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GRASP55, a second mammalian GRASP protein involved in the stacking of Golgi cisternae in a cell-free system

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We have identified a 55 kDa protein, named GRASP55 (Golgi reassembly stacking protein of 55 kDa), as a component of the Golgi stacking machinery. GRASP55 is homologous to GRASP65, an *N*-ethylmaleimide-sensitive membrane protein required for the stacking of Golgi cisternae in a cell-free system. GRASP65 exists in a complex with the vesicle docking protein receptor GM130 to which it binds directly, and the membrane tethering protein p115, which also functions in the stacking of Golgi cisternae. GRASP55 binding to GM130, could not be detected using biochemical methods, although a weak interaction was detected with the yeast two-hybrid system. Cryo-electron microscopy revealed that GRASP65, like GM130, is present on the *cis*-Golgi, while GRASP55 is on the *medial*-Golgi. Recombinant GRASP55 and antibodies to the protein block the stacking of Golgi cisternae, which is similar to the observations made for GRASP65. These results demonstrate that GRASP55 and GRASP65 function in the stacking of Golgi cisternae.

Keywords: cisternae/GM130/Golgi/GRASP65/stacking

Introduction

In mammalian cells, the Golgi apparatus is composed of a highly ordered parallel array of cisternae which form a stacked structure typically found in the perinuclear region of the cell (Rambourg and Clermont, 1997). The major functions of the Golgi apparatus are thought to be in the synthesis of the complex carbohydrate structures that are attached to many cellular and secretory proteins and lipids, and in the sorting of these proteins and lipids to their correct subcellular destinations (Mellman and Simons, 1992). The ordered structure of the Golgi apparatus is thought to reflect the requirement for these enzymes and of the protein sorting machinery to be compartmentalized to allow a specific series of post-translational modifications and sorting reactions to be carried out (Farquhar, 1985). In recent years a number of studies have identified components that act in the fusion of Golgi membranes to give rise to cisternae, and in the subsequent stacking of these cisternae to form stacks. These studies have used *in vitro* assays in which the Golgi apparatus can be disassembled and reassembled under defined conditions, thus allowing the identification of components important for different aspects of Golgi structure (Acharya *et al.*, 1995; Rabouille *et al.*, 1995a). One cell-free system (Rabouille *et al.*, 1995a) has exploited the disassembly of the Golgi apparatus into many small vesicles and membrane fragments during cell division (Lucocq *et al.*, 1987, 1989). In this system, isolated Golgi membranes are treated with mitotic cell cytosol to generate a population of mitotic Golgi fragments (MGFs) that can reassemble into stacked Golgi membranes when incubated under the correct conditions. The *N*-ethylmaleimide (NEM)-sensitive factor (NSF), its cofactors the soluble NSF attachment proteins (SNAPs) and the vesicle tethering protein p115 act in conjunction with p97 and its cofactor p47 to rebuild cisternae from MGFs (Rabouille *et al.*, 1995b, 1998).

Electron microscopic studies on cells and isolated Golgi membranes have described filamentous structures, bridging the gaps between adjacent cisternae, that may be components of a structural exoskeleton helping to hold them together (Franke *et al.*, 1972; Cluett and Brown, 1992). There are many proteins that are candidates for components of these filamentous structures. One group of proteins is the golgins, a large family of coiled-coil proteins including GM130 and giantin originally described as autoantigens, either peripherally or integrally associated with the Golgi apparatus (Chan and Fritzler, 1998). Another group of proteins are more often thought of as components of the actin cytoskeleton; these are the recently identified Golgi-localized isoforms of ankyrin and spectrin (Beck *et al.*, 1994; Devarajan *et al.*, 1996; Stankewich *et al.*, 1998). Studies using the system for the reassembly of stacked Golgi apparatus membranes from MGFs have begun to provide some clues as to the nature of the filamentous bridge structures. When MGFs are pre-treated with the alkylating agent NEM they can reassemble in the presence of either interphase cytosols or the purified membrane fusion components NSF, SNAPs, p115 and p97 to give single cisternae, but not stacks of cisternae (Rabouille *et al.*, 1995a,b). This observation was used to identify GRASP65 (Golgi reassembly stacking protein of 65 kDa), an NEM-sensitive membrane component required for the stacking of cisternae (Barr *et al.*, 1997). Two other membrane components are known to be required for stacking, GM130 and giantin, something that is distinct from their role in the NSF pathway of membrane fusion (Shorter and Warren, 1999). GRASP65 exists in Golgi membranes as part of a complex with GM130 (Barr *et al.*, 1998), which acts as the receptor for p115 during the docking of vesicles with Golgi membranes (Nakamura

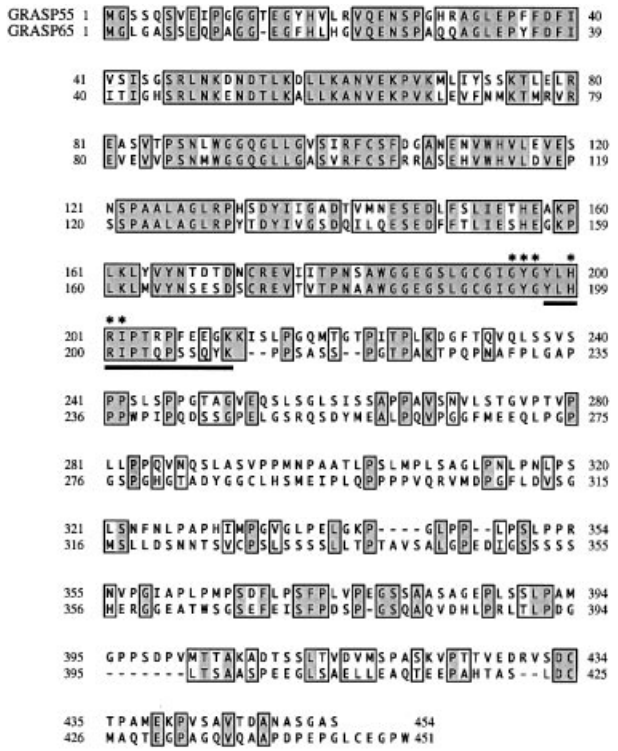

Fig. 1. Comparison of the GRASP55 and GRASP65 sequences. Alignment of the GRASP55 (AF110267) and GRASP65 (AF015264) sequences; shading indicates identity and boxed residues are conserved. Residues in GRASP65 important for GM130 binding are indicated by asterisks. Underlined residues indicate the peptide used to raise the FBA19 antiserum.

et al., 1997; Sönnichsen *et al.*, 1998). During vesicle docking, p115 is thought to bind to giantin on the vesicle, and GM130 on the target Golgi membrane (Sönnichsen *et al.*, 1998), thus tethering the two membranes together and allowing specific membrane fusion to occur. A recent study has shown that p115 also functions upstream of GRASP65 in the stacking of Golgi cisternae (Shorter and Warren, 1999). The p115–GM130–GRASP65 complex together with giantin might therefore act in a specialized docking pathway bringing cisternae together at the *cis*-face of the Golgi apparatus, then handing them over to other protein complexes that would hold later Golgi cisternae together.

Results

Cloning of a mammalian GRASP65-related protein

During the course of previous studies on GRASP65 a number of antibodies have been raised against either the full-length protein expressed in bacteria, or synthetic peptides. One of these antibodies, FBA19, raised against the sequence YLHRIPTQPSSQYK (underlined in Figure 1), which is conserved amongst the known forms of GRASP65 from various yeasts and *Caenorhabditis elegans*, recognizes 65 and 55 kDa proteins in rat liver Golgi membranes, as shown below. Other antibodies to GRASP65 that recognize epitopes in the less well conserved C-terminal domain of the protein recognize only a 65 kDa protein in Golgi membranes (Barr *et al.*, 1997). This led us to believe that there might be a second form of GRASP65, a view supported by the existence of expressed sequence tags (ESTs) with only partial homology to the known rat GRASP65 sequence. A RACE cloning strategy was adopted in order to obtain a clone for the putative GRASP65 homologue, using nested primers designed from a mouse testis EST (DDBJ/EMBL/GenBank accession number AA061790). To obtain 5′ and 3′ clones corresponding to this GRASP65-related protein, nested primer pairs TR1 to TR4 and adaptor primers AP-1 and AP-2 were used. These clones were sequenced and a new pair of primers designed to amplify the full open reading frame. Analysis of the sequence and predicted open reading frame of this clone revealed that it had a high level of homology, but not identity with GRASP65 in the first 212 amino acids, and after this point became highly divergent (Figure 1). The cDNA encodes a 454 amino acid protein with a predicted molecular weight of 55 kDa, confirmed by *in vitro* translation (Figure 6A, lane 9) and Western blotting with specific antibodies (Figure 3A, lane 1), and it was therefore named GRASP55. Like GRASP65, it has a consensus site for myristoylation at the N-terminus and could be anchored to membranes by means of this modification (Barr *et al.*, 1997). The sequence in this N-terminal first 21 amino acids is slightly divergent in the two proteins, and GRASP55 has an insertion at position 14, the only point in the first 212 amino acids at which the two proteins are not co-linear. This raises the possibility that they are not targeted to the same regions of the Golgi apparatus, or that their interaction with membranes is differentially regulated. Comparison of the first 212 amino acids of GRASP65 and 213 amino acids of GRASP55 reveals that 66% of residues are identical and 14% conserved in this region, with the residues currently known to be important for GM130 binding being identical (asterisks in Figure 1; Barr *et al.*, 1998). The region against which the FBA19 antibody was raised is partially conserved between the two proteins, YLHRIPTRPFEEGK in GRASP55 and YLHRIPTQPSSQYK in GRASP65 (underlined in Figure 1), thereby explaining why it gives rise to two bands in purified Golgi membranes.

