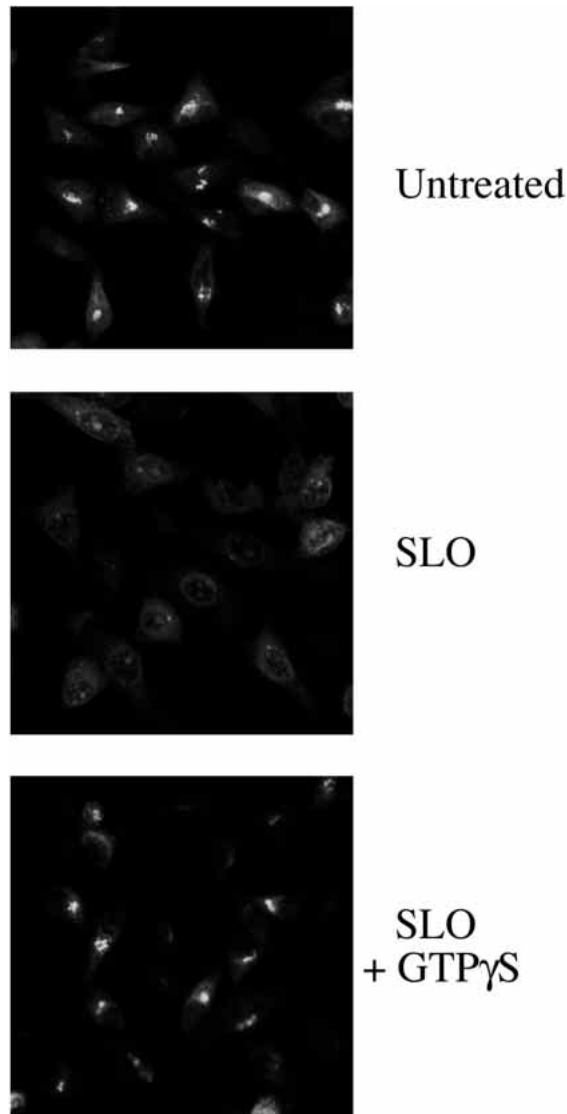
Activators of G proteins, such as GTP $\gamma$ S, have previously been shown to stabilise membrane association of p230 (Gleeson et al., 1996). We also examined whether the binding of GFP-p230-C98aa to Golgi membranes was affected by GTP $\gamma$ S. HeLa cells expressing GFP-p230-C98aa were treated at 4°C with streptolysin O (SLO) to permeabilise the cell surface membrane, washed and then incubated in the presence or absence of GTP $\gamma$ S for 30 minutes at 37°C. Incubation of SLO-permeabilised HeLa cells in the absence of GTP $\gamma$ S resulted in a cytoplasmic distribution of GFP-p230-C98aa with very little GFP fusion protein remaining in the Golgi region (Fig. 10). In contrast, incubation of SLO treated HeLa cells with GTP $\gamma$ S resulted in fluorescence associated with the Golgi (Fig. 10). Similar results were obtained for endogenous p230 in transfected HeLa cells (not shown), consistent with the behaviour of p230 in untransfected HeLa cells (Gleeson et al., 1996). Thus GFP-p230-C98aa behaves in a similar fashion to endogenous p230 in the presence of GTP $\gamma$ S, and the regulation of p230 binding by G proteins thus appears to be governed by the C terminus of the molecule.

## DISCUSSION

Numerous peripheral membrane proteins have been identified that are associated with the cytoplasmic face of Golgi membranes; however, the basis for the specific membrane localisation for most of these proteins has not been defined. p230 is a peripheral membrane protein specifically associated with the TGN of mammalian cells. The association of p230 with non-clathrin-coated buds/vesicles at the TGN indicates a role for this molecule in vesicular transport (Gleeson et al., 1996). In this report we have identified a 42-amino-acid C-terminal domain of p230 responsible for the specific targeting to Golgi membranes. This conclusion was based firstly on the observation that removal of the C-terminal 98 amino acids from p230 results in loss of Golgi association, and secondly on deletion and alanine scanning mutagenesis, which demonstrated that a minimum 42-amino-acid region close to the C-terminus of p230 is sufficient to target a GFP fusion protein efficiently to the Golgi apparatus.

