Golgi matrix proteins interact with p24 cargo receptors and aid their efficient retention in the Golgi apparatus

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The Golgi apparatus is a highly complex organelle comprised of a stack of cisternal membranes on the secretory pathway from the ER to the cell surface. This structure is maintained by an exoskeleton or Golgi matrix constructed from a family of coiled-coil proteins, the golgins, and other peripheral membrane components such as GRASP55 and GRASP65. Here we find that TMP21, p24a, and gp25L, members of the p24 cargo receptor family, are present in complexes with GRASP55 and GRASP65 in vivo. GRASPs interact directly with the cytoplasmic domains of specific p24 cargo receptors depending on their oligomeric state, and mutation of the GRASP binding site in the cytoplasmic tail of one of these, p24a, results in it being transported to the cell surface. These results suggest that one function of the Golgi matrix is to aid efficient retention or sequestration of p24 cargo receptors and other membrane proteins in the Golgi apparatus.

Introduction

The Golgi apparatus is an organelle on the secretory pathway required for the processing of complex sugar structures on many proteins and lipids, and for the sorting of these proteins and lipids to their correct subcellular destinations (Farquhar and Palade, 1998). It is comprised of a series of cisternal membranes organized into a stacked structure, the first or cis-face receives material from the ER, whereas the final or trans-cisternae packages it for delivery to the plasma membrane and endocytic pathways (Farquhar and Palade, 1998). This structure is maintained by an exoskeleton or Golgi matrix which has been proposed to be required for linking cisternae together into the characteristic Golgi stack structure, and also for vesicle recognition during membrane traffic (Warren and Malhotra, 1998; Seeman et al., 2000).

The most studied components of the Golgi matrix are p115, the GRASP65–GM130 complex, and an integral membrane protein, giantin (Linstedt and Hauri, 1993; Nakamura et al., 1995; Sapperstein et al., 1995; Barr et al., 1997; Shorter and Warren, 1999). GRASP65 was identified in a screen for factors involved in the stacking of cisternae, and later shown to be a specific binding partner of GM130 required to target it to the Golgi (Barr et al., 1998). GM130 in turn is a receptor for p115, required for tethering vesicles to their target membrane (Barroso et al., 1995; Nakamura et al., 1997). More recently, the formation of Golgi stacks from cisternae in vitro was found to require p115, discrete from its membrane fusion function, and giantin (Shorter and Warren, 1999). GM130 and p115 also make interactions with giantin during vesicle docking, and potentially during cisternal stacking (Shorter and Warren, 1999; Dirac-Svejstrup et al., 2000; Lesa et al., 2000). The network of interactions between these proteins may be regulated by the rab GTPases, as both p115 and GM130 have been shown to interact with the active or GTP form of rab1 (Allan et al., 2000; Moyer et al., 2001; Weide et al., 2001). Recruitment of p115 to vesicles destined to fuse with the cis-Golgi is mediated via the interaction with rab1 (Allan et al., 2000), whereas the consequences of rab1 binding to GM130 are unknown. A second GRASP complex, containing GRASP55 and the coiled-coil protein golgin-45, exists in Golgi membranes (Shorter et al., 1999; Short et al., 2001). Like the GRASP65–GM130 complex, this complex also binds a rab GTPase and is required for the maintenance of normal Golgi structure and protein transport (Short et al., 2001).

In addition to the Golgi matrix proteins discussed above, some membrane proteins are of potential importance to Golgi structure. One such group is the medial-Golgi enzymes, found to specifically bind to the Golgi matrix although the mechanism remains uncharacterized (Slusarewicz et al.,...
Another is the p24 family of cargo receptors, identified as major transmembrane components of vesicles recycling between the ER and Golgi complex, and as potential structural Golgi proteins in mammalian cells (Wada et al., 1991; Stamnes et al., 1995; Rojo et al., 1997). All p24 proteins share a common structure, with a short cytoplasmic domain containing binding signals for the COP-I and COP-II vesicle coat complexes, plus a lumenal domain with potential secretory cargo binding capabilities (Fiedler et al., 1996; Sohn et al., 1996; Dominguez et al., 1998; Muniz et al., 2000). At least one p24 protein, TMP21, is an essential gene in mammals, and a heterozygous deletion shows reduced levels of the protein and a partially disrupted Golgi apparatus (Denzel et al., 2000). However, in yeast, all p24 family members can be deleted with little or no effect on protein transport or secretory pathway morphology (Springer et al., 2000).

