Mapping the interaction between GRASP65 and GM130, components of a protein complex involved in the stacking of Golgi cisternae

Francis A.Barr[1], Nobuhiro Nakamura[2] and Graham Warren

Cell Biology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

[2]Present address: Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-12, Japan

[1]Corresponding author
e-mail: f.barr@europa.lif.icnet.uk

The nature of the complex containing GRASP65, a membrane protein involved in establishing the stacked structure of the Golgi apparatus, and GM130, a putative Golgi matrix protein and vesicle docking receptor, was investigated. Gel filtration revealed that GRASP65 and GM130 interact in detergent extracts of Golgi membranes under both interphase and mitotic conditions, and that this complex can bind to the vesicle docking protein p115. Using *in vitro* translation and site-directed mutagenesis in conjunction with immunoprecipitation, the binding site for GRASP65 on GM130 was mapped to the sequence xxNDxxxIMVI-COOH at the C-terminus of GM130, a region known to be required for its localization to the Golgi apparatus. The same approach was used to show that the binding site for GM130 on GRASP65 maps to amino acids 189–201, a region conserved in the mammalian and yeast proteins and reminiscent of PDZ domains. Using green fluorescent protein (GFP)-tagged reporter constructs, it was shown that one essential function of the interaction between GRASP65 and GM130 is in the correct targeting of the two proteins to the Golgi apparatus.
Keywords: GM130/Golgi apparatus/Golgi localization/ GRASP65/PDZ domains

Introduction

The Golgi apparatus can be visualized under the light microscope as a reticulum adjacent to the nucleus in animal cells (Golgi, 1898; Burke *et al*., 1982). This reticulum consists of a number of flattened disc-shaped cisternae aligned in parallel to form stacked structures linked at their edges by tubules (Rambourg and Clermont, 1990). Morphological studies have shown that filamentous material linking adjacent Golgi cisternae can be visualized under the electron microscope in a wide variety of plant and animal cells (Mollenhauer, 1965; Amos and Grimstone, 1968; Franke *et al*., 1972). Biochemical studies showed that these cross-bridges were proteinaceous, and that proteolytic treatment can disrupt the stacked organization of isolated Golgi stacks (Cluett and Brown, 1992), presumably by digestion of these filamentous cross-

bridges. Subsequently, a putative intercisternal Golgi matrix was isolated from purified rat liver Golgi stacks, and was shown to be able to bind to enzymes of the *medial/ trans*-Golgi, but not to those of the *cis*-Golgi and *cis*-Golgi network (Slusarewicz *et al*., 1994). One component of this matrix, GM130, was identified, and turned out to be a member of a family of autoantigens possessing extensive coiled-coil motifs (Nakamura *et al*., 1995). Many of these autoantigens are peripheral or integral membrane proteins associated with the Golgi apparatus (Kooy *et al*., 1992; Fritzler *et al*., 1993, 1995; Linstedt and Hauri, 1993; Seelig *et al*., 1994; Nakamura *et al*., 1995; Misumi *et al*., 1997), and thus are candidates for components of an intercisternal Golgi matrix (Barr and Warren, 1997). Further characterization of GM130 revealed that it acts as a receptor for p115 (Nakamura *et al*., 1997), a factor involved in the tethering of Golgi transport vesicles prior to their fusion (Waters *et al*., 1992; Sapperstein *et al*., 1995). GM130 is phosphorylated in mitosis and can no longer bind p115, an observation that has been linked to the block in vesicle traffic during cell division (Levine *et al*., 1996; Nakamura *et al*., 1997). How this role of GM130 in vesicle docking is related to its putative function in a structural matrix of the Golgi is unclear at present.

Recently, we identified a 65 kDa membrane-associated protein, GRASP65, involved in the reassembly of Golgi stacks in a cell-free system (Barr *et al*., 1997). Analysis of the sequence of GRASP65 revealed a central domain conserved between yeast and mammals, and a less well conserved C-terminal domain containing many serine and proline residues possibly involved in cell-cycle regulation (Barr *et al*., 1997). GRASP65 is thought to be associated with membranes by means of an N-terminal myristic acid anchor, and is found in a complex with GM130 (Barr *et al*., 1997). It could thus provide a link between vesicle traffic and cisternal stacking. We therefore set out to define the interactions between GRASP65, GM130 and p115 in an attempt to understand better the roles of these proteins in cisternal stacking. This work uses a combination of biochemical studies on purified Golgi membranes, together with deletion and point mutagenesis of GRASP65 and GM130, to demonstrate and characterize the direct interaction between these two proteins and the role this might play in their targeting to Golgi membranes.

Results

Association of GRASP65 and GM130 under both interphase and mitotic conditions

We have shown previously that GRASP65 and GM130 can be co-immunoprecipitated from a detergent extract of purified rat liver Golgi membranes, and that both GRASP65 and GM130 are present in high molecular
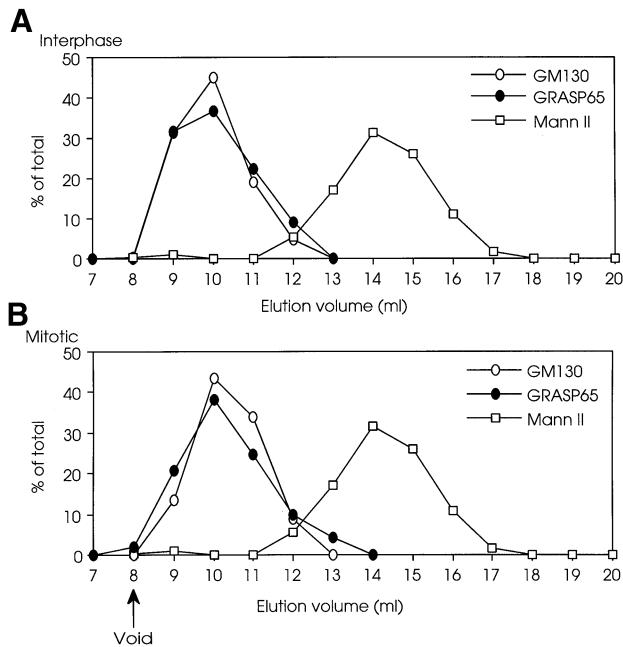
Fig. 1. GM130 and GRASP65 exist as a complex of ~1.2 MDa in detergent extracts of Golgi membranes under interphase and mitotic conditions. Purified rat liver Golgi membranes were incubated with either interphase (**A**) or mitotic (**B**) HeLa cytosol for 60 min. The membranes were recovered and extracted in IP buffer, this extract was then fractionated by gel filtration over Superose 6. Aliquots of each fraction were analysed by SDS–PAGE and Western blotting with antibodies to either GM130 (○), GRASP65 (●) or mannosidase II (□).

Fig. 2. Direct interaction of GRASP65 with GM130 and binding of the GM130–GRASP65 complex to p115 beads. (**A**) Purified rat liver Golgi membranes (10 μg) were extracted in IP buffer and fractionated by SDS–PAGE, and the proteins were transferred to nitrocellulose membranes. These were probed with 1, 10 or 50 μg/ml of histidine-tagged GRASP65 and an anti-histidine tag monoclonal antibody, then stripped by washing in 200 mM glycine pH 2.5 and reprobed with antibodies to GM130. (**B**) Purified rat liver Golgi membranes were extracted in IP buffer and then used in binding assays with either control or p115-coated beads. Aliquots of the total (RLG extract, 5% of total), and half of the material bound to the control and p115 beads were analysed by SDS–PAGE and Western blotting with antibodies to GM130 and GRASP65.