To determine the expression patterns of GRASP65 and GRASP55, Northern blots on a panel of rat tissues were performed using a combination of probes specific or common to the two mRNAs (Figure 2). A probe specific for GRASP65 recognized a message at the correct size in all tissues, although only very faintly in spleen (Figure 2A). As previously reported, a second message of ~1.5 kb was observed in testis. The GRASP55-specific probe detected two messages of slightly smaller size than seen by the GRASP65 probe, again present in all tissues (Figure 2B). The lower band of this doublet was especially noticeable in liver, but was visible in other tissues upon longer exposure of the blot. In testis, the lower band of the doublet was more prominent than the upper band, in contrast to other tissues, and a second, smaller message of ~1.4 kb was also observed. A probe common to both GRASP65 and GRASP55 gave a complex pattern of bands corresponding to the addition of the signals seen with the GRASP65- and GRASP55-specific probes (Figure 2C). An actin probe used as a control for the loading of the Northern blot gave the expected pattern of hybridization (Figure 2D). Therefore, messages for GRASP65 and GRASP55 would appear to be present in all tissues

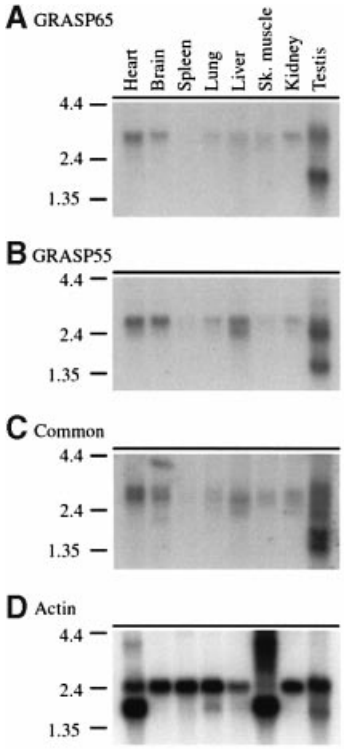

Fig. 2. GRASP55 and GRASP65 are both ubiquitously expressed. A Northern blot of the rat tissues indicated in the figure was hybridized with probes specific for (**A**) GRASP65, (**B**) GRASP55 and (**C**) a probe common to both GRASP65 and GRASP55. As a control the blot was also hybridized with an actin-specific probe (**D**).

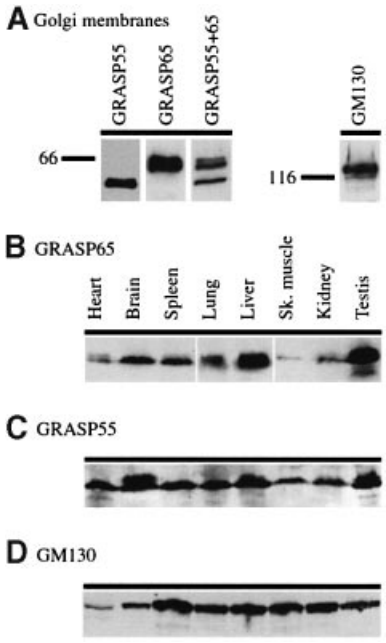

Fig. 3. Tissue Western blots for GRASP55 and GRASP65. (**A**) Purified Golgi membranes were Western blotted for GRASP55, polyclonal FBA34, GRASP65, monoclonal 7E10, GRASP55 + GRASP65, polyclonal FBA19 or GM130, polyclonal MLO-7). Western blots of the tissues indicated in the figure were probed with these antibodies specific for either GRASP65 (**B**), GRASP55 (**C**) or GM130 (**D**).

tested, with multiple possible splice variants being present in testis.

To confirm that the proteins are actually expressed, Western blots were performed using protein extracts from these tissues. These Western blots were probed with antibodies specific for GRASP65, GRASP55 and GM130 (Figure 3). To demonstrate that these antibodies are in fact specific for the GRASP65 and GRASP55, purified rat liver Golgi membranes were blotted with these and with an antibody that sees both proteins. In Golgi membranes, the sheep polyclonal FBA34 recognizes a 55 kDa protein, the 7E10 monoclonal to GRASP65 sees a 65 kDa protein and the FBA19 antibody recognizes both GRASP65 and GRASP55 (Figure 3A). The antibody to GRASP65 detected a protein of 65 kDa in all tissues, with a fainter second band in testis at ~60 kDa possibly corresponding to the second messenger RNA seen in this tissue (Figure 3B). Similar results were obtained with an antibody to GRASP55; this detected a 55 kDa band in all tissues (Figure 3C). Blotting for the GRASP65 partner protein GM130 revealed that this protein is also present in all the tissues examined (Figure 3D). Together, these data demonstrate that GRASP55 is ubiquitously expressed in mammalian tissues, and therefore, like GRASP65, could act in the stacking of Golgi cisternae.

Localization of GRASP55 to the Golgi apparatus

The N-terminal domain of GRASP65 when fused to green fluorescent protein (GFP) can target to the Golgi apparatus, something that is abolished by mutations in its GM130 binding site (Barr *et al.*, 1998). Given the similarity between the N-terminal domains of GRASP65 and GRASP55, it was likely that GRASP55 would also target the Golgi apparatus. To test this, full-length GRASP65 and full-length GRASP55 fused to GFP were transfected into HeLa cells either singly or together (Figure 4). When HeLa cells were transfected with constructs for either rat GRASP65 (Figure 4A) or GRASP55–GFP (Figure 4B), a pattern typical of the perinuclear ribbon-like structure of the Golgi apparatus was observed. To confirm that this was indeed the Golgi apparatus, equivalent samples were stained with antibodies to the Golgi marker protein GM130, and GRASP65 (Figure 4C), or GRASP55–GFP fluorescence visualized (Figure 4D). Comparison of the individual staining patterns reveals that GRASP55, like GRASP65 and GM130, is localized to the Golgi apparatus. Cells were also transfected with both GRASP65 and GRASP55–GFP to allow a direct comparison of the distributions of these two proteins (Figure 4E). Comparison of the two images shows that GRASP65 and GRASP55 have similar distributions within the Golgi apparatus. The targeting of GRASP65 to the Golgi apparatus requires its N-terminal myristoylation site, and a series of residues in the domain which is involved in binding to GM130 (Barr *et al.*, 1998). To find out if the targeting of GRASP55 was similar to that of GRASP65, point mutants were constructed analogous to those known to abolish GRASP65 targeting. When the glycine at position 2 was mutated to alanine, GRASP55 G2A, the protein was found to accumulate in the cytoplasm with only a very weak signal for the Golgi apparatus (Figure 4F). Mutations of two residues that cause a complete loss of binding to GM130 and of Golgi targeting in GRASP65, the G196A and H199A mutations (Barr *et al.*, 1998), caused only a

