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dGRASP Localization and Function in the Early Exocytic Pathway in *Drosophila* S2 Cells[D]

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The de novo model for Golgi stack biogenesis predicts that membrane exiting the ER at transitional ER (tER) sites contains and recruits all the necessary molecules to form a Golgi stack, including the Golgi matrix proteins, p115, GM130, and GRASP65/55. These proteins leave the tER sites faster than Golgi transmembrane resident enzymes, suggesting that they act as a template nucleating the formation of the Golgi apparatus. However, the localization of the Golgi matrix proteins at tER sites is only shown under conditions where exit from the ER is blocked. Here, we show in *Drosophila* S2 cells, that dGRASP, the single *Drosophila* homologue of GRASP65/55, localizes both to the Golgi membranes and the tER sites at steady state and that the myristoylation of glycine 2 is essential for the localization to both compartments. Its depletion for 96 h by RNAi gave an effect on the architecture of the Golgi stacks in 30% of the cells, but a double depletion of dGRASP and dGM130 led to the quantitative conversion of Golgi stacks into clusters of vesicles and tubules, often featuring single cisternae. This disruption of Golgi architecture was not accompanied by the disorganization of tER sites or the inhibition of anterograde transport. This shows that, at least in *Drosophila*, the structural integrity of the Golgi stacks is not required for efficient transport. Overall, dGRASP exhibits a dynamic association to the membrane of the early exocytic pathway and is involved in Golgi stack architecture.

INTRODUCTION

The Golgi apparatus has a unique and almost universal membrane architecture. Its basic component, the flattened membrane bound compartment called cisterna, is found in almost all eukaryotic species with the exception of some primitive protists. In most eukaryotes, cisternae are arranged in a parallel manner to form a stack, on each side of which is found one tubular-vesicular network, the *cis*- and *trans*-Golgi network.

Two opposing models have been proposed to explain the biogenesis of this unique organelle, the Golgi matrix model and the de novo Golgi formation model. According to the first, the Golgi apparatus is an autonomous organelle built on a preexisting template, as suggested by data from a variety of organisms. For instance, microsurgically created peripheral cytoplasts from mammalian cells that do no longer contain any Golgi membrane failed to produce a Golgi apparatus, although they contained a significant amount of ER membranes (Pelletier *et al.,* 2000). Furthermore, studies on the Golgi stack duplication in two protozoa have provided support to a template-mediated mechanism of Golgi formation, both in *Trypanosoma brucei* (He *et al.,* 2004) and *Toxoplasma gondii* (Pelletier *et al.,* 2002).

In mammalian cells, the template is proposed to be a Golgi matrix (Slusarewicz *et al.,* 1994; Shorter and Warren, 2002) formed by Golgi matrix proteins, which comprise golgins, a group of long coiled-coil proteins localizing in the Golgi membranes, such as p115 and GM130, and the Golgi reassembly and stacking proteins GRASP65 and GRASP55 (Barr and Short, 2003). All these proteins have been implicated in the building and/or maintenance of the Golgi stack architecture, and the role of p115 in the structural integrity of the Golgi apparatus is the best established (Nakamura *et al.,* 1997; Shorter and Warren, 1999; Alvarez *et al.,* 1999, 2001; Puthenveedu and Linstedt, 2001, 2004; Kondylis and Rabouille, 2003).

GRASP65 and GRASP55 were originally identified as cisternal stacking factors (Barr *et al.,* 1997; Shorter *et al.,* 1999) and were shown to act as Golgi receptors for GM130 and golgin45, respectively (Barr *et al.,* 1998; Short *et al.,* 2001). GRASP65 is anchored on the Golgi membranes by myristoylation of the glycine residue at position 2 (Barr *et al.,* 1997) and forms dimers that could directly stack neighboring cisternae by forming transoligomers (Shorter and Warren, 1999; Wang *et al.,* 2003, 2005). This oligomerization is mediated by the N-terminal GRASP domain and is regulated by phosphorylation of serine/threonine residues at the C-terminal half of the protein during mitosis (Preisinger *et al.,* 2005; Wang *et al.,* 2005).