weight fractions by gel filtration, indicating that they may be part of the same large complex (Nakamura *et al.*, 1995; Barr and Warren, 1997). During mitosis, the interactions between Golgi cisternae are lost and the cisternae become fragmented into many small vesicles and tubules (Thyberg and Moskalewski, 1985; Lucocq *et al.*, 1987). The putative GRASP65–GM130 complex is thought to be involved in these events and we believed that it might undergo some cell cycle-regulated changes in size and composition, since it is known that both these proteins are phosphorylated during mitosis (Barr *et al.*, 1997; Nakamura *et al.*, 1997). To investigate this possibility further, detergent extracts of Golgi membranes pre-treated with either interphase or mitotic cytosols were fractionated by gel filtration over a Superose 6 column, and the fractions analysed by SDS–PAGE and Western blotting with specific antibodies (Figure 1). Comparison of the distributions of GM130 and GRASP65 under interphase (Figure 1A) and mitotic (Figure 1B) conditions showed that the peak positions of both GM130 and GRASP65 were very similar under both conditions tested (Figure 1A and B, fraction 10), corresponding to an apparent molecular mass of ~1.2 MDa. The only indication that a change in the composition or conformation of the GRASP65–GM130 complex had occurred was a shift in the shoulder of the peaks from the left under interphase conditions(Figure 1A, fraction 9) to the right under mitotic conditions (Figure 1B, fraction 11). As a control that should not undergo any cell cycle changes in molecular weight during gel filtration, the Golgi mannosidase II (Mann II) was investigated and found to gel filter at 200–250 kDa (Figure 1, fractions 14 and 15) under both interphase and mitotic conditions as expected for a dimer
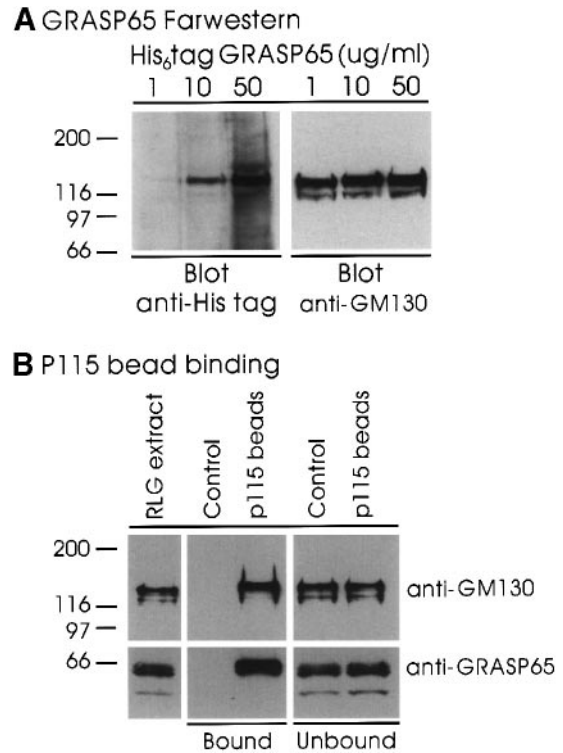
of a 120 kDa, mainly globular protein (Moremen *et al.*, 1991; Nakamura *et al.*, 1995). These data support the idea that GRASP65 and GM130 are present as part of a stable complex in Golgi membranes, under both interphase and mitotic conditions.

Direct interaction of GRASP65 with GM130 and binding of the GM130–GRASP65 complex to the vesicle docking protein p115

To provide evidence that GRASP65 binds directly to GM130, far Western analyses were performed. A detergent extract of Golgi membranes was fractionated by SDS–PAGE, and the proteins transferred to nitrocellulose membranes; these were probed with recombinant histidine-tagged GRASP65 (Figure 2A, left panel), then stripped to remove the bound GRASP65 and reprobed with antibodies to GM130 (Figure 2A, right panel). Recombinant GRASP65 bound to a protein of 130 kDa, and this binding increased with increasing concentrations of probe. Western blotting with antibodies to GM130 revealed that the protein recognized by the recombinant GRASP65 had the same molecular weight as GM130. This provides evidence that GRASP65 directly interacts with a 130 kDa protein in Golgi membranes that is likely to be GM130.

GM130 is known to function as a receptor for the

vesicle transport protein p115, thought to be involved in the tethering of membranes. It has been shown that this interaction is prevented in mitosis by phosphorylation of the N-terminus of GM130 (Nakamura *et al.*, 1997). The relationship between this function of GM130 in vesicle traffic and that of GRASP65 in establishing the stacked cisternal structure of the Golgi apparatus is unclear. One possible explanation is that two functionally discrete pools of GM130 exist in Golgi membranes, one complexed with GRASP65 and the other with p115. To investigate this, p115 binding assays were performed (Figure 2B), using a detergent extract of Golgi membranes incubated with control beads or beads on which purified rat p115 had been immobilized as described previously (Nakamura *et al.*, 1997). Aliquots of the total extract, and the bound and unbound fractions from either control or p115 bead-containing incubations were analysed by SDS–PAGE and Western blotting with antibodies to GM130 and GRASP65. Reactivity corresponding to GM130 and GRASP65 was found to bind to p115 (Figure 2B, p115 beads) but not to control beads (Figure 2B, Control), and the ratio between the GM130 and GRASP65 signals was found to be similar to that observed in the starting extract (Figure 2B, RLG extract). In previous studies using p115 beads, GRASP65 was not detected since it does not stain well with Coomassie Blue, and is in the same region of the gel as an abundant contaminating protein, serum albumin (see Figure 1 in Nakamura *et al.*, 1997). It is unlikely, therefore, that two pools of GM130 exist distinguished by their ability to bind p115. This result supports the view that the complex of GRASP65–GM130–p115 functions in both vesicle docking and cisternal stacking.

Reconstitution of the interaction between GRASP65 and GM130 in an in vitro assay

In an attempt to characterize the complex containing GRASP65 and GM130, it was decided to see if it could be reconstituted, at least in part, with only these two proteins, using coupled *in vitro* transcription–translation in conjunction with immunoprecipitation. *In vitro* translation reactions were carried out with plasmids encoding either GRASP65 (Figure 3, lane 1) or GM130 (Figure 3, lane 2) alone, or GRASP65 and GM130 together (Figure 3, lane 3). Aliquots of the total reactions were analysed by SDS–PAGE and autoradiography; the position of GM130 is marked by an open triangle and that of GRASP65 by an asterisk (Figure 3A). As expected, the production of GM130 and GRASP65 depended on the presence of their respective plasmids. To assay for an interaction between the two proteins, immunoprecipitations were performed from these *in vitro* translation reactions with antibodies to GM130 or GRASP65. Analysis of the bound material by SDS–PAGE and autoradiography revealed that antibodies to GM130 precipitated GM130 (Figure 3B, lane 2) but not GRASP65 (Figure 3B, lane 1), and antibodies to GRASP65 precipitated GRASP65 (Figure 3C, lane 1) but not GM130 (Figure 3C, lane 2). If GRASP65 was translated *in vitro* together with GM130, it was found to be precipitated by antibodies to GM130 (Figure 3B, lane 3) and, conversely, GM130 was found to be immunoprecipitated by antibodies to GRASP65 (Figure 3C, lane 3). Using this method, a complex between GRASP65 and GM130 can be reconstituted with only these two proteins,
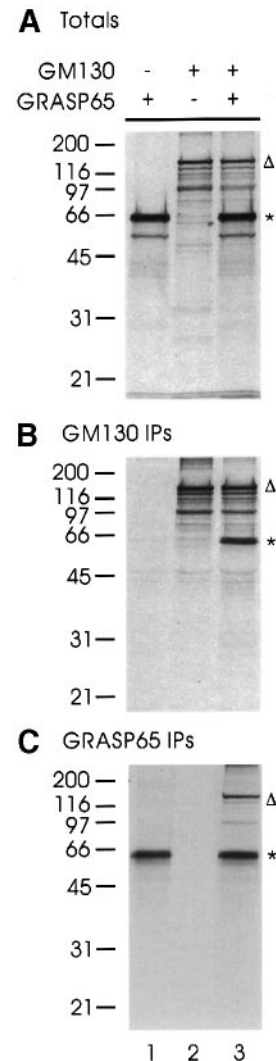

Fig. 3. Reconstituting the interaction of GM130 with GRASP65. Coupled *in vitro* transcription–translation assays were performed using the indicated plasmids encoding either GRASP65 (lane 1) or GM130 (lane 2), or both GRASP65 and GM130 (lane 3). (**A**) Aliquots of the total *in vitro* translation reactions were analysed by SDS–PAGE and autoradiography. Immunoprecipitations were performed using antibodies to GM130 (**B**) and GRASP65 (**C**), with either 5 or 10 µl of the single and co-*in vitro* translates, respectively. The immunoprecipitated material was analysed by SDS–PAGE and autoradiography. The positions of GM130 and GRASP65 are marked by a triangle and an asterisk, respectively.

and thus their interaction is amenable to further analysis *in vitro*.

GRASP65 binds to the C-terminus of GM130

To define the binding site for GRASP65 on GM130, a series of N- and C-terminal deletion constructs in GM130 were constructed (Figure 4). These constructs were then used in the *in vitro* translation and immunoprecipitation assay together with a wild-type GRASP65 construct. Analysis of the total *in vitro* translation reactions by SDS–PAGE and autoradiography revealed that all constructs were expressed equally, taking into account the different number of methionines present in the various deletion constructs (Figure 5A). Deletions from the N-terminus of GM130 to position 441 and 690 (Figure 5B, lanes 9 and 10) had no effect on the interaction with GRASP65. The
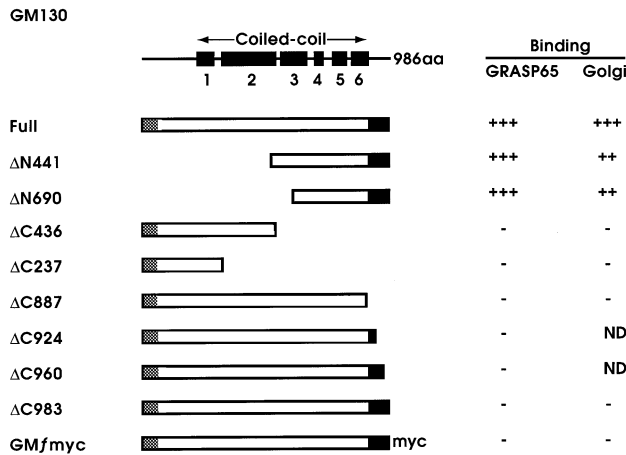
Fig. 4. Structure of GM130 and deletion constructs. Analysis of the sequence of GM130 reveals a central coiled-coil region consisting of six predicted coiled-coil domains, boxed and numbered. Deletion constructs were designed taking this predicted structure into account and are described according to the position of their first (ΔN) or last (ΔC) amino acid in full-length GM130. The light and dark shaded regions of the deletion constructs indicate the p115-binding site at the N-terminus and the region previously shown to be involved in Golgi targeting at the C-terminus. GM*f*myc is identical to full-length GM130 with a myc epitope tag added at the C-terminus. A summary of the binding experiments (Figure 5) and the ability to target Golgi membranes is shown on the right, ND indicates not done (Nakamura *et al.*, 1997).