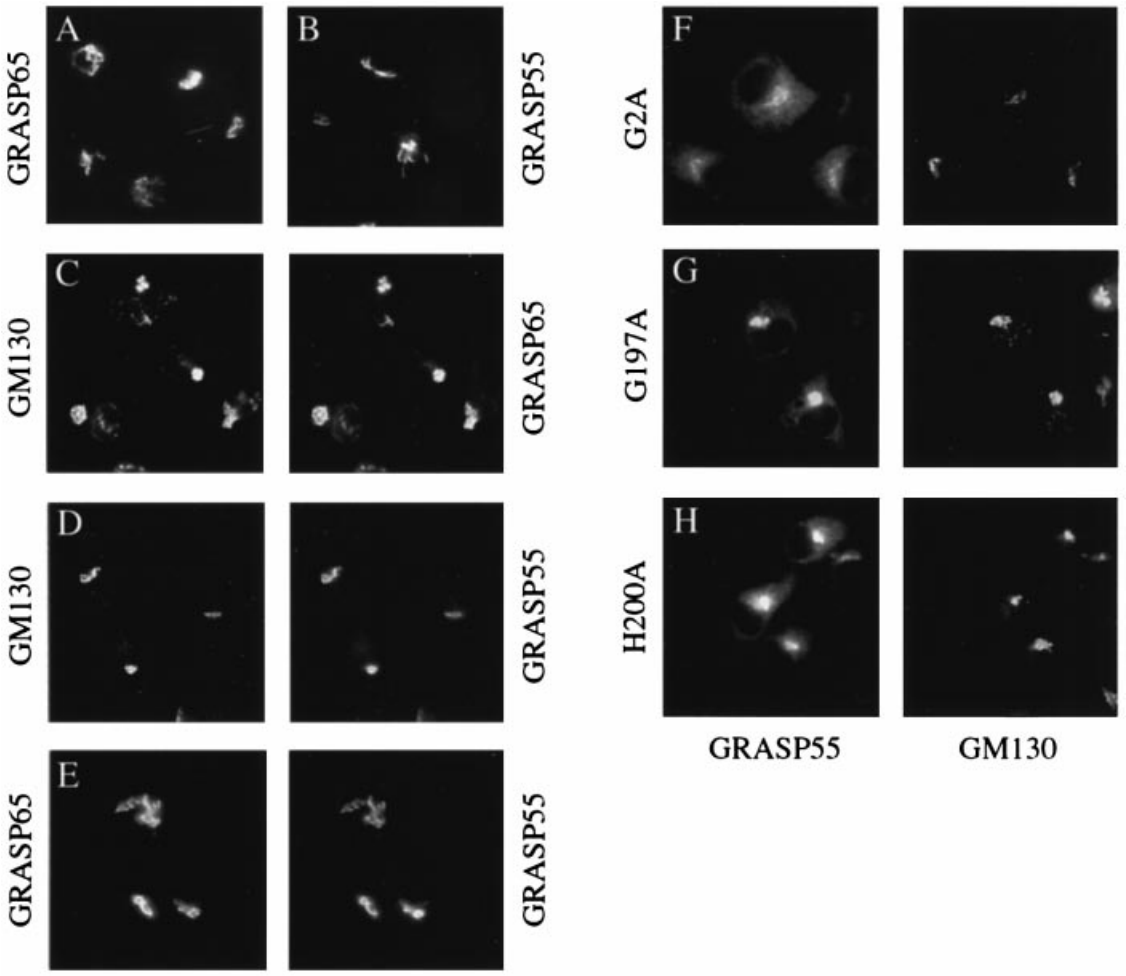

Fig. 4. Localization of GRASP55 to the Golgi apparatus by immunofluorescence. HeLa cells were transfected with rat GRASP65 (**A** and **C**), rat GRASP55–GFP (**B** and **D**), rat GRASP65 and GRASP55–GFP (**E**) or the GRASP55–GFP mutants G2A (**F**), G197A (**G**) and H200A (**H**). Cells were processed for immunofluorescence with antibodies to rat GRASP65 only (A and E), rat GRASP65 and GM130 (C) or GM130 only (D, F–H). GFP fluorescence was used to visualize GRASP55 (B, D–H).

partial defect in Golgi targeting when introduced into GRASP55 G197A (Figure 4G) and H200A (Figure 4H), seen as an increased diffuse cytoplasmic fluorescence relative to the wild-type protein (Figure 4B). These data show that GRASP55 localizes to the Golgi apparatus, and that this requires the N-terminal myristoylation site. Unlike GRASP65, mutations in the putative GM130 binding region have only a small effect on the Golgi localization of GRASP55, implying that this does not require interaction with a target protein at this site, or that the mutations have no effect on binding of GRASP55 to its target protein.

To determine where GRASP55 and GRASP65 were localized within the Golgi apparatus, antibody labelling on cryo-sections of HeLa cells transfected with rat GRASP65 and GRASP55–GFP was performed (Figure 5). Under the electron microscope it could be seen that the labelling for both proteins was over the Golgi apparatus, consistent with the localization at the light microscope level. GRASP65 labelling was typically seen over the *cis*-face of the stack (Figure 5A), although it cannot be ruled out that it is present further into the stack but is simply not accessible to antibodies. The observation that GRASP65 only becomes accessible to the small alkylating agent NEM after treatment of stacked Golgi membranes with mitotic cytosol indicates that it is sequestered in some protein complex in these structures, and indicates that it may not be easily accessible to some antibodies under native conditions. GRASP55 labelling was found to be more over the stack with some labelling over the *cis*-face of the Golgi (Figure 5B), and was at a level similar to GRASP65. Double-labelling experiments confirm that GRASP65 labelling (Figure 5C and D, large gold particles), marked by the arrows, is over the *cis*-face of the Golgi stack, while GRASP55 labelling (Figure 5C and D, small gold particles) is seen over the stack and at the *cis*-Golgi cisternae. The labelling efficiency for GRASP65 in the double-labelling experiments was lower than that seen for GRASP55–GFP, and also lower than for GRASP65 detected by a polyclonal antibody in single-labelling experiments. This might be explained by the use of a monoclonal antibody to detect GRASP65, which by definition sees only a single epitope, in the double-labelling experiments, as opposed to the use of a polyclonal antibody, likely to recognize multiple epitopes, to detect GRASP55–GFP. In order to be able to compare the localizations of GRASP65 and GRASP55 within the Golgi apparatus, the distributions of gold particles corresponding to antibody labelling of the proteins were quantitated. Due