The drug brefeldin A disrupts the Golgi apparatus and causes Golgi enzymes, but not putative structural components such as the GRASPs and GM130, to be relocated to the ER (Nakamura et al., 1995; Seeman et al., 2000). Under conditions in which the Golgi is first disrupted with brefel-
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din A and then allowed to recover while protein transport from the ER is blocked, a Golgi-like structure forms that lacks Golgi enzymes but contains Golgi matrix proteins (Seeman et al., 2000). However, under the electron microscope, these structures lack some key features of normal Golgi membranes, such as well-defined stacked cisternae. Therefore, the Golgi matrix must interact with additional factors, either integral membrane proteins or specific lipids to organize Golgi membranes. Therefore, we decided to investigate whether GRASPs could provide a link between Golgi matrix components and transported integral membrane proteins.

Results and discussion

Analysis of purified GRASP55 complexes by electrophoresis on nonreducing polyacrylamide gels showed that in addition to GRASP55 and golgin-45, distinct protein bands at 21 and 25 kD were visible (Fig. 1 A). Mass spectrometric tryptic fingerprinting revealed these proteins to be members of the p24 cargo receptor family (Fig. 1 A). Comparing the intensity of the Coomassie blue–stained GRASP55 and p24 protein bands suggests a stoichiometric complex of GRASP55 and TMP21, assuming equal staining efficiency and allowing for the difference in molecular masses. Western blotting of GRASP55 and GRASP65 immunoprecipitates with antibodies specific to p24a and gp25L showed that they are in complexes with both GRASPs (Fig. 1 B). These are specific complexes, as GRASP55 immunoprecipitates did not contain GRASP65, and vice versa (Fig. 1 C). To find out if GRASPs are present in complexes containing both golgins and p24 proteins, immunoprecipitates of golgin-45 and GM130 were Western blotted for p24a, gp25L, and the GRASPs. This revealed that GM130 immunoprecipitates contained both p24 proteins tested for, whereas golgin-45 immunoprecipitates did not (Fig. 1 B), despite the fact that GRASP55 was easily detected (Fig. 1 C). In summary, GRASP65 is found in a complex containing both GM130 and p24 proteins, whereas GRASP55 may exist in two discrete complexes containing either golgin-45 or p24 proteins.

If the presence of p24 proteins in the purified GRASP complexes is relevant for GRASP function, there should be an interaction between the cytoplasmic domains of these proteins with GRASP55 and GRASP65. To investigate this we used the yeast two-hybrid system, fusing the cytoplasmic domains of different p24 proteins as baits, and full-length GRASP55 and GRASP65 as prey. This screen revealed no
interaction between any of the p24 proteins tested or TGF-α with either GRASP, despite the fact that GM130 showed an interaction with the GRASPs (Fig. 2, A and B). One property these proteins share is that they exist as dimers or oligomers within the cell (Dominguez et al., 1998; Kuo et al., 2000), suggesting that oligomeric state might be important for their recognition by GRASPs. To oligomerize the cytoplasmic domains, a coiled coil was added to the two-hybrid bait constructs. This resulted in positive signals being obtained for both GRASPs with p24a, p24b, and TGF-α, in addition to GM130 in the growth (Fig. 2, A and B) and lacZ reporter gene assays (Fig. 2 C). The coiled-coil domain used gave no interaction with GRASPs on its own (unpublished data), and a number of the p24 proteins tested failed to interact with GRASPs, even with the coiled coil. Purified GRASP55 and GRASP65 were able to bind directly to the cytoplasmic domains of p24a, p24b, TGF-α, and the GM130 COOH terminus immobilized at high density on the surface of agarose beads (Fig. 2 D), consistent with the two-hybrid results. No binding was observed to a glutathione S-transferase (GST)* control, and only weak binding to other p24 proteins (Fig. 2 D). The only discrepancy between the two approaches was that the TMP21 cytoplasmic tail, which did not show an interaction in two-hybrid system bound to GRASP55 in the protein-binding assay. Therefore, GRASP55 and GRASP65 directly interact with the p24a and p24b cytoplasmic domains, and this recognition is apparently dependent on the oligomeric state of the signal. GRASP55 may also be able to bind to the TMP21 cytoplasmic domain.