Fig. 5. GRASP65 binds to the C-terminus of GM130. (**A**) and (**B**) coupled *in vitro* transcription–translation assays were performed using plasmids encoding both GRASP65 and GM130 (lane 1), GRASP65 with the GM130 N- and C-terminal deletions ΔC887, ΔC436, ΔC237, ΔC924, ΔC960, ΔC983, GM*f*myc, ΔN441 and ΔN690 (lanes 2–10), and either GRASP65 (lane 11) or GM130 (lane 12) alone. (**C**) Coupled *in vitro* transcription–translation assays were performed using plasmids encoding GRASP65, and either GM130 (WT) or GM130 alanine point mutations scanning from amino acids 976 to 986 (D976–I986). Immunoprecipitations were performed using antibodies to GM130 (B) and (C) with either 5 or 10 μl for single and co-*in vitro* translations, respectively. Aliquots of the total (A) and immunoprecipitated material were analysed by SDS–PAGE and autoradiography (B and C). The position of GRASP65 is marked by an asterisk in (A) and (B). Densitometric analysis of the amount of co-immunoprecipitating GRASP65 was performed in (C). The means, normalized to GM130 for each point, of three separate experiments are shown. Bars indicate the standard error.

weaker signal for GRASP65 and GM130ΔN690 is due to a decreased efficiency in the immunoprecipitation rather than a lack of interaction between the two proteins, since the major epitope recognized by the polyclonal antiserum used for these experiments is deleted in this GM130 construct. Since the N-terminal 75 amino acids contain the binding site for p115, this indicated that the interaction of GRASP65 with GM130 is distinct from that of p115. Deletions from the C-terminus of GM130 to positions 887, 436 and 237, known to abolish the targeting of GM130 to the Golgi apparatus (Nakamura *et al.*, 1997), also resulted in the loss of binding to GRASP65 (Figure 5B, lanes 2–4). The molecular weight of the GM130ΔC436 construct is similar to that of GRASP65, and it runs immediately below it on SDS–PAGE, giving the appearance of a very broad band in the total *in vitro* translation (Figure 5A, lane 3). Careful examination of the immunoprecipitation (Figure 5B, lane 3) reveals that the upper part of this broad band corresponding to GRASP65 is missing, indicating that there is no interaction of GRASP65 with this GM130 deletion construct. Introduction of a myc tag at the extreme C-terminus (GM*f*myc) also had the same effect, abolishing both binding to GRASP65 (Figure 5B, lane 8) and targeting to the Golgi apparatus (N.Nakamura unpublished data). Together, these data suggested that the binding site for GRASP65 lay in the last 100 amino acids of GM130 including the free C-terminus, and it was decided to focus on this region more closely. Additional deletion constructs in GM130 were made in which the last 62, 26 or three amino acids were removed by the introduction of in-frame stop codons. None of the constructs GM130ΔC924, ΔC960 or ΔC983 was able to interact with GRASP65 (Figure 5B, lanes 5–7), confirmin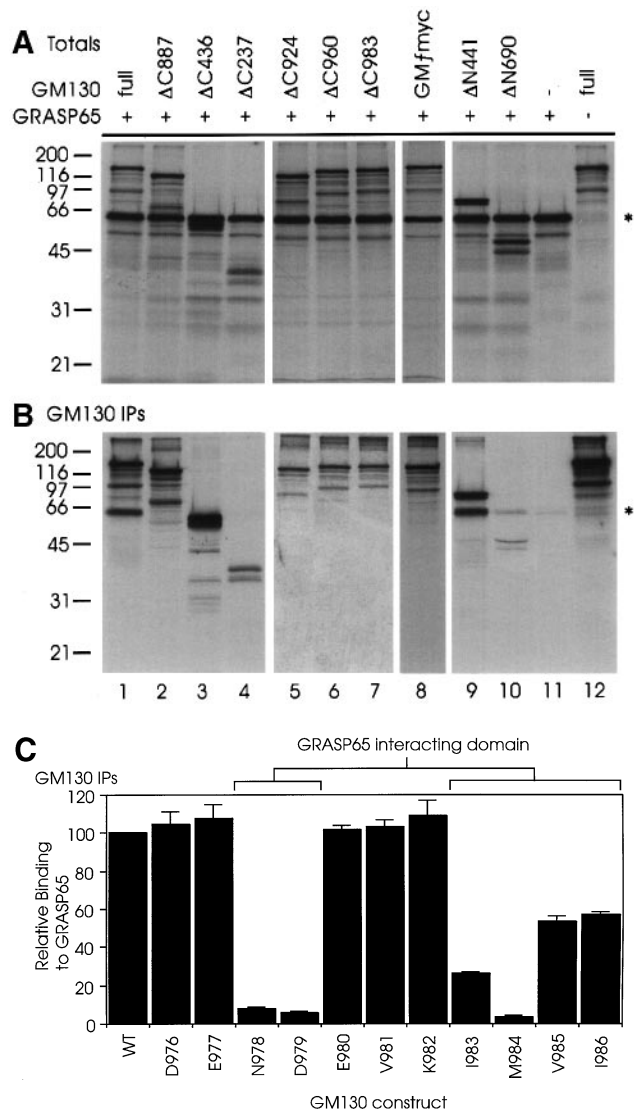g that the binding site lay in this region. Alanine-scanning mutagenesis of the last 11 amino acids of GM130 from D976 to I986 was then performed in order to define the GRASP65-binding site. These GM130 constructs were then used in the *in vitro* translation assay; quantitations of such experiments are shown in Figure 5C. From this analysis, it can be seen that the four hydrophobic amino acids Ile–Met–Val–Ile lying at the C-terminus, in conjunc-
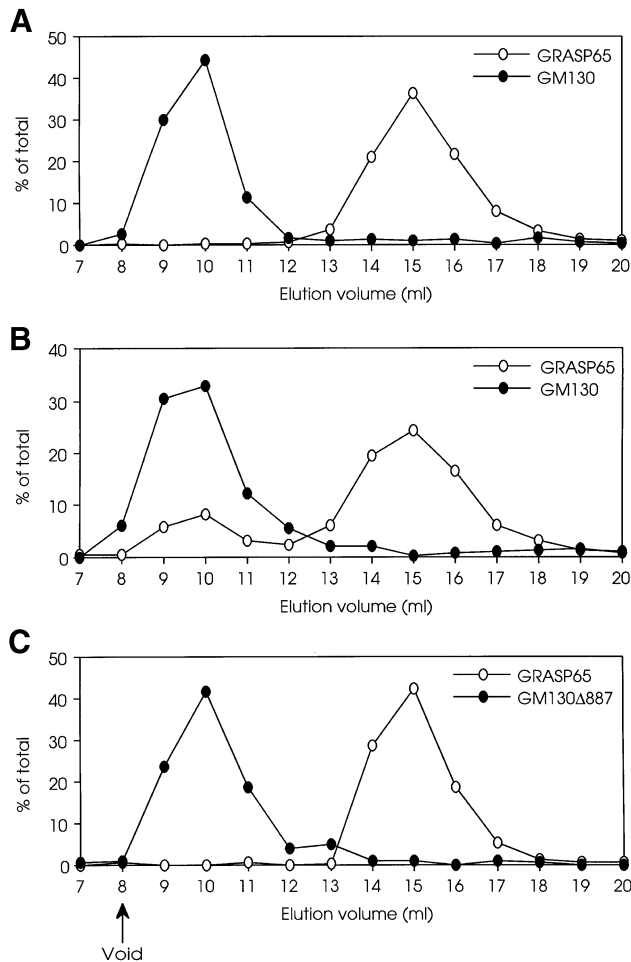
Fig. 6. Analysis of *in vitro* translated GM130–GRASP65 complexes by gel filtration. (**A**) Separate *in vitro* transcription–translation assays were performed using plasmids encoding either GRASP65 (GRASP65, ○) or wild-type GM130 (GM130, ●). These assays were then cooled on ice and mixed. *In vitro* transcription–translation assays were performed using plasmids encoding GRASP65 (GRASP65, ○) together with (**B**) wild-type GM130 (GM130, ●) and (**C**) GM130 deleted from amino acid 887 to the end (GM130ΔC887, ●). The *in vitro* translation reactions (A–C) were then fractionated over Superose 6, collecting 1 ml fractions. Aliquots of each fraction were analysed by SDS–PAGE and autoradiography, then densitometric analysis of the distribution of each protein was performed and the results plotted as a percentage of the total.

tion with the Asn–Asp pair at position 978–979, form the site of GRASP65 interaction with GM130.