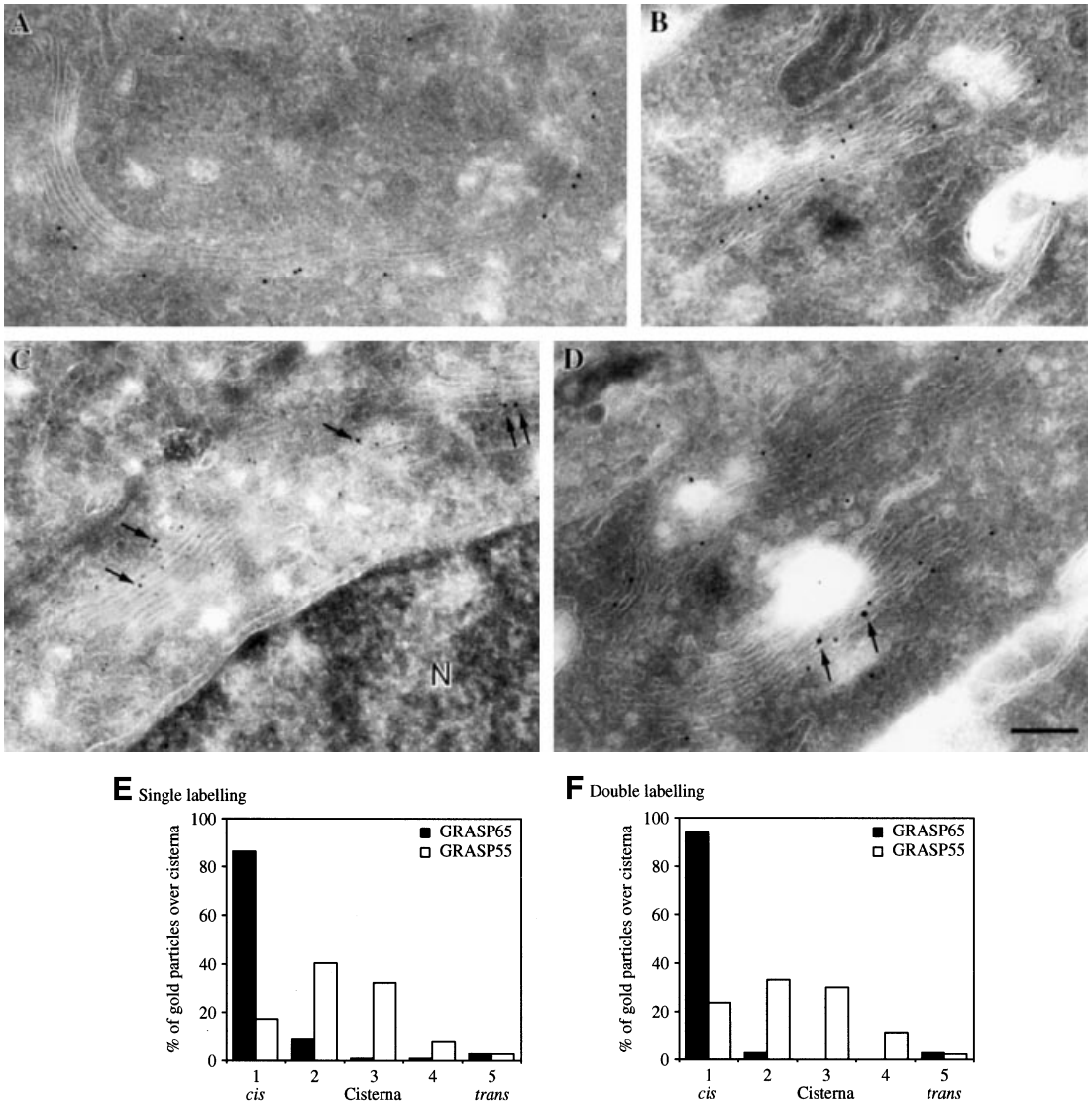

Fig. 5. Localization of GRASP55 and GRASP65 within the Golgi apparatus by cryo-electron microscopy. HeLa cells transfected with rat GRASP65 and GRASP55–GFP were processed for cryo-electron microscopy. Cryosections were labelled with a rabbit polyclonal FBA31 or the 7E10 monoclonal antibody to rat GRASP65, or a polyclonal antibody to GFP to detect GRASP55–GFP. A panel of images is shown: (**A**) single FBA31 labelling for GRASP65; (**B**) single GFP labelling for GRASP55; (**C** and **D**) double labelling for GRASP65 with 7E10, large gold particles marked by arrows, and GRASP55 with anti-GFP, small gold particles. N marks the nucleus in (C) and the scale bar denotes 0.25 μM. Distributions of gold particles over Golgi cisternae were quantitated for GRASP65 and GRASP55 in both single (**E**) and double (**F**) labelling experiments. The percentage of gold particles is plotted for each Golgi cisterna from *cis* (1) to *trans* (5).

to the differences in labelling efficiencies discussed above this was carried out for both the single- and double-labelling experiments, and the results compared. For the single labellings, the distribution of gold particles over 22 (GRASP65; 133 gold particles) and 20 (GRASP55; 147 gold particles), Golgi regions with a defined *cis* to *trans* polarity were quantitated. Plotting these distributions as a function of cisterna reveals that GRASP65 (Figure 5E, shaded bars) is present over the first cisterna, while GRASP55 (Figure 5E, open bars) is mainly present over the second and third cisternae. For the double labellings, the distribution of gold particles for GRASP65 (34 gold particles) and GRASP55 (97 gold particles) over 14 Golgi regions with a defined *cis* to *trans* polarity were quantitated. Plotting these distributions as a function of cisterna reveals that GRASP65 (Figure 5F, shaded bars) is present over the first cisterna, while GRASP55 (Figure 5F, open bars) is mainly present over the second and third cisternae. From these data it appears that GRASP65 is located at the *cis*-face of the Golgi, while GRASP55 is found more over the stack. This is consistent for GRASP65 with the localization of its binding partner GM130, to the *cis*/*medial*-Golgi at the electron microscope level (Nakamura *et al.*, 1995).

GRASP65 and GRASP55 binding to GM130

Given the high degree of homology between the two proteins in the region previously shown to be important for binding to GM130, it seemed likely that, like GRASP65, GRASP55 would interact with GM130. To test this, the same *in vitro* transcription–translation system developed to demonstrate and characterize the interaction between GRASP65 and GM130 was used (Barr *et al.*, 1998). *In vitro* translation reactions were carried out with plasmids

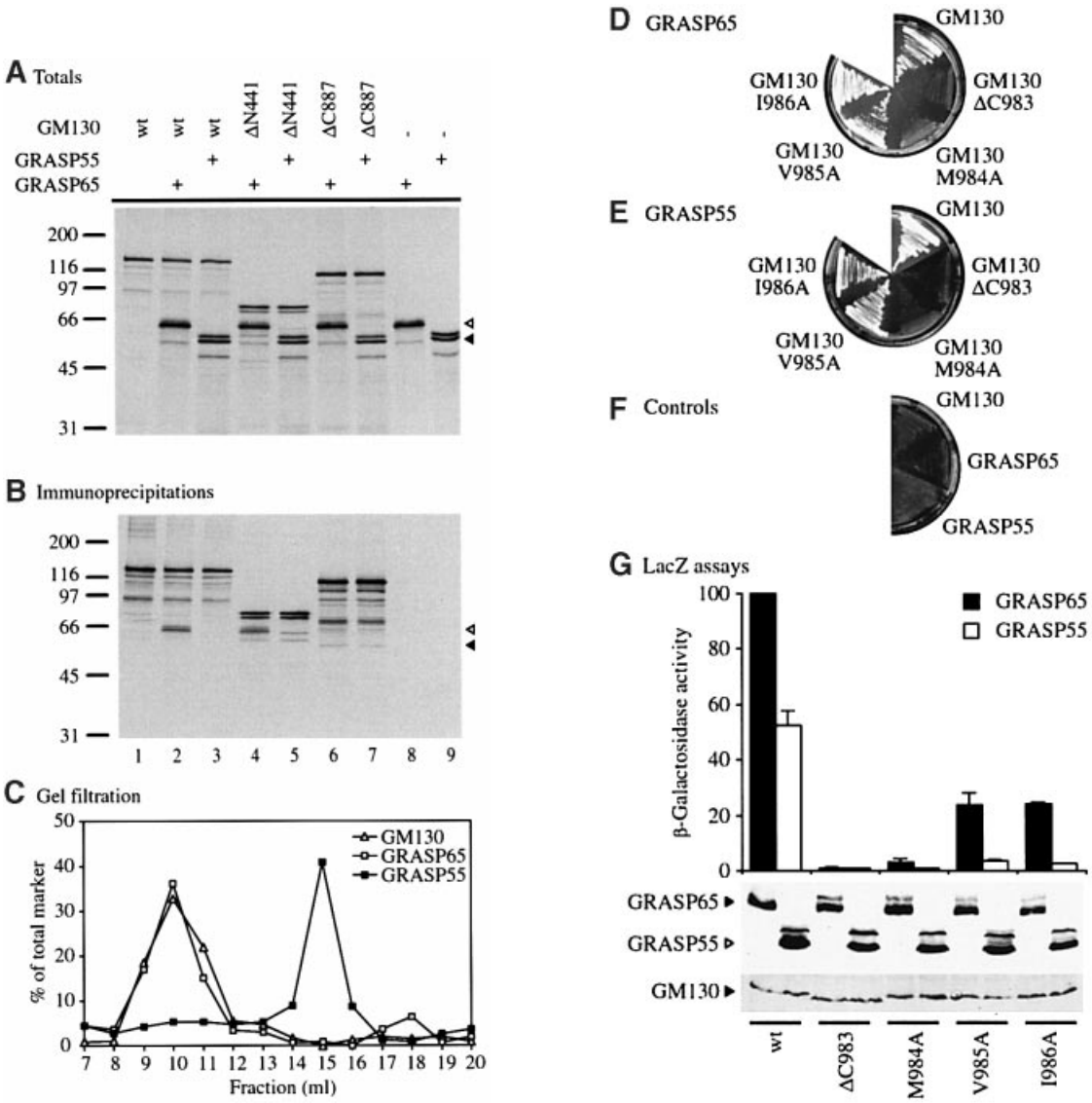

Fig. 6. GRASP65 and GRASP55 binding to GM130. Transcription–translation assays were performed using plasmids encoding GM130, GRASP65 or GRASP55 alone, or GRASP65 and GRASP55 with full-length GM130 or the N- and C-terminal deletions ΔN441 and ΔC887. Immunoprecipitations were performed using antibodies to GM130 with either 5 or 10 μl for single and co-*in vitro* translations, respectively. Aliquots of the total (**A**) and immunoprecipitated (**B**) material were analysed by SDS–PAGE and autoradiography. The positions of GRASP65 and GRASP55 are marked by an open and closed triangle, respectively, in (A) and (B). (**C**) Golgi membranes (10 μg), were extracted in buffer 20 mM HEPES–KOH pH 7.3, 200 mM KCl, 0.5% (w/v) Triton X-100. This extract was then fractionated by gel filtration over Superose 6, collecting 1 ml fractions. Aliquots of each fraction were analysed by SDS–PAGE and Western blotting with antibodies to either GM130 MLO-7 (open triangles), GRASP65 7E10 (open squares) or GRASP55 FBA32 (closed squares). The distribution of each marker is plotted as a percentage in a given fraction of the total signal for that marker. Two-hybrid assays were carried out using GRASP65 (**D**) and GRASP55 (**E**) as the bait constructs. Wild-type GM130 C-terminal domain, a C-terminal deletion ΔC887 and three point mutants M984A, V985A and I986A were used as prey constructs. Controls using only GRASP65, GRASP55 and GM130 together with the appropriate empty vector are shown in (**F**). To test for two-hybrid interactions, strains were streaked on to synthetic media lacking leucine, tryptophan, histidine and adenine. Representative examples of such plates are shown in (D), (E) and (F). The strength of these two-hybrid interactions was quantitated using liquid assays for β-galactosidase (**G**). Strains were Western blotted for the GRASP65 and GRASP55 prey constructs, and the GM130 bait constructs (G).