We then sought to define the nature of the GRASP binding site in the cytoplasmic domain of the p24a protein. Visual inspection of the p24a sequence revealed that it ends with two valine residues, identical to TGF-α and similar to GM130. Mutation of these residues to alanine reduced the interaction of p24a with GRASP55 and GRASP65 in the growth (Fig. 3, A and B) and lacZ reporter two-hybrid assays (Fig. 3, C and D), reminiscent of previous observations that a hydrophobic signal at the extreme COOH terminus of GM130 is needed for it to bind GRASP65 (Barr et al., 1998). Alteration of the adjacent arginine residues had no effect on recognition of the signal by either GRASP, whereas a double mutant behaved identically to the double valine to alanine mutation (Fig. 3, A–C). Interactions were again only observed with oligomeric p24a tail constructs. Protein binding assays confirmed that point mutations reducing the two-hybrid interaction between the GRASPs and the p24a cytoplasmic tail also decrease the interaction between the respective proteins (Fig. 3 D). Therefore, GRASP55 and GRASP65 directly recognize a signal in the cytoplasmic tail of p24a comprised of the two valines at its extreme COOH terminus, and whereas the adjacent arginines are not important, the contribution of other residues to this signal cannot be excluded.

If the interaction of p24 proteins with GRASPs is important for their function, disruption of the GRASP binding signal should perturb their localization or transport. Mutation of the GRASP binding site in p24a caused the protein to appear at the cell surface even at low expression levels, whereas the wild-type protein was efficiently retained inside the cell (Fig. 4 A). Quantitation revealed that surface levels of p24a increased fourfold after inactivation of the GRASP binding site (Fig. 4 B). A double mutation in which the

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*Abbreviations used in this paper: GFP, green fluorescent protein; GST, glutathione S-transferase.
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COP II transport signal was abolished by mutating the two phenylalanines in the p24a cytoplasmic tail to alanine reverted this effect (Fig. 4 A). The COP II binding mutation alone was trapped in the ER and some adjacent vesicular structures as expected (Fig. 4 A) (Fiedler et al., 1996). Mutating the two arginines alone had little effect on the transport of p24a to the cell surface, whereas combining the GRASP binding site and arginine motif mutations gave a reduced level of transport to the cell surface to half that of the GRASP binding mutant alone (Fig. 4, A and B), indicating a transport rather than a retention defect. Therefore, the GRASP binding site in p24a is required for its efficient retention in the Golgi apparatus, but not for p24a transport to the Golgi or retention in the ER. This signal acts at the level of the Golgi, as a COP II transport signal mutant is trapped in the ER, and combining this with the GRASP binding mutant reduces cell surface levels of p24a.

We initially purified p24 proteins in a complex with GRASP55 from Golgi membranes, and subsequent analysis revealed that GRASP65 also binds to p24 proteins. It is known that four p24 proteins, gp25L, p24a, TMP21, and p27, form a stoichiometric complex (Füllekrug et al., 1999), consistent with our observation that three of these p24 proteins are found in association with GRASP55 and GRASP65. The basis for this association appears to be an interaction between the cytoplasmic tail of p24a and the GRASPs. We also found that p26/p24b, which is not present in the gp25L-containing complex, can interact with GRASPs in the two-hybrid system. Interestingly, the GRASP-binding p24 proteins, p24a and p24b, have been shown previously not to interact with COP I (Dominguez et al., 1998). Therefore, our observations may help to explain how complexes containing these two p24 proteins are retained in the Golgi, as GRASP interaction was only seen when the p24 cytoplasmic tail sequences were oligomerized, and no binding was observed with monomeric p24 tails. This may be a mechanism by which GRASPs can distinguish p24 protein complexes in the Golgi apparatus from those present in the ER. A relevant observation in this regard is that complexes containing up to 50 copies of the p24 proteins exist in Golgi membranes (Dominguez et al., 1998). Therefore, specific interaction of Golgi matrix proteins with p24 proteins in post-ER membranes could be an avidity effect, mediated by virtue of the approximately tenfold higher concentration (compared with the ER) of transported proteins, and presumably cargo receptors, in the Golgi apparatus (Quinn et al., 1984).

The transmembrane precursor of TGF-α also binds to GRASP55 in the Golgi, an interaction necessary for its correct localization and transport (Kuo et al., 2000). We show that GRASP65 can also bind to the TGF-α cytoplasmic do-

tail, or mutants abolishing the GRASP binding site, VV→AA, the phenylalanine and arginine motifs, FF→AA and RR→AA, or combinations thereof, FFVV→AA and RRVV→AA. Total fluorescence was visualized with GFP, and cell-surface fluorescence by an antibody to GFP detected with a CY3-coupled secondary antibody. (B) Extent of p24a transport to the cell surface was measured for wild-type and mutant p24a tails constructs. This ratio does not approach unity due to the different dyes used to measure surface and total fluorescence. Mean and standard deviation are plotted, n = 20. Bar, 20 μm
main, raising the possibility that GRASPs could bind to a variety of transmembrane cargo proteins via their cytoplasmic domains. Therefore, interaction with Golgi matrix proteins may be a general sequestration mechanism to regulate the transport and retention of integral membrane proteins within the Golgi apparatus.