Gel filtration of in vitro translated GM130 and GRASP65

To confirm that the complex formed by the *in vitro* translation of GM130 and GRASP65 was related to that present in Golgi membranes, it was fractionated by gel filtration over Superose 6 as described previously (Figure 1). For this purpose, three sets of assays were performed, with *in vitro* translation of: GM130 and GRASP65 individually, followed by their mixing on ice (Figure 6A); GM130 and GRASP65 together (Figure 6B); and GM130 lacking the last 100 amino acids (GM130ΔC887) and GRASP65 together (Figure 6C). When GM130 and GRASP65 were *in vitro* translated separately then mixed on ice, they gave two clearly resolved peaks by gel

filtration, GM130 at fraction 10 (Figure 6A, ●) and GRASP65 at fraction 15 (Figure 6A, ○). From the column calibration, these elution positions would correspond to apparent mol. wts of 1.2 MDa for GM130 and 200 kDa for GRASP65. When GM130 and GRASP65 were *in vitro* translated together and fractionated by gel filtration, the peak of GM130 was shifted slightly to the left (Figure 6B, ●) and a second peak of GRASP65 was present at fraction 10 (Figure 6B, ○) consisting of ~25% of the total GRASP65. Since GRASP65 is present in molar excess over GM130, the peak at fraction 15 (Figure 6B, ○) is still present. This is consistent with the formation of a stable interaction between GRASP65 and GM130 in the *in vitro* translation assay, as was also shown by immunoprecipitation with specific antibodies. Finally, when GM130ΔC887 and GRASP65 were *in vitro* translated together and fractionated by gel filtration, they gave two clearly resolved peaks by gel filtration, GM130 at fraction 10 (Figure 6C, ●) and GRASP65 at fraction 15 (Figure 6C, ○). This is consistent with the lack of an interaction between GRASP65 and GM130ΔC887 in the *in vitro* translation assay, as was also shown using immunoprecipitation with specific antibodies (Figure 5B). Gel filtration confirms the results obtained by immunoprecipitation implicating the C-terminus of GM130 in the direct binding of GRASP65, and shows that these two proteins alone could form the 1.2 MDa complex present in detergent extracts of Golgi membranes.

GM130 binds to a conserved region of GRASP65

To define the binding site for GM130 on GRASP65, a series of N- and C-terminal deletion constructs in GRASP65 were constructed (Figure 7A). These constructs were then used in the *in vitro* translation and immunoprecipitation assay together with a wild-type GM130 construct. Analysis of the total *in vitro* translation reactions by SDS–PAGE and autoradiography revealed that all constructs were expressed equally, taking into account the different number of methionines present in the various deletion constructs (Figure 8A). Deletions of the C-terminus of GRASP65 to position 237 and 314 (GRASP65ΔC237 and ΔC314) had little effect on the interaction with GM130 (Figure 8B, lanes 3 and 4). The decreased amount of the GRASP65ΔC314 immunoprecipitating with GM130 (Figure 8B, lane 4) may be due to incorrect folding of this protein, since larger deletions, for example to amino acid 237 (Figure 8B, lane 3), interact with GM130 to the same extent as the full-length GRASP65 (compare lanes 1 and 3). Deletions of the C-terminus of GRASP65 to position 152 (GRASP65ΔC152) and the N-terminus to position 198 (GRASP65ΔN198) resulted in the loss of binding to GM130 (Figure 8B, lanes 2 and 5). These data suggested that the binding site for GM130 lay in the 90 amino acids of GRASP65 from amino acids 112 to 202, and it was decided to study this region, domain 2, more closely. A double deletion from the N- and C-termini comprising only domain two of GRASP65 (GRASP65ΔN112ΔC202) was able to interact with GM130, indicating that this domain alone contains the binding site for GM130 (Figure 8B, lane 6). Interestingly, this region of GRASP65 is in the part of the protein most highly conserved between different organisms, including mammals and yeasts (Barr *et al.*, 1997). In
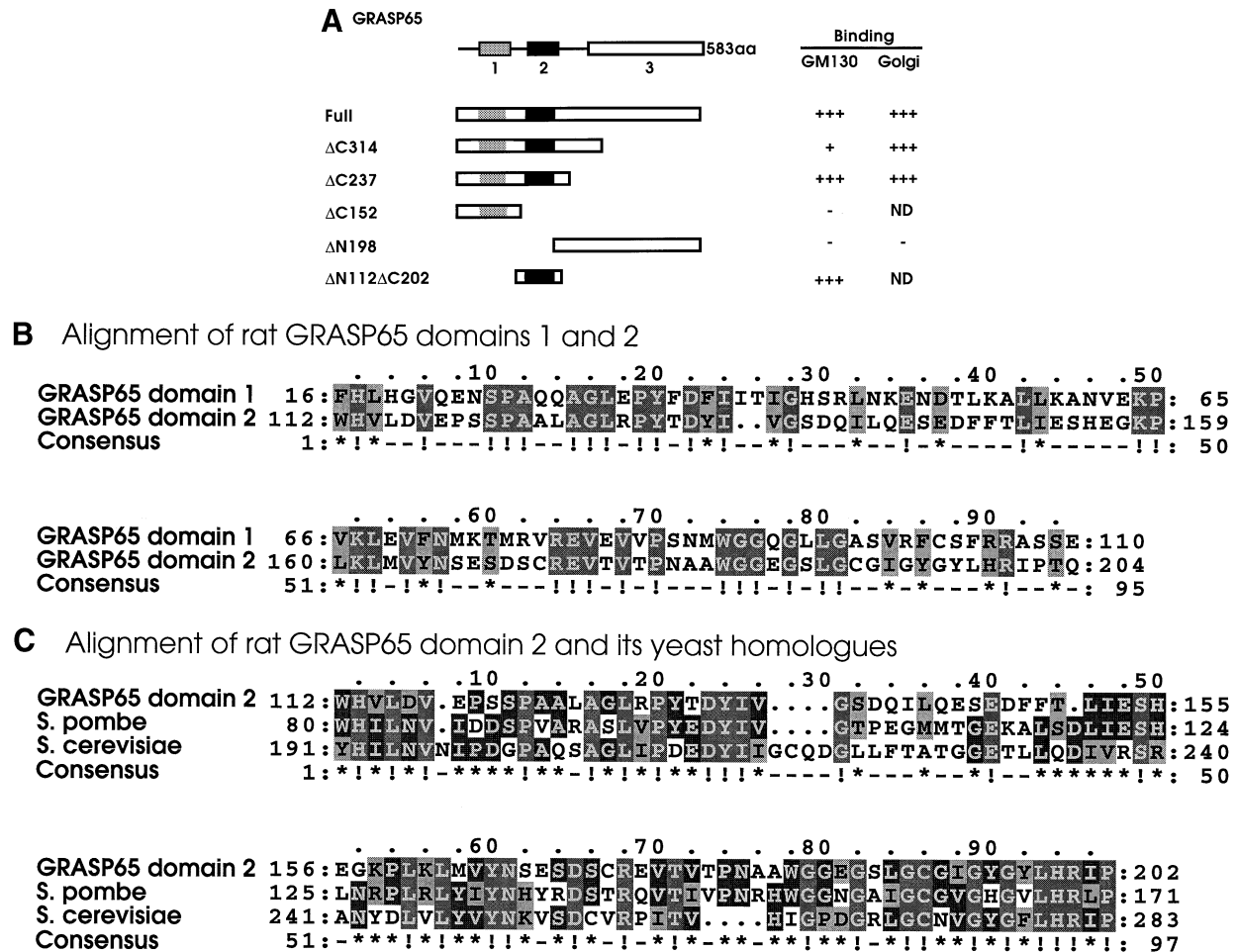
A GRASP65

	Binding	
	GM130	Golgi
Full	+++	+++
ΔC314	+	+++
ΔC237	+++	+++
ΔC152	-	ND
ΔN198	-	-
ΔN112ΔC202	+++	ND

B Alignment of rat GRASP65 domains 1 and 2

```
                      .   .   .  .10  .   .   .  .20  .   .   .  .30  .   .   .  .40  .   .   .  .50
GRASP65 domain 1  16:FHLHGVQENSPAQQAGLEPYFDFIITIGHSRLNKENDTLKALLKANVEKP: 65
GRASP65 domain 2 112:WHVLDVEPSSPAALAGLRPYTDYI..VGSDQILQESEDFFTLIESHEGKP:159
Consensus          1:*!*--!---!!!--!!!-!!-!*!--*!---*--!-*----!*-----!!: 50


                      .   .   .  .60  .   .   .  .70  .   .   .  .80  .   .   .  .90  .  .
GRASP65 domain 1  66:VKLEVFNMKTMRVREVEVVPSNMWGCQGLLGASVRFCSFRRASSE:110
GRASP65 domain 2 160:LKLMVYNSESDSCREVTVTPNAAWGCEGSLGCGIGYGYLHRIPTQ:204
Consensus         51:*!!-!*!--*---!!!-!-!---!!!-!-!!--*-*----*!--*-: 95
```