encoding wild-type, and ΔN441 and ΔC887 deletions of GM130, GRASP65 and GRASP55 as indicated in the legend to Figure 6. Aliquots of the total reactions were analysed by SDS–PAGE and autoradiography, the position of GRASP65 is marked by an open triangle and that of GRASP55 by a filled triangle (Figure 6A and B). To assay for an interaction between GM130 and GRASP65 or GRASP55, immunoprecipitations were performed from these *in vitro* translation reactions with antibodies to GM130. Analysis of the bound material revealed that antibodies to GM130 precipitated GM130 (Figure 6B, lane 1) but not GRASP65 or GRASP55 (Figure 6B, lanes 8 and 9). If GRASP65 was translated together with GM130 it was found to be co-precipitated by antibodies to GM130 (Figure 6B, lane 2), whereas under the same conditions GRASP55 was not co-precipitated with GM130 (Figure 6B, lane 3). Deletion of the N-terminus of GM130, ΔN441, had no effect on the binding of GRASP65 to GM130 (Figure 6B, lane 4), while deletion of three amino acids from the C-terminus of GM130, ΔC887, abolished this interaction (Figure 6B, lane 6). Again, GRASP55 was unable to bind either of these truncated forms of GM130

(Figure 6B, lanes 5 and 7). Therefore, while GRASP65 can specifically bind to the C-terminus of GM130, GRASP55 is unable to under these conditions. To investigate this further, we decided to see if GRASP55 exists in a complex with GM130 and GRASP65 when isolated from stacked Golgi membranes. Golgi membranes were extracted in a salt- and detergent-containing buffer, fractionated by gel filtration, and the distributions of GRASP65, GRASP55 and GM130 determined by Western blotting (Figure 6C). GM130 (Figure 6C, open triangles) and GRASP65 (Figure 6C, open squares) were found to co-fractionate by gel filtration, existing as a complex of ~1200 kDa, as reported previously (Barr *et al.*, 1998). In contrast, GRASP55 (Figure 6C, closed squares) behaved as a 200 kDa protein by gel filtration, clearly resolved from GRASP65 and GM130. These observations are consistent with GRASP55 existing as part of a complex discrete from that containing GM130 and GRASP65. Because the residues known to be important for GM130 binding in GRASP65 are conserved in GRASP55, the inability to detect an interaction between GRASP55 and GM130 was unexpected. The two most likely possible explanations of this are that GRASP55 either does not bind to GM130, or that it binds to GM130 with much lower affinity than GRASP65. We decided to use the yeast two-hybrid to see if there is a low affinity interaction between GRASP55 and GM130 that was not detected with biochemical approaches. The interaction between GRASP65 and GM130 was used as a control, since this has been well characterized by other methods. A GRASP65 construct was transformed into a yeast reporter strain together with plasmids for wild-type GM130, or forms unable to bind (GM130ΔC983, M984A) or reduced in their ability to bind (GM130V985A, I986A) GRASP65 (Figure 6D). These strains were tested for the ability to grow under conditions that select for the activation of the reporter genes, indicative of a two-hybrid interaction (Figure 6D). Wild-type GM130 or mutants (Figure 6D, GM130V985A, I986A) with 50% reduced binding to GRASP65 were able to grow, indicating that these proteins could interact with GRASP65. Mutations that abolish the ability of GM130 to bind to GRASP65 (Figure 6D, GM130ΔC983, M984A) could not grow, indicating there was no longer any interaction between these forms of GM130 and GRASP65. A GRASP55 construct was transformed into a yeast reporter strain together with the plasmids for GM130 used above (Figure 6E). Wild-type GM130 or mutants (Figure 6E, GM130V985A, I986A) with 50% reduced binding to GRASP65 were able to grow, indicating that these proteins could interact with GRASP55. Mutations that abolish the ability of GM130 to bind to GRASP65 (Figure 6E, GM130ΔC983, M984A) could not grow, indicating that there was no longer any interaction between these forms of GM130 and GRASP55. Controls where either the empty bait or prey plasmids were tested for their ability to activate wild-type GM130, GRASP55 or GRASP65 constructs were all unable to grow on selective media (Figure 6F). These results demonstrate that GRASP55 can interact with GM130, and it does so via the same C-terminal signal as that recognized by GRASP65. In order to obtain a semi-quantitative measure of the strength of the interaction between GRASP55 and GM130, relative to that seen between GRASP65 and GM130, liquid culture assays for β-galactosidase were performed (Figure 6G). As before, the mutations that abolished binding of GM130 to GRASP65 and GRASP55 gave no signal in this assay (Figure 6G, GM130ΔC983, M984A). Wild-type GM130 gave a signal with both GRASP65 and GRASP55; however, the magnitude was 2-fold greater with GRASP65 than with GRASP55. Mutations that reduce the binding of GM130 to GRASP65 gave ~25% of the wild-type GM130 signal (Figure 6G, GM130V985A, I986A), consistent with the observed 50% reduction in binding observed by co-immunoprecipitation (Barr *et al.*, 1998). These same mutations in GM130 reduced the signal seen with GRASP55 to $<5\%$ of the wild-type GRASP65 signal (Figure 6G, GM130V985A, I986A). Empty vector controls using the constructs shown in Figure 6F gave the same signal as a background control using buffer alone (data not shown). Western blotting of these strains with antibodies to GM130 to detect the bait constructs, and the HA-epitope present in the GRASP55 and GRASP65 prey constructs was carried out to ensure that the different bait and prey constructs were expressed equally. The blots show that the GRASP55 and GRASP65 prey constructs (upper panel, Figure 6G), and the different GM130 bait constructs (lower panel, Figure 6G), respectively, were expressed at similar levels. These data suggest that GRASP55 can bind to GM130 in the yeast two-hybrid system, but that it does so with a lower affinity than GRASP65. Taking the biochemical data together with the two-hybrid results, it is unlikely that GRASP55 interacts with GM130 *in vivo*. The two-hybrid data do indicate that a low affinity interaction between GRASP55 and GM130 can occur, and that this is dependent on the same signal as that recognized by GRASP65. It is therefore likely that GRASP55 recognizes a C-terminal hydrophobic signal present in another protein, related to the one found in GM130.