High-level expression of the GFP fusion protein resulted in the loss of Golgi localisation of endogenous p230. As the Golgi markers  $\beta$ -COP and p200 were not perturbed in cells expressing high levels of the GFP fusion protein, a likely explanation is that the 42-amino-acid C-terminal domain of p230 prevents the recruitment of endogenous p230 to TGN membranes by competing for the same membrane target structures. In addition, since GFP-p230-C98 fusion protein prevents endogenous p230 from binding to Golgi membranes, it is unlikely that the GFP fusion protein can form oligomers with endogenous p230. This conclusion is further supported by





**Fig. 10.** Effect of GTP $\gamma$ S on Golgi localisation of GFP-p230-C98 in SLO-permeabilised HeLa cells. HeLa cells stably expressing GFP-p230-C98 were either untreated or 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 GTP $\gamma$ S (SLO+GTP $\gamma$ S). Cells were fixed and images collected by confocal fluorescence microscopy.

the observation that the GFP fusion protein does not coprecipitate full-length p230. Moreover we have demonstrated that the cytosolic form of GFP-p230-C98 is monomeric (C. van Vliet and P. A. Gleeson, unpublished observations). Together these results indicate that the Golgi localisation of p230 reflects an intrinsic property of the sequences of the C-terminal domain of the molecule.

Alanine scanning mutagenesis indicated the requirement for a 42 residue stretch at the C terminus of p230 to provide efficient Golgi targeting. Blocks of 6-alanine substitutions throughout this domain resulted in loss of Golgi localisation of the GFP fusion protein. This result indicates that the conformation of the 42-residue domain is critical for correct targeting, a conclusion supported by the observation that the

efficiency of Golgi localisation was increased with the presence of additional flanking sequences. From the amino acid sequence, this domain is predicted to form an  $\alpha$ -helix (Erllich et al., 1996). Replacement of blocks of 6 residues with alanine may perturb the helical structure of this domain and thereby influence its function in targeting.

The GFP-p230-C98aa fusion protein binds to Golgi membranes in transfected cells with similar characteristics to endogenous p230. The immunofluorescence data of stably transfected HeLa cells showed that GFP-p230-C98aa accumulates on Golgi membranes in SLO-permeabilised cells in the presence of GTP $\gamma$ S, whereas in the absence of the G protein activator the majority of the GFP fusion protein was in the cytosol. Therefore, G proteins are involved in the recruitment of the cytoplasmic GFP-p230-C98aa to the Golgi membranes, a behaviour similar to endogenous p230 in HeLa cells (Gleeson et al., 1996). Furthermore, previous studies have shown that p230 is brefeldin A-sensitive and that the rate of dissociation of p230 from HeLa Golgi membranes is relatively slow, compared with the rapid dissociation (<2 minutes) of other proteins such as  $\beta$ -COP. Significantly, GFP-p230-C98aa was demonstrated to be brefeldin A-sensitive with a slow rate of dissociation from HeLa Golgi membranes similar to endogenous p230. As the Golgi binding of the GFP-p230-C98aa fusion protein displays similar characteristics to endogenous p230, the brefeldin A sensitivity and regulation by G proteins must be mediated via the interaction of the Golgi-targeting domain of p230 with the membrane-binding structures. However the nature of these structures is not known at this stage.

A number of peripheral membrane proteins, found on the cytoplasmic surface of Golgi membranes, have been identified, including those considered to be important in the organisation of Golgi membranes, for example matrix proteins, cytoskeletal-associated proteins and proteins implicated in membrane transport. Recently, the C-terminal domain of the cis-Golgi matrix protein, GM130, a protein with an extensive coiled-coil domain, has been shown to be responsible for Golgi localisation (Nakamura et al., 1997) and the pleckstrin homology domain of the peripheral membrane oxysterol-binding protein is responsible for specific localisation to the trans-Golgi (Levine and Munro, 1998). However there is no amino acid similarity between the C-terminal domain of GM130 and the pleckstrin homology domain, and p230.

Although the localisation and behaviour of p230 suggests a role in vesicular transport, the precise function of this molecule remains unresolved. The identification of the Golgi targeting sequence of p230 provides avenues for exploring its function. For example, the GFP-p230 fusion protein should allow visualisation of the movement of p230 decorated TGN vesicles. Also the ability to saturate the p230 binding sites provides the potential to generate a dominant negative p230 mutant by the overexpression of the minimum p230 targeting sequence in stably transfected cells. This approach may interfere with the recruitment of endogenous p230 to the TGN, and inhibit function, providing insights into the role of p230 in membrane traffic out of or into the TGN.

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