C Alignment of rat GRASP65 domain 2 and its yeast homologues

```
                      .   .   .  .10  .   .   .  .20  .   .   .  .30  .   .   .  .40  .   .   .  .50
GRASP65 domain 2 112:WHVLDV.EPSSPAALAGLRPYTDYIV....GSDQILQESEDFFT.LIESH:155
S. pombe          80:WHILNV.IDDSPVARASLVPYEDYIV....GTPEGMMTGEKALSDLIESH:124
S. cerevisiae    191:YHILNVNIPDGPAQSAGLIPDEDYLIGCQDGLLFTATGGETLLQDIVRSR:240
Consensus          1:*!*!*!-****!**-!*!*!**!!!*----!*---*---*!--******!*: 50


                      .   .   .  .60  .   .   .  .70  .   .   .  .80  .   .   .  .90  .   .  .
GRASP65 domain 2 156:EGKPIKLMVYNSESDSCREVTVTPNAAWGGEGSLGCGIGYCYLHRIP:202
S. pombe         125:LNRPLRLYIYNHYRDSTRQVTIVPNRHWGGNGAIGCGVGHGVLHRLP:171
S. cerevisiae    241:ANYDLVLYVYNKVSDCVRPITV....HIGPDGRLGCNVGYGFLHRIP:283
Consensus         51:-***!*!**!!*-*!*-!-*!*-**-**!**!-*!!**!*!*!!!*!: 97
```

Fig. 7. Structure of GRASP65 and deletion constructs. (**A**) Analysis of the sequence of rat GRASP65 reveals three distinct features. Domain 2 is conserved from mammals to yeast, an imperfect repeat of this sequence forms domain 1, while domain 3 is a Ser/Pro-rich sequence at the C-terminus. Deletion constructs were designed taking this predicted structure into account, and are described according to the position of their first (ΔN) or last (ΔC) amino acid in full-length GRASP65. The light and dark shaded boxes indicate domains 1 and 2, respectively. A summary of the binding experiments (Figure 8) is shown on the right. (**B**) Alignment of domain 2 of rat GRASP65 conserved from mammals to yeast from amino acids 112 to 202 with domain 1, its imperfect repeat from amino acids 16 to 108 of rat GRASP65. Asterisks and light shading indicate conserved residues, while exclamation marks and dark shading indicate identical residues. (**C**) Alignment of domain 2 of rat GRASP65 with the yeast homologues indicates that they are 40–50% identical and 50–60% conserved in this region. All alignments were produced in the GCG PILEUP program (Devereux *et al.*, 1984) and were formatted using MacBOXSHADE.

the rat GRASP65, the conserved sequence comprising Trp112–Pro202 (Figure 7A, domain 2), is repeated imperfectly from amino acids Phe16 to Ser108 (Figure 7A, domain 1). The deletion analysis indicates that domain 1, the imperfectly repeated sequence of domain 2, is unable to bind to GM130, since the GRASP65ΔC152 deletion possessing domain 1 but lacking domain 2 does not interact with GM130. Alignment of the conserved domain of rat GRASP65 from Trp112 to Pro202 containing the binding site for GM130 with this imperfectly repeated sequence from Phe16 to Ser108 reveals that they are similar throughout their entire length (Figure 7B). Since the deletion mutagenesis of GRASP65 indicates that the binding site for GM130 lies after amino acid 152, the differences in the alignment from this point on are likely to explain why the conserved domain but not its repeated form constitute a binding site for GM130. The least conserved part of the alignment between the conserved domain and its repeat coming after amino acid 152 of GRASP65 lies between amino acids 194 and 202, with only one of 12 amino acids being identical (Figure 7B).

This region is, however, conserved from rat GRASP65 domain 2 to the yeast homologues (Figure 7C). It was decided, therefore, to focus on the region lying between Cys191 and Pro202 of GRASP65, together with some additional conserved flanking residues. Alanine-scanning mutagenesis of the 22 amino acids of GRASP65 from Pro179 to Pro202, excluding the alanine residues at 181 and 182, was then performed in order to define the GM130-binding site. These GRASP65 constructs were then used in the *in vitro* translation assay; quantitations of such experiments are shown in Figure 8C. From this analysis, it can be seen that the key residues for the interaction of GRASP65 with GM130 are from Gly194 to Ile201 and that these lie in the least conserved region of the repeated domain as predicted. To provide further evidence that the effects of the mutations in GRASP65 define a specific binding site for GM130, and do not grossly alter the structure of the protein, gel filtration analysis of all the constructs used was performed. *In vitro* translated full-length, N- and C-terminal deletions and the alanine-scanning point mutant forms of GRASP65 were
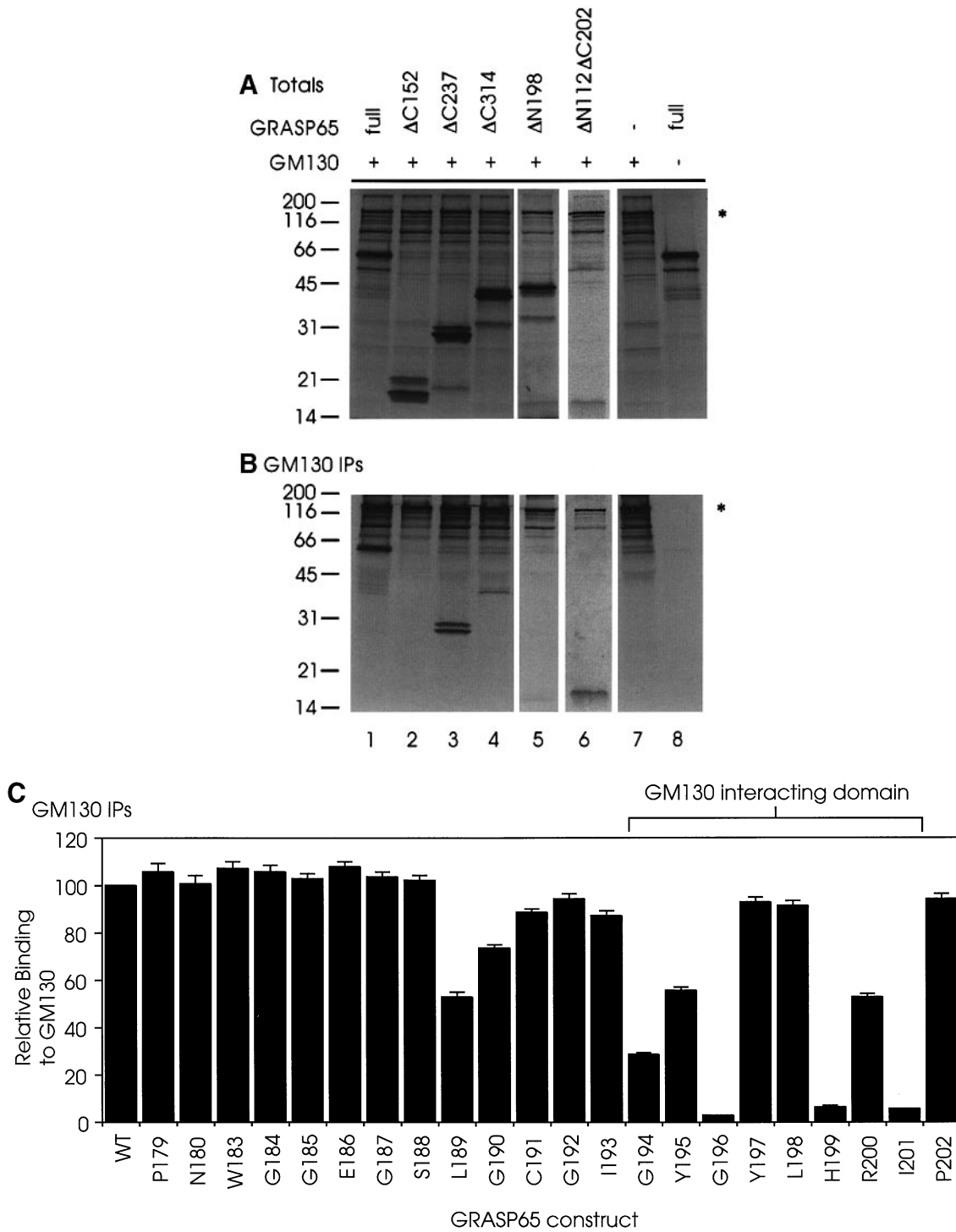
Fig. 8. GM130 binds to a conserved region of GRASP65. (**A**) and (**B**) Coupled *in vitro* transcription–translation assays were performed using plasmids encoding both GM130 and GRASP65 (lane 1), GM130 with the GRASP65 N- and C-terminal deletions ΔC152, ΔC237, ΔC314, ΔN198 and ΔN112ΔC202 (lanes 2–6), and either GM130 (lane 7) or GRASP65 (lane 8) alone. (**C**) Coupled *in vitro* transcription–translation assays were performed using plasmids encoding GM130, and either GRASP65 (WT) or GRASP65 alanine point mutations scanning from amino acids 179 to 202 (P179–P202). Immunoprecipitations were performed using antibodies to GM130 (B) and (C) with either 5 or 10 μl for single and co- *in vitro* translations, respectively. Aliquots of the total (A) and immunoprecipitated material were analysed by SDS–PAGE and autoradiography (B and C). The position of GM130 is marked by an asterisk in (A) and (B). Densitometric analysis of the amount of co-immunoprecipitating GRASP65 was performed in (C), the means of three separate experiments for each point are shown. Bars indicate the standard error.