GRASP55 functions in the stacking of Golgi cisternae in a cell-free system

To provide evidence that GRASP55 functions in the mechanism by which Golgi cisternae come together to form stacks, the effects of soluble recombinant GRASP55 in a cell-free system for Golgi reassembly were tested. Similar experiments were used to demonstrate that GRASP65 functions in stacking, and that its point of action is downstream of p115 (Barr *et al.*, 1997; Shorter and Warren, 1999). Stacked Golgi membranes were treated with mitotic cytosol for 20 min to give rise to a population of unstacked short cisternae and vesicles (Figure 7, M20). These MGFs were then either left untreated or treated with 1.2 μM GRASP65 or GRASP55, followed by incubation with rat liver cytosol for 60 min to allow the regrowth and stacking of cisternae. Reassembly of control MGFs (Figure 7A, M20R60) gave stacks with predominantly two to three cisternae per stack, as expected. When the MGFs were pre-incubated with either the recombinant forms of GRASP65 or GRASP55, Golgi stacks were not formed. In these cases a population of single cisternae and some vesicles was observed (Figure 7A, M20R60 + 1.2 μM GRASP65; M20R60 + 1.2 μM GRASP55), similar to that seen when MGFs are pre-treated with the alkylating agent NEM (Barr *et al.*, 1997). This effect was not due to a decrease in the amount of membrane being

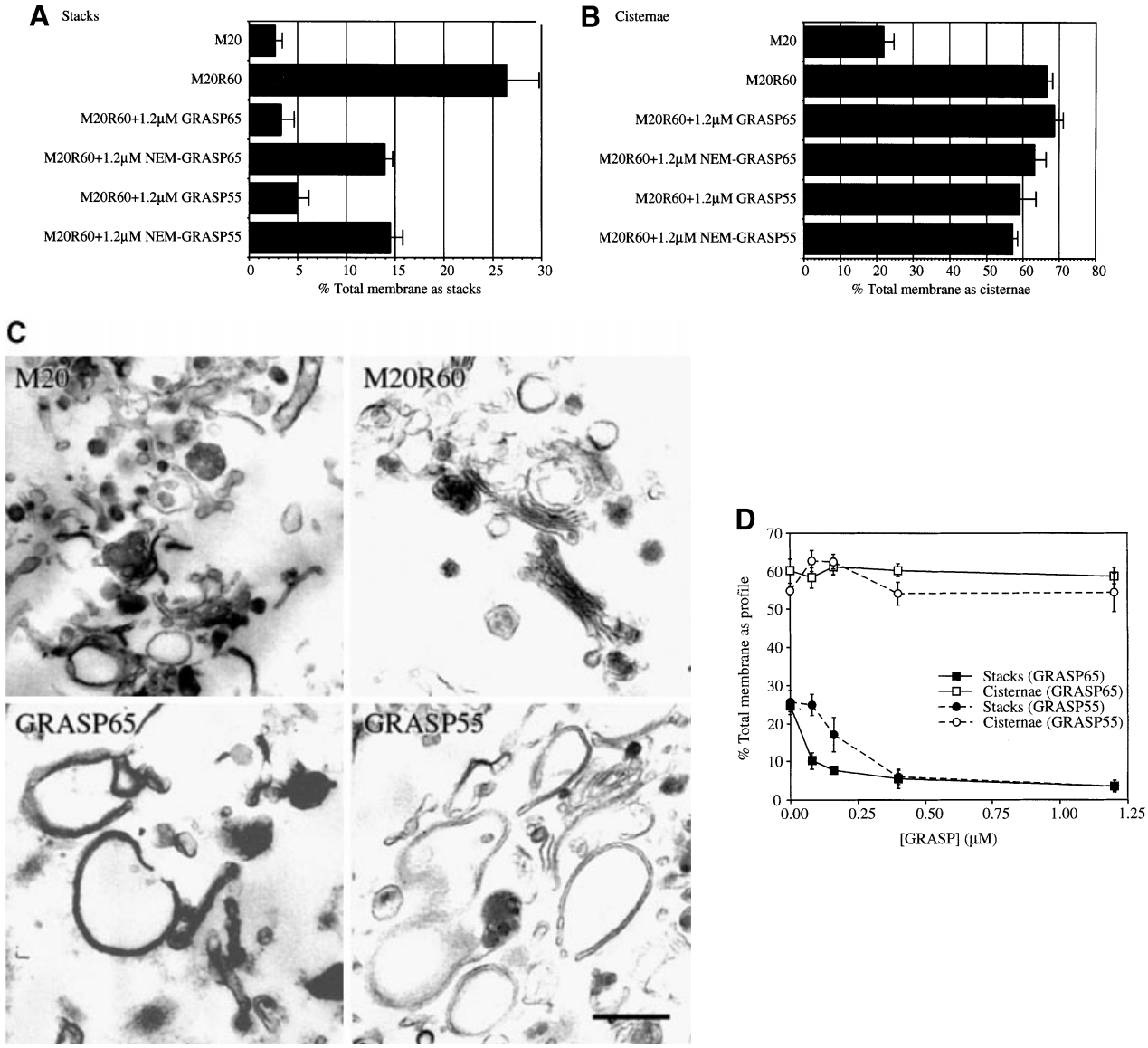

Fig. 7. Stacking of Golgi cisternae is blocked by recombinants forms of GRASP55 and GRASP65. Golgi membranes were treated with mitotic cytosol for 20 min to disassemble their stacked structure (M20). These MGFs were used for 60 min reassembly reactions in the absence (M20R60) or presence of 1.2 μM soluble recombinant forms of GRASP65 (M20R60 + 1.2 μM GRASP65) and GRASP55 (M20R60 + 1.2 μM GRASP55). Reactions were also performed using the same amount of NEM-treated recombinant forms of GRASP65 (M20R60 + 1.2 μM NEM–GRASP65) and GRASP55 (M20R60 + 1.2 μM NEM–GRASP55). The amount of membrane present in stacks of two or more cisternae (**A**), or present in all cisternae (**B**) was quantitated and expressed as a percentage of the total amount of membrane. Representative images of the different reassembly reactions are shown, scale bar denotes 0.5 μM (**C**). MGFs were used for 60 min reassembly reactions in which 0.1, 0.2, 0.4 and 1.2 μM of either GRASP55 or GRASP65 were added. The amount of membrane present in stacks of two or more cisternae (closed symbols) or present in all cisternae (open symbols) was quantitated and expressed as a percentage of the total amount of membrane for both GRASP55 (circles) and GRASP65 (squares) (**D**).

incorporated into cisternae, since this was not significantly changed in the presence or absence of soluble recombinant GRASP65 or GRASP55 (Figure 7B, M20R60; M20R60 + 1.2 μM GRASP65; M20R60 + 1.2 μM GRASP55). Representative images of these different conditions are shown in Figure 7C, and clearly demonstrate the effects of GRASP55 and GRASP65 in blocking the formation of stacks but not cisternae. To find out if GRASP55 is an NEM-sensitive component of the Golgi stacking machinery like GRASP65, reassembly experiments were performed with NEM-treated recombinant proteins. When the recombinant GRASP55 and GRASP65 were pre-treated with NEM prior to their addition to the reassembly assay (Figure 7A, M20R60 + 1.2 μM NEM–GRASP65; M20R60 + 1.2 μM NEM–GRASP55), their respective abilities to inhibit stack formation were decreased by ~50%. This was not due to any change in the amount of membrane incorporated into cisternae, which was unchanged (Figure 7B, M20R60 + 1.2 μM NEM–GRASP65; M20R60 + 1.2 μM NEM–GRASP55) compared with the incubations where untreated recombinant GRASP55 and GRASP65 were added (Figure 7B, M20R60; M20R60 + 1.2 μM GRASP65; M20R60 + 1.2 μM GRASP55). These results are consistent with