In addition, several recent studies have suggested that GRASP65 is also crucial for cell cycle regulation (Sutterlin *et al.,* 2002; Preisinger *et al.,* 2005), apoptosis (Lane *et al.,* 2002), and growth (Yoshimura *et al.,* 2005). Regarding this latter role, the mitotically Cdk1-phosphorylated serine 277 of GRASP65 is also phosphorylated in interphase by ERK kinase, and this is enhanced by the addition of epidermal growth factor (EGF) (Preisinger *et al.,* 2005; Yoshimura *et al.,* 2005).

The second model of Golgi stack biogenesis, the de novo Golgi formation, considers the Golgi apparatus as an outgrowth of ER exit sites or transitional ER (tER) sites, the

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specialized ER subdomains where cargo proteins destined for the Golgi apparatus are packaged into COPII-coated vesicles (Bannykh *et al.*, 1996; Antonny and Schekman, 2001). This model proposes that the membranes exiting the tER sites contain all the necessary molecular information to trigger the building of a functional Golgi apparatus by a mechanism of self-organization (Zaal *et al.*, 1999; Ward *et al.*, 2001; Bevis *et al.*, 2002; Glick, 2002; Altan-Bonnet *et al.*, 2004). This model has been supported by experimental observations on GRASP65. First, live cell imaging studies of GFP-tagged GRASP65 have shown that it exhibits a dynamic association on and off the Golgi membranes (Marra *et al.*, 2001; Ward *et al.*, 2001). Second, using either reagents that block ER-to-Golgi transport, such as Sar1p and Arf1 mutants, or the drugs brefeldin A and H89, GRASP65 was reported to undergo cycling between the Golgi apparatus and the ER exit sites, similar to other golgins (Miles *et al.*, 2001; Ward *et al.*, 2001; Puri and Linstedt, 2003; Stroud *et al.*, 2003; Kasap *et al.*, 2004). Overall, these results have been taken as evidence that GRASP proteins along with other Golgi matrix proteins could be recruited to the membranes exiting the tER sites and differentiate them into a Golgi apparatus (Glick, 2002; Altan-Bonnet *et al.*, 2004).

These two models have recently been reconciled to propose that the biogenesis of the Golgi apparatus occurs through a self-organizing nucleation mechanism. GM130, GRASP65, and GRASP55 were shown to leave the tER sites faster than the Golgi enzymes upon removal of ER exit blocks (Puri and Linstedt, 2003; Kasap *et al.*, 2004), suggesting that Golgi matrix proteins could be able to form a template facilitating the acquisition of Golgi stack morphology.

In *Drosophila*, Golgi stacks exhibit a close spatial association with tER sites, forming tER-Golgi units (Kondylis and Rabouille, 2003; Herpers and Rabouille, 2004), comparable to those observed in *Pichia* (Rossanese *et al.*, 1999; Mogelsvang *et al.*, 2003), *Trypanosoma* (He *et al.*, 2004), and plants (DaSilva *et al.*, 2004).

Here, we show that the single *Drosophila* GRASP homologue, dGRASP, exhibits a steady state localization to both the Golgi membranes and the tER sites under normal growth conditions in *Drosophila* cells and tissues. When depleted from S2 cells by RNAi, alone or in combination with dGM130, the fly homologue of GM130, the Golgi stack architecture was quantitatively disrupted into Golgi clusters, as suggested from its role in mammalian cells. However, the tER organization remained unaffected, as well as the anterograde transport from the ER to the plasma membrane through the Golgi clusters. This suggests that Golgi stack integrity is not necessary for anterograde transport.

Our results indicate that dGRASP, while exhibiting dynamic properties, regulates the acquisition/maintenance of the Golgi stacked morphology. This does not, however, exclude additional functions in signaling and cell cycle regulation.

MATERIALS AND METHODS

Preparation of Double-stranded RNA

A 559-base pair 5′ fragment of dGRASP cDNA was amplified by PCR from a reverse transcriptase reaction of total RNA from *Drosophila* Kc cells using the 5′ primer atagaattcatgggctcgagccac and the 3′ primer caggcggtattcggcttgat. This fragment was cloned into the pCR II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. The clone 3.5 was used to PCR amplify a 587-base pair template bearing T7 ends using the 5′ primer TAATACGACTCACTATAGGGAGA(T7)-agccacagcatccat, and the 3′ primer T7-