analysed by gel filtration over Superose 6 in 200 mM KCl, and the apparent molecular masses were calculated from their elution positions relative to standard marker proteins. The results of such an analysis are summarized in Table I, and indicate that wild-type GRASP65 and the

GRASP65Δ314 deletion behave as dimers or trimers under these conditions. All of the alanine-scanning mutants were found to be indistinguishable from the wild-type GRASP65, indicating that these proteins are also forming dimers or trimers. The GRASP65Δ237 deletion lacking

Table I. Analysis of wild-type and mutant forms of GRASP65 by gel-filtration

GRASP65 construct	Predicted mol. wt (kDa)	Gel filtration mol. wt (kDa)	Predicted oligomeric state
GRASP65 full-length	65	203 ± 31	trimer/dimer
GRASP65Δ314	35	117 ± 15	trimer/dimer
GRASP65Δ237	26	39 ± 6	dimer/monomer
GRASP65Δ152	17	22 ± 3	monomer
GRASP65 alanine-scanning mutants	65	203 ± 35	trimer/dimer

the predicted elongated tail, domain 3, forms dimers, whereas the GRASP65Δ152 deletion lacking domains 2 and 3 only forms monomers. While it is possible that di- or trimerization of GRASP65 is important for its binding to GM130, the observations presented here support the conclusion that the deletion and alanine-scanning point mutations in GRASP65 have defined a binding site for GM130 on this protein.

Targeting of the GRASP65–GM130 complex to the Golgi apparatus

Having defined the sites at which GM130 and GRASP65 interact, we then investigated the role of this interaction in the targeting of the two proteins to the Golgi apparatus. Transient transfection studies were carried out using either the tail region, residues 816–986, of GM130 fused to the C-terminus of green fluorescent protein (GFP), or the first 202 amino acids of GRASP65 fused to the N-terminus of GFP. The same mutations that disrupted the interactions between the two proteins were then introduced into these constructs, and the effects these had on the targeting of each protein were examined.

When fused to GFP, the wild-type GM130 tail is able to target correctly to the Golgi apparatus (Figure 9A), whereas deletion of the last three amino acids of the tail abolishes this (Figure 9B). Alanine-scanning point mutations through the tail of GM130 from amino acids 978 to 986 reveal that the ability to bind GRASP65 (Figure 5C) correlates with the targeting of the corresponding GFP reporter construct to the Golgi apparatus (Figure 9C–K). Point mutations that lowered the binding of GM130 to GRASP65 (GM130 V985A and I986A) resulted in an intermediate phenotype, with a small amount of Golgi staining and a large soluble pool of the GFP reporter construct (Figure 9J and K). When fused to GFP, the wild-type N-terminal 202 amino acids of GRASP65 are able to target correctly to the Golgi apparatus (Figure 9L), whereas mutation of the glycine at position two of GRASP65 to abolish the myristoylation site prevents this (Figure 9M). Point mutations at amino acids 196 and 199, abolishing the ability of GRASP65 to bind to GM130 (Figure 8C), prevent its localization to the Golgi apparatus (Figure 9N and O). This indicates that the *N*-myristoylation site and the domain defined as the binding site for GM130 are important for targeting GRASP65 to the Golgi apparatus.

Therefore, the interaction between GRASP65 and GM130 is required not only for them to form a stable complex, but also for their correct localization to the Golgi apparatus.

Discussion

A protein complex involved in Golgi transport, vesicle docking and the stacking of Golgi cisternae

We have shown that the 1.2 MDa complex present in Golgi membranes containing GRASP65 and GM130 is able to interact with the vesicle transport factor p115 under interphase conditions. Using far Western blotting and an *in vitro* translation system, we were able to demonstrate a direct interaction between GM130 and GRASP65, and to map the binding sites on both proteins. For GM130, the region interacting with GRASP65 proved to be at the extreme C-terminus and to correlate with the region previously shown to be involved in the targeting of GM130 to the Golgi apparatus. This indicates that one function of GRASP65 is in the Golgi targeting of GM130. Previously, using a functional assay for the identification of proteins involved in the stacking of Golgi cisternae, we found GRASP65 and, GM130. The fact that GM130 and GRASP65 directly interact and were identified by this approach implies that they may both play a role in the stacking of Golgi cisternae. The known function of p115 as a factor tethering Golgi transport vesicles to their target membranes and the role of GM130 as one of its receptors suggests that they may also function in the process by which cisternae form stacks. In addition to tethering vesicles during docking events, p115 might also, in conjunction with GRASP65, link cisternae together during the process of stacking, allowing the establishment of more stable cisternal cross-bridges, analogous to the SNARE pair formation during vesicle docking.

The interaction of GRASP65 with GM130 is reminiscent of the binding of PDZ domains to their ligands

The binding site for GM130 on GRASP65 was mapped to a region between amino acids 194 and 201 in the most conserved part of GRASP65 which extends from amino acids 112 to 202, and is imperfectly repeated between amino acids 16 and 108. Within the region identified as the binding site for GM130 is the sequence Gly–Tyr–Gly–Tyr (GYGY) that is reminiscent of the sequence Gly–Leu–Gly–Phe (GLGF), a conserved motif of the PDZ domain (Ponting *et al.*, 1997). It should be noted that this motif is not absolutely conserved, and exists in many different variants (Ponting *et al.*, 1997). The binding of GRASP65 to the last four hydrophobic amino acids of GM130 is reminiscent of the interaction of PDZ domain-containing proteins with the extreme C-terminal four amino acids of their target proteins (Saras and Heldin, 1996). Specific PDZ domains have been shown to bind
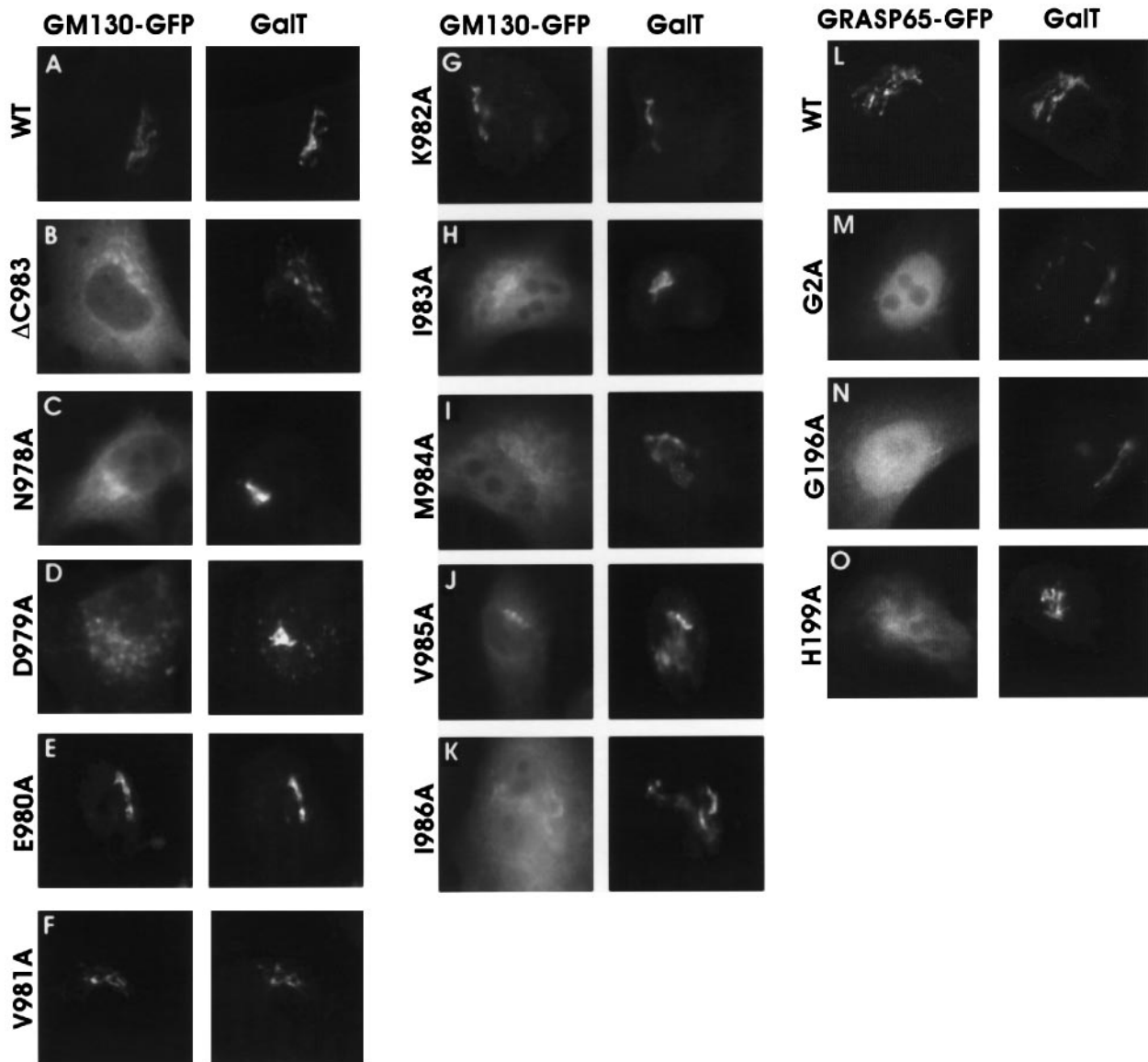
Fig. 9. Targeting of GRASP65 and GM130 GFP reporter proteins to the Golgi apparatus. The wild-type GM130 tail (**A**), the GM130ΔC983 deletion (**B**) or alanine point mutants of residues 978–986 of GM130 (**C–K**) were expressed as GFP fusion proteins in HeLa cells using transient transfection. Wild-type GRASP65 (**L**) and the G2A, G196A and G199A GRASP65 point mutations (**M–O**) were expressed as GFP fusion proteins in HeLa cells using transient transfection. Cells were fixed and stained with an antibody to β-1,4-galactosyltransferase (GalT). The GFP fluorescence is shown on the left and the GalT immunofluorescence is shown on the right of each pair of images.