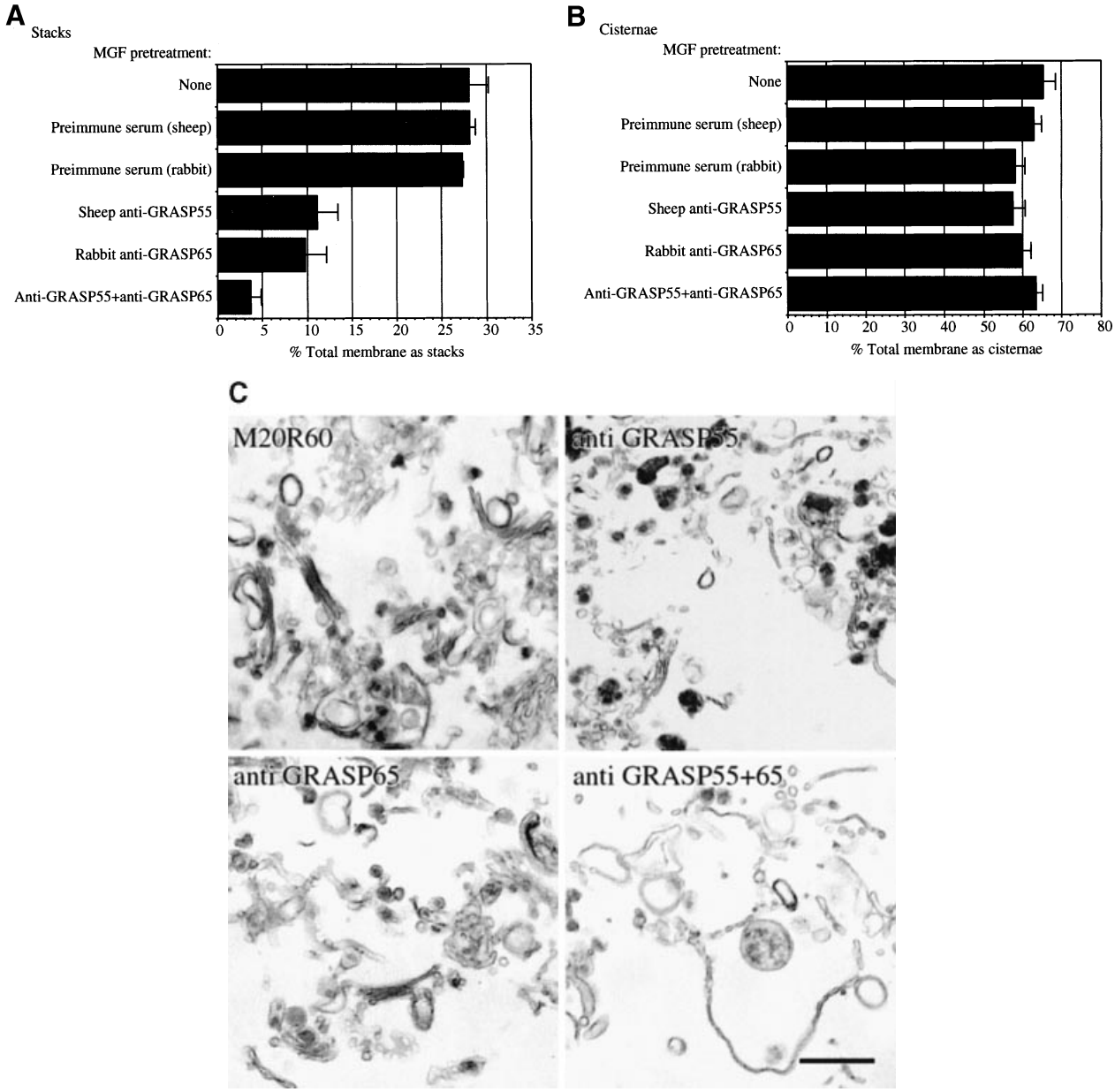

Fig. 8. Antibodies to GRASP55 and GRASP65 block the stacking of Golgi cisternae. Golgi membranes were treated with mitotic cytosol for 20 min to disassemble their stacked structure. These MGFs were left untreated (none), treated with pre-immune sera for GRASP55 (sheep), GRASP65 (rabbit), immune sera to GRASP55 (sheep anti-GRASP55), GRASP65 (rabbit anti-GRASP65), or GRASP55 and GRASP65 (anti-GRASP55 + anti-GRASP65), then used for 60 min reassembly reactions. The amount of membrane present in stacks of two or more cisternae (**A**), or present in all cisternae (**B**) was quantitated and expressed as a percentage of the total amount of membrane. (**C**) Representative images of the different reassembly reactions are shown. Scale bar denotes 0.5 μM.

GRASP55 being an NEM-sensitive Golgi stacking protein. Titration experiments were performed to ascertain the relative potencies of recombinant GRASP65 and GRASP55 in blocking the formation of Golgi stacks in this cell-free system. Since 1.2 μM of each recombinant protein seemed able to block stacking almost completely, progressively lower amounts of the two proteins were titrated into the assay. These experiments revealed that the maximal inhibition was similar in both cases, but that GRASP55 is less potent that GRASP65 in blocking the formation of stacks; the IC_{50} is 0.25 μM compared with 0.1 μM (Figure 7D, closed squares; closed circles). This effect was not due to an inhibition of membrane fusion, since this was not changed by the addition of either recombinant GRASP65 or GRASP55 (Figure 7D, open squares and open circles, respectively).

To provide evidence that the endogenous GRASP55 acts during stacking, the effects of GRASP55-specific antibodies on the formation of Golgi stacks were examined in the cell-free system for Golgi reassembly. In these experiments, MGFs were either left untreated, treated with pre-immune or immune sera for antibodies specific for GRASP55 or GRASP65, followed by incubation with rat liver cytosol for 60 min to allow the regrowth and stacking of cisternae. Reassembly of control MGFs (Figure 8A, none), or MGFs treated with the pre-immune sera for the GRASP55 (Figure 8A, pre-immune sheep serum) or GRASP65 (Figure 8A, pre-immune rabbit serum)

antibodies gave stacks with predominantly two to three cisternae per stack, as expected. When the MGFs were pre-incubated with antibodies for either GRASP55 (Figure 8A, sheep anti-GRASP55) or GRASP65 (Figure 8A, rabbit anti-GRASP65), the number of Golgi stacks formed was decreased by ~60%. Treatment of MGFs with antibodies to both GRASP55 and GRASP65 (Figure 8A, anti-GRASP55 + anti-GRASP65) resulted in a >80% inhibition of stack formation. These effects were not due to a decrease in the amount of membrane being incorporated into cisternae, since this was not significantly changed from the control value by the presence of any of the immune or pre-immune sera tested (Figure 8B). Representative images of these different conditions shown in Figure 8C demonstrate the effects of GRASP55 and GRASP65 in blocking the formation of stacks but not cisternae. The simplest explanation for these results is that endogenous GRASP65 and GRASP55 act together in the pathway by which cisternae come together to form stacks.

Discussion

In this study we have shown that GRASP55, a GRASP65-related protein from mammals, also functions in the formation of Golgi stacks. The data presented here indicate that GRASP65 and GRASP55 have similar functions in establishing, and possibly maintaining, the interactions between adjacent cisternae in a cell-free system, although whether or not they do so *in vivo* will require further experiments. Unlike GRASP65, GRASP55 does not exist in a stable complex with GM130, since we were unable to detect an interaction between the two proteins by biochemical methods and could only demonstrate a weak interaction between GRASP55 and the C-terminus of GM130 using the yeast two-hybrid system. In addition, GRASP65 was shown to be present on mainly *cis*-Golgi cisternae by cryo-electron microscopy, similar to the distribution of GM130 (Nakamura *et al.*, 1995), whereas GRASP55 was found predominantly over the *medial*- and *trans*-cisternae. The basis for this differential localization could be explained if GRASP55 is in a complex with a protein other than GM130 that then targets to a different region of the Golgi apparatus. Supporting this idea are experiments showing that GRASP65 and GM130 need to bind to one another to target efficiently to the Golgi apparatus (Barr *et al.*, 1998). Arguing against this are the data showing that mutations in the conserved binding region of GRASP55 have little effect on its Golgi targeting, indicating it might not require interaction with another protein at this site for correct localization. Identification of the proteins binding to GRASP55 should allow this point to be addressed.

Why do mammalian cells have two members of the GRASP family of proteins while an organism such as the yeast *Saccharomyces cerevisiae* has only one? Mammalian cells do have a more complex Golgi structure than this yeast; typically there are between three and five cisternae in a stack with extensive tubular networks at the *cis*- and *trans*-faces, and many small vesicles associated with the edges of the cisternae (Rambourg and Clermont, 1997). Yeast possess clearly defined Golgi cisternae, but serial sectioning and electron microscopy have shown that only 40% of the cisternae are in these structures at any one time (see Table 1 in Preuss *et al.*, 1992). The more complex array of membrane-bound compartments in mammalian cells is also reflected in the diversity of other proteins involved in membrane transport; for example, there are many more members of the SNARE family of proteins in mammalian cells than in yeast (Advani *et al.*, 1998). The presence of two GRASP proteins may reflect the more complex organization of the Golgi apparatus, or a requirement for additional regulation of the structure of this organelle in mammals. Consistent with this argument are the localizations of GRASP65 and GRASP55 to distinct subcompartments of the Golgi apparatus.

During the reassembly of the Golgi complex, following its mitotic disassembly in a cell-free system, there is an initial phase during which cisternae are rebuilt from the MGFs (Rabouille *et al.*, 1995a). These cisternae then align and dock with one another to form stacks in a process that depends on the presence of p115 (Shorter and Warren, 1999). It is at this point that the function of p115 in the reassembly of the Golgi apparatus must diverge from that of simply tethering membranes prior to membrane fusion, as we believe is the case during vesicle transport. If this were not the case, then one would expect Golgi cisternae to dock and fuse with one another, rather than form stacks. There must therefore be additional factors acting either to divert the tethering complexes away from the SNARE-mediated membrane fusion pathway, or clamping them so that they stably bridge the gap between adjacent cisternae. This might involve the formation of a matrix or lattice-like structure from the many coiled-coil proteins associated with the Golgi complex (Chan and Fritzler, 1998). Evidence has been presented for such a structure existing on Golgi membranes, and furthermore that GM130 is a component of this matrix (Slusarewicz *et al.*, 1994; Nakamura *et al.*, 1995). One possibility is that GRASP65 and GRASP55 function in the nucleation of such a structural matrix, and thus divert the p115-dependent tethering machinery away from the SNARE-mediated membrane fusion pathway. The inhibition of stack formation by recombinant forms of these two proteins could be due to competition for interactions with the endogenous membrane associated GRASP proteins. These interactions must be with membrane associated components of MGFs, since stacks can reassemble from these membranes with the addition of purified membrane fusion components (Shorter and Warren, 1999). Our current hypothesis is that the tethering of cisternae mediated by p115 and its two membrane receptors, giantin and GM130, leads to their initial docking and alignment. Following this, the GRASP proteins mediate lateral interactions with other membrane-associated components, which results in the formation of stable cross-bridges between the cisternae.