Materials and methods

Materials

Antibodies to GRASP55, GRASP65, GM130, and golgin-45 were described previously (Barr et al., 1997; Shorter et al., 1999; Short et al., 2001); antibodies were raised in rabbits against the peptides ECFFERTS-GTKMGG from p24a, and KCFHEPDGMVTI from gp25L. The plasmid encoding green fluorescent protein (GFP-tagged p24a was a gift from Dr. R. Blum (Institut für Physiologie, Ludwig Maximilians Universität, München, Germany). The Max-Planck-Institute of Biochemistry’s Oligonucleotide Synthesis Laboratory ( Martinsried, Germany) provided DNA oligonucleotides.

Purification and analysis of GRASP55 complexes

GRASP55 complexes were purified as described previously (Short et al., 2001). Aliquots of total extract and bound protein complexes were analyzed by SDS-PAGE on 10% minigels under nonreducing conditions. Proteins were extracted from Coomassie blue-stained gel slices and digested with sequencing-grade trypsin (Promega) for analysis of the peptide fragments by MALDI-TOF ( Reflex III; Bruker) and probability-based database searching (Perkins et al., 1999).

Yeast two-hybrid and protein binding assays

Standard molecular biology techniques were used for the construction of all constructs, and all constructs were confirmed by DNA sequencing (Medigenomix). Double-stranded oligonucleotides corresponding to the cytoplasmic domains of the p24 proteins, TGF-α, and the COOH terminus of GM130 were inserted into the EcoRI and BamHI sites of pGBT. Coiled coil-containing constructs were made by inserting a PCR-amplified fragment corresponding to amino acids 755–974 of GM130 into the EcoRI site of pbGT9. The full-length rat GRASP55 and GRASP65 constructs in pACT2 were described previously (Shorter et al., 1999). Yeast two-hybrid assays were performed as described previously (Short et al., 2001). Measurements of lacZ reporter gene expression were performed according to the CLONTECH Yeast Protocol handbook, and the results are expressed in BL21(DE3) for pGAT2 constructs at 37 °C for 3 h, or JM109 for pGAT2 constructs at 23 °C for 18 h. Fusion proteins were expressed from E. coli pQE32 constructs at 23 °C for 3 h, or JM109 for pGAT2 constructs at 37 °C for 3 h, or JM109 for pQE32 constructs at 23 °C for 18 h. Fusion proteins were purified on nitrocellulose-attached−nickel agarose according to the manufacturer’s instructions (QIAGEN), dialyzed against PBS, and then stored at −80°C. For each binding assay, 5 μg of GST fusion protein was bound to 10 μl glutathione-Sepharose (Amersham-Pharmaica Biotech). These beads were incubated in the presence of 10 ng/μl GRASP55 or GRASP65 in a total volume of 200 μl PNT (25 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.1% TX-100) for 1 h at 4°C on a rotating wheel. Beads were washed three times with 400 μl PNT, and then bound proteins eluted with sample buffer for analysis by SDS-PAGE and Western blotting. Bound GRASP proteins were detected using a monoclonal antibody to the hemaglutinin epitope or the 7F10 monoclonal antibody to GRASP65, and GST fusion proteins with a polyclonal antibody to GST.

Microscopy and protein transport assays

GFP-tagged p24a expression constructs were made by amplification of the p24a luminal and transmembrane domains using primers that replaced the cytoplasmic domain with the required mutant sequence. These constructs were then cloned back into the GFP-p24 expression vector (Blum et al., 2000). HeLa cells plated on glass coverslips were transfected with a plasmid encoding GFP-tagged p24a, or mutants thereof, for 18 h at 37°C. The cells were then fixed with 4% paraformaldehyde in PBS. Cell surface p24a was detected with a sheep antibody to GFP attached to the luminal domain and a donkey anti–sheep secondary coupled to Cy3 (Jackson ImmunoResearch Laboratories), and total p24a by GFP fluorescence. Images were collected using a Zeiss Axioplan-2 with 63× Plan Apochromat objective equipped with a 1300 by 1030 pixel cooled-CCD camera (Princeton Instruments), and Metaview software (Universal Imaging Corp.). The ratio of surface to total measured fluorescence was used to normalize the amount of p24a at the cell surface.

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