specific peptide sequences *in vitro*, which explains their specificities *in vivo* (Songyang *et al.*, 1997). Typically, they act as adaptors in the assembly of multifunctional protein complexes involved in signalling events at the surfaces of cellular membranes (Cowburn, 1996; Ponting *et al.*, 1997), for example the binding of PSD-95, a guanylate kinase, to the N-terminus of the NMDA receptor 2B (Kornau *et al.*, 1995). In the case of GRASP65, this domain is involved in the targeting to the Golgi membrane of GM130, a molecule that would otherwise be cytosolic. It should be noted that although the PDZ domain has been found only in multicellular animals, there is evidence for the presence of PDZ-like domains capable of binding C-terminal tetrapeptide sequences in yeasts, plants and bacteria (Ponting, 1997).

The most conserved region of GRASP65 forms part of a domain required for the binding of GM130, and localiz-

ation to the Golgi apparatus. GRASP65, although lacking a transmembrane domain, is *N*-myristoylated (Barr *et al.*, 1997). This modification is necessary for its interaction with membranes (Figure 9M), but not its specific localization to the Golgi apparatus (Johnson *et al.*, 1994; Bhatnagar and Gordon, 1997). One possibility is that the GRASP65–GM130 complex targets specifically to the Golgi apparatus by interaction of the GRASP65 N-terminal domains 1 and 2 with Golgi-specific proteins possessing transmembrane domains. Alternatively, it is possible that GM130 can interact with a Golgi-specific transmembrane protein only when bound to GRASP65, and that this explains how the GRASP65–GM130 complex targets only to Golgi membranes. We currently are investigating these possibilities.

In the yeast homologues of rat GRASP65, the sequence implicated in the binding of GM130 is highly conserved,

indicating that these proteins may be binding to the C-terminus of a GM130 homologue in these organisms. Analysis of the *Saccharomyces cerevisiae* genome reveals no protein that possesses the C-terminal sequence in GM130 that is recognized by GRASP65. This implies that the changes (Figure 7C) from rat (GIGYGYLHRI) to *Schizosaccharomyces pombe* (GVGHGVLHRL) and *S.cerevisiae* (NVGYGFLHRI) GRASP65 have altered its specificity, such that it recognizes a different tetrapeptide signal, something that has also been observed for classical PDZ domains (Ponting *et al.*, 1997).

Stoichiometry of GRASP65 and GM130

Fractionation of the complex containing GM130 and GRASP65 in detergent extracts of Golgi membranes by gel filtration gave an apparent molecular mass of 1.2 MDa, similar to that obtained for the complex reconstituted by *in vitro* translation of the two proteins. Since GM130, based on its sequence, is thought to form a long rod-like structure similar to the myosin heavy chain (Nakamura *et al.*, 1995), these values are unlikely to represent the true molecular weight of the complex. It is known that elongated rod-like proteins have anomalous behaviour when analysed by gel filtration, compared with the globular protein standards used to calibrate the column, and hence give much higher apparent molecular weights (Ackers, 1970). Consistent with this interpretation is the observation that *in vitro* translated GM130 behaves almost exactly the same as GM130 from Golgi membranes on gel filtration, irrespective of the presence of GRASP65. Thus, from this analysis, it is not possible to estimate the stoichiometry of GRASP65 and GM130 or make any statements as to the presence of other proteins in the complex isolated from Golgi membranes. Under the conditions used, no p115 or giantin were found co-fractionating with this complex, although it should be noted that the extraction conditions were chosen to disrupt low-affinity interactions in order to simplify the analysis of the GRASP65–GM130 complex. Consideration of the interaction of this complex with p115 does give some clues as to the stoichiometry of GM130 and GRASP65. It is known that p115 forms a parallel homodimer comprising a C-terminal coiled-coil rod and a globular N-terminal domain (Sapperstein *et al.*, 1995). The N-terminus of GM130 binds to this p115 homodimer (Nakamura *et al.*, 1997), and is therefore likely to be a dimer itself, something also suggested by its sequence similarity with other proteins either known or thought to form coiled-coil homodimers (Nakamura *et al.*, 1995). It is probable that GRASP65 is also a dimer, an interpretation supported by the behaviour of *in vitro* translated GRASP65 as a protein of 200 kDa, corresponding to a dimer or trimer based on its mol. wt of 65 kDa by SDS–PAGE. This view is supported by observations that PDZ domains can be important for dimerization, and the fact that the form of GRASP65 lacking its PDZ-like domain (Table I, GRASP65Δ152) behaves as a monomer.

Therefore, the minimal complex of GRASP65 and GM130 is likely to consist of a dimer of GRASP65 binding to the C-terminus of a coiled-coil dimer of GM130. This would result in the orientation of the GM130 rod such that the N-terminal p115-binding site projected away from the membrane, possibly enhancing its function in the capture and tethering of vesicles in docking and perhaps cisternal membranes during Golgi stack formation.

Materials and methods

Antibodies
Antibodies were as follows: NN5-1 rabbit polyclonal (Nakamura *et al.*, 1995) and mouse monoclonal 2C10 (Nakamura *et al.*, 1997) raised against the 97 kDa fragment of GM130; mouse monoclonal 53FC3 to Mann II (Burke *et al.*, 1982); and rabbit polyclonals FBA30 and 31 to GRASP65.

SDS–PAGE, Western and far Western blotting
Protein samples were solubilized in SDS–PAGE sample buffer, boiled for 3 min and analysed on 10 and 12% SDS–polyacrylamide gels (Blobel and Dobberstein, 1976). Western blotting was performed using a semi-dry blotter onto Hybond-C (Amersham Life Science, UK). Blocking and antibody incubations were performed in phosphate-buffered saline (PBS) plus 10% (w/v) low fat skim milk powder. All secondary antibodies or streptavidin were horseradish peroxidase (HRP) conjugates (Tago, Buckingham, UK), detected using ECL (Amersham Life Science, UK). Far Westerns were performed in PBS plus 4% (w/v) low fat skim milk powder and 0.2% (w/v) Triton X-100. Blocking incubations were performed for 12–18 h at 4°C, and binding was carried out for 8 h at 4°C. Antibody incubations were for 20 min at room temperature, and all washes were three times for 5 min at room temperature. Detection was using ECL plus (Amersham Life Science, UK).

Constructs
The GM130 constructs in pBluescriptII have been described previously (Nakamura *et al.*, 1997). GRASP65 constructs were made using the Quickchange method (Stratagene, UK) for point mutations, PCR mutagenesis to introduce stop codons for C-terminal deletions, or convenient restriction sites for N-terminal deletions in the pcDNA3.1 mammalian expression vectors (Invitrogen). GFP-tagged constructs were constructed in the pEGFP mammalian expression vectors (Clontech Laboratories Inc.). Details of the primers used are available from the corresponding author.

In vitro transcription–translation
Constructs for *in vitro* transcription–translation were in pcDNA3.1+ for GRASP65 and pBluescriptII for GM130. Reactions of 50 μl were performed with the T7 polymerase for GRASP65 and the T3 polymerase for GM130, according to the manufacturer's instructions (Promega) using 0.5 μg of plasmid DNA and methionine minus amino acid mix plus 4 μl of [^{35}S]L-methionine (typically 1400 Ci/mmol and 11 mCi/ml, ICN Pharmaceuticals Inc.). For *in vitro* translation reactions involving both GRASP65 and GM130 constructs, 50 μl reactions were set up for each construct, these were incubated separately for 60 min at 30°C, then mixed and incubated for a further 2 h at 30°C.