Materials and methods

Materials

Laboratory reagents were from Sigma, Fisher Scientific, New England Biolabs and Qiagen. Antibodies used in this study were as follows: rabbit polyclonals NN5-1 and MLO-7 raised against the rat GM130 from N.Nakamura and M.Lowe; rabbit polyclonal FBA19 raised against the peptide YLHRIPTQPSSQYK; mouse monoclonal 7E10 and rabbit polyclonals FBA30 and FBA31 all raised against recombinant GRASP65; a rabbit polyclonal antisera FBA32, and a sheep antisera FBA34 raised against recombinant GRASP55; the H-7 monoclonal to the HA-epitope (Santa Cruz).

Cloning of GRASP55 and protein expression

GRASP55 was cloned using a RACE protocol and specific nested primers as follows: TR1, GGTACCCCCATTACTCCTCTTAAGGATGGG; TR2, CAGAGGTCCAGCTGTCTTCAGTCAGTCCTCC; TR3, GGAGGACTGACTGAAGACAGCTGGACCTCTG; TR4, CCCATCCTTAAGAGGAGTAATGGGGGTACC. Rat testis cDNA was used as the template for the first round of 5′ and 3′ RACE, the AP1 and AP2 cDNA adaptor primers were as described in the Marathon RACE protocol (Clontech). All reactions were performed for 25 cycles with annealing temperatures and elongation times of 60°C and 2 min, respectively, with the polymerase mix and buffer 1 from the Expand PCR system (Boehringer Mannheim). Primers TR12, CCGGCTGGGTCGTACGGATCGCCCGCCG and TR10, ACTTGGTGGGAGACAAGCCTTCAGACTC were used to obtain the full open reading frame of GRASP55, which was TA-cloned into pCRII-TOPO (Invitrogen) and sequenced. The DDBJ/EMBL/GenBank accession number for the rat GRASP55 sequence is AF110267. Mutagenesis of GRASP55 was performed using the Quickchange method (Stratagene) to introduce alanine codons at the required positions. For expression in bacteria, GRASP55 was subcloned into pTrcHisA (Invitrogen) and GRASP65 into pQE31 (Qiagen), to create N-terminally His-tagged constructs. Purified His-tagged GRASP55 and GRASP65 were gel-filtered using a Superose-6 HR10/30 column (Pharmacia Biotech) equilibrated in 25 mM HEPES–KOH pH 7.2, 150 mM KCl to remove low molecular weight contaminants and to exchange the buffer.

Northern and Western blotting

DNA probes were as follows: GRASP65-specific probe, *Bam*HI–*Sma*I fragment of the GRASP65 cDNA; GRASP55-specific probe, *Mlu*I–*Eco*RI fragment of the GRASP55 cDNA; GRASP common probe, equal amounts of the *Eco*RI–*Bam*HI fragment of the GRASP65 cDNA and the *Eco*RI–*Eco*RI fragment of the GRASP55 cDNA. All probes were labelled using Rediprime (Amersham Life Sciences). A multiple rat tissue Northern blot (Clontech) loaded with ~2.0 μg poly(A) plus RNA from the tissues indicated in the appropriate figure legend, was hybridized for 2 h at 65°C in rapid hybridization buffer (Amersham Life Sciences) with the appropriate DNA probe. After hybridization, the filter was rinsed in 2× SSC plus 0.1% (w/v) SDS, washed twice for 15 min in the same buffer at 42°C, rinsed in 2× SSC then exposed to film. For Western blotting, fresh tissue samples from one female Sprague–Dawley rat were washed three times with 10 ml of ice cold PBS, then 0.6 g of each was finely chopped with a razor blade. The chopped tissue was placed into a ceramic mortar and a small volume of liquid nitrogen added. Using a ceramic pestle the frozen tissue was ground to a fine powder, resuspended in 4 ml of sample buffer and boiled for 5 min, then centrifuged at 2000 *g* for 10 min to pellet any insoluble material.

Yeast two-hybrid assays

All protocols were performed as described in the yeast protocols handbook (Clontech). Prey constructs comprising the full-length GRASP65 and GRASP55 were made in pACT2 (Bartel *et al.*, 1993). Bait constructs, comprising the last 230 amino acids of wild-type GM130, or the ΔC983, M984A, V985A and I986A GM130 mutants were made in pGBT9 (Li *et al.*, 1994). The yeast two-hybrid reporter strain PJ69-2A (James *et al.*, 1996) was transformed with the various bait and prey constructs, or with the empty vectors as controls and plated on synthetic media lacking tryptophan and leucine. Positive colonies were tested for the ability to grow on synthetic media lacking tryptophan, leucine, histidine and adenine, which would indicate an interaction between the bait and prey proteins. For semi-quantitative β-galactosidase assays, bait and prey plasmids were transformed into the Y187 reporter strain (Harper *et al.*, 1993). Positive transformants were grown in synthetic media lacking tryptophan and leucine until the cultures reached log-phase growth, then semi-quantitative assays for β-galactosidase assays performed. Enzyme activity is expressed as β-galactosidase units, relative amount of substrate hydrolysed/min/mg protein.

Cell-free system for Golgi disassembly and reassembly

Rat liver Golgi membranes, mitotic and rat liver cytosols were prepared as described previously (Rabouille *et al.*, 1995; Hui *et al.*, 1998). Mitotic Golgi disassembly and subsequent reassembly reactions, processing for EM and stereology were carried out as in Shorter and Warren (1999). Briefly, rat liver Golgi membranes were treated with mitotic cytosol for 20 min to generate MGFs. Reassembly reactions were carried out with these MGFs for 60 min at 37°C with rat liver cytosol, an ATP regeneration system and the amounts of recombinant GRASP55 and GRASP65 indicated in the figure legends. NEM treatments were carried out as follows: 50 μl of GRASP (0.4 mg/ml final concentration; in 25 mM HEPES–KOH pH 7.2, 150 mM KCl) were treated with 1 mM NEM for 15 min on ice. The reaction was quenched by addition of dithiothreitol (DTT) to 2 mM and left on ice for 30 min, prior to addition to assay. NEM quenched with DTT was used as the control treatment. For antibody inhibition experiments, MGFs were treated for 15 min on ice with either 2 μl of the appropriate pre-immune serum, 1 μl of the FBA31 or FBA34 antibodies plus 1 μl of the opposing pre-immune serum, or 1 μl each of FBA31 and FBA34.

Immunofluorescence, cryo-electron microscopy and stereology

Transfection and immunofluorescence of HeLa cells was carried out as described previously (Barr *et al.*, 1998). For cryo-electron microscopy, HeLa cells were plated at 30% density in 6 cm dishes, left to attach for 12 h, then transfected using 2.0 μg DNA from the appropriate construct with Effectene (Qiagen). After 18 h, cells from two 6 cm dishes were scraped from the dish, fixed with 0.2% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer pH 7.4, and prepared for cryosectioning and immunostaining (Slot *et al.*, 1991). Immunolabelling was performed using a 1:5 dilution of the 7E10 monoclonal antibody and a 1:50 dilution of the anti-GFP polyclonal antibody. These were detected using gold particles coupled to an anti-rabbit antibody and protein A, respectively. The distributions of GRASP55 and GRASP65 over the Golgi apparatus in single and double-labelling experiments were determined as follows. For each Golgi apparatus, the *cis* to *trans* polarity was defined using the presence of clathrin coated buds to identify the *trans*-Golgi network. Gold particles within 30 nm of the membrane of a particular cisterna were assigned to that cisterna, numbered 1 to 5 or greater, from *cis* to *trans*. The number of gold particles found over a particular cisterna was expressed as a percentage of the total number of gold particles present.

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