Immunoprecipitations
Immunoprecipitations were performed in IP buffer [20 mM HEPES–KOH pH 7.3, 200 mM KCl, 0.5% (w/v) Triton X-100] using 4 μl of the appropriate antiserum and either 10 μl of packed protein A– or protein G–Sepharose (Pharmacia) for rabbit polyclonal and mouse monoclonal antibodies, respectively. After binding for 60 min at 4°C, the beads were washed four times with 500 μl of IP buffer, eluted with 30 μl of SDS–PAGE sample buffer, and the eluate analysed as appropriate.

p115 binding assay
Golgi membranes (10 μg) were extracted in 200 μl of IP buffer for 15 min on ice then centrifuged at 14 000 r.p.m. for 2 min in an Eppendorf microfuge to remove any insoluble material. Binding assays were performed in a total volume of 200 μl using 10 μl of packed p115 beads and 200 μl of Golgi membrane extract. After rotating for 60 min at 4°C to allow any interactions to form, the beads were washed four times with 500 μl of IP buffer, eluted with 30 μl of SDS–PAGE sample buffer, and the eluate analysed by SDS–PAGE and Western blotting.

Cell-free incubations
Standard disassembly assays were carried out using interphase or mitotic HeLa cytosols at a final concentration of 10 mg/ml and 50 μg of purified rat liver Golgi membranes, as described previously (Misteli and Warren, 1994). After incubations with cytosol, Golgi membranes were recovered

by centrifugation through a 1 ml cushion of 0.5 M sucrose in 100 mM potassium phosphate, pH 6.7, at 55 000 r.p.m. for 20 min at 4°C in the TLS55 rotor.

Gel filtration

Samples to be analysed by gel filtration were adjusted to 500 µl and injected on to a Superose 6 HR10/30 column (Pharmacia) pre-equilibrated in 20 mM HEPES–KOH pH 7.3, 200 mM KCl, 0.25% (w/v) Triton X-100. Fractions of 1.0 ml were collected at a flow rate of 0.3 ml/min. Aliquots of each fraction were analysed by SDS–PAGE and either Western blotting or autoradiography as appropriate.

Transient transfections and immunofluorescence

HeLa cells were plated at 30% density on glass coverslips in 2 cm wells and left to attach for 12 h. They were then transfected using lipofectin (Gibco-BRL, UK) according to the manufacturer's instructions. After 28 h, the cells were fixed in 3% paraformaldehyde and processed for immunofluorescence with appropriate antibodies.

Acknowledgements

References

Ackers,G.K. (1970) Analytical gel chromatography of proteins. *Adv. Protein Chem.*, **24**, 343.

Amos,W.B. and Grimstone,A.V. (1968) Intercisternal material in the Golgi body of *Trichomonas*. *J. Cell Biol.*, **38**, 466–477.

Barr,F.A. and Warren,G. (1997) Disassembly and reassembly of the Golgi apparatus. *Semin. Cell Dev. Biol.*, **7**, 505–510.

Barr,F.A., Puype,M., Vandekerckhove,J. and Warren,G.B. (1997) GRASP65, a protein involved in the stacking of Golgi cisternae. *Cell*, **91**, 253–262.

Bhatnagar,R.S. and Gordon,J.I. (1997) Understanding covalent modification of proteins by lipids: where cell biology and biophysics mingle. *Trends Cell Biol.*, **7**, 14–20.

Blobel,G. and Dobberstein,B. (1976) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed immunoglobulin light chains on membrane-bound ribosomes of murine melanoma. *J. Cell Biol.*, **67**, 1621–1628.

Burke,B., Griffiths,G., Reggio,H., Louvard,D. and Warren,G. (1982) A monoclonal antibody against a 135-K Golgi membrane protein. *EMBO J.*, **1**, 1621–1628.

Cluett,E.B. and Brown,W.J. (1992) Adhesion of Golgi cisternae by proteinaceous interactions: intercisternal bridges as putative adhesive structures. *J. Cell Sci.*, **103**, 773–784.

Cowburn,D. (1996) Adaptors and integrators. *Structure*, **4**, 1005–1008.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Franke,W.W., Kartenbeck,J., Krien,S., VanderWoude,W.J., Scheer,U. and Morre,D.J. (1972) Inter- and intracisternal elements of the Golgi apparatus. A system of membrane-to-membrane cross-links. *Z. Zellforsch. Mikrosk. Anat.*, **132**, 365–380.

Fritzler,M.J., Hamel,J.C., Ochs,R.L. and Chan,E.K. (1993) Molecular characterization of two human autoantigens: unique cDNAs encoding 95- and 160-kD proteins of a putative family in the Golgi complex. *J. Exp. Med.*, **178**, 49–62.

Fritzler,M.J., Lung,C.C., Hamel,J.C., Griffith,K.J. and Chan,E.K. (1995) Molecular characterization of Golgin-245, a novel Golgi complex protein containing a granin signature. *J. Biol. Chem.*, **270**, 31262–31268.

Golgi,C. (1898) Sur la structure des cellules nerveuses. *Arch. Ital. Biol.*, **30**, 60–71.

Johnson,D.R., Bhatnagar,R.S., Knoll,L.J. and Gordon,J.I. (1994) Genetic and biochemical studies of protein *N*-myristoylation. *Annu. Rev. Biochem.*, **63**, 869–914.

Kooy,J., Toh,B.H., Pettitt,J.M., Erlich,R. and Gleeson,P.A. (1992) Human autoantibodies as reagents to conserved Golgi components. Characterization of a peripheral, 230-kDa compartment-specific Golgi protein. *J. Biol. Chem.*, **267**, 20255–20263.

Kornau,H.C., Schenker,L.T., Kennedy,M.B. and Seeburg,P.H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science*, **269**, 1737–1740.

Levine,T.P., Rabouille,C., Kieckbusch,R.H. and Warren,G. (1996) Binding of the vesicle docking protein p115 to Golgi membranes is inhibited under mitotic conditions. *J. Biol. Chem.*, **271**, 17304–17311.

Linstedt,A.D. and Hauri,H.P. (1993) Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell*, **4**, 679–693.

Lucocq,J.M., Pryde,J.G., Berger,E.G. and Warren,G. (1987) A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.*, **104**, 865–874.

Misteli,T. and Warren,G. (1994) COP-coated vesicles are involved in the mitotic fragmentation of Golgi stacks in a cell-free system. *J. Cell Biol.*, **125**, 269–282.

Misumi,Y., Sohda,M., Yano,A., Fujiwara,T. and Ikehara,Y. (1997) Molecular characterization of GCP170, a 170-kDa protein associated with the cytoplasmic face of the Golgi membrane. *J. Biol. Chem.*, **272**, 23851–23858.

Mollenhauer,H.H. (1965) An intercisternal structure in the Golgi apparatus. *J. Cell Biol.*, **24**, 504–511.

Moremen,K.W., Touster,O. and Robbins,P.W. (1991) Novel purification of the catalytic domain of Golgi α-mannosidase II. *J. Biol. Chem.*, **266**, 16876–16885.

Nakamura,N., Rabouille,C., Watson,R., Nilsson,T., Hui,N., Slusarewicz,P., Kreis,T.E. and Warren,G. (1995) Characterization of a *cis*-Golgi matrix protein, GM130. *J. Cell Biol.*, **131**, 1715–1726.

Nakamura,N., Lowe,M., Levine,T.P., Rabouille,C. and Warren,G. (1997) The vesicle docking protein p115 binds GM130, a *cis*-Golgi matrix protein, in a mitotically regulated manner. *Cell*, **89**, 445–455.

Ponting,C.P. (1997) Evidence for PDZ domains in bacteria, yeast, and plants. *Protein Sci.*, **6**, 464–468.

Ponting,C.P., Phillips,C., Davies,K.E. and Blake,D.J. (1997) PDZ domains: targeting signalling molecules to sub-membranous sites. *BioEssays*, **19**, 469–479.

Rambourg,A. and Clermont,Y. (1990) Three-dimensional electron microscopy: structure of the Golgi apparatus. *Eur. J. Cell Biol.*, **51**, 189–200.

Sapperstein,S.K., Walter,D.M., Grosvenor,A.R., Heuser,J.E. and Waters,M.G. (1995) p115 is a general vesicular transport factor related to the yeast endoplasmic reticulum to Golgi transport factor Uso1p. *Proc. Natl Acad. Sci. USA*, **92**, 522–526.

Saras,J. and Heldin,C.H. (1996) PDZ domains bind carboxy-terminal sequences of target proteins. *Trends Biochem. Sci.*, **21**, 455–458.

Seelig,H.P., Schranz,P., Schroter,H., Wiemann,C. and Renz,M. (1994) Macrogolgin—a new 376 kD Golgi complex outer membrane protein as target of antibodies in patients with rheumatic diseases and HIV infections. *J. Autoimmun.*, **7**, 67–91.

Slusarewicz,P., Nilsson,T., Hui,N., Watson,R. and Warren,G. (1994) Isolation of a matrix that binds medial Golgi enzymes. *J. Cell Biol.*, **124**, 405–413.

Songyang,Z. *et al.* (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science*, **275**, 73–77.

Thyberg,J. and Moskalewski,S. (1985) Microtubules and the organization of the Golgi complex. *Exp. Cell Res.*, **159**, 1–16.

Waters,M.G., Clary,D.O. and Rothman,J.E. (1992) A novel 115-kD peripheral membrane protein is required for intercisternal protein transport in the Golgi stack. *J. Cell Biol.*, **118**, 1015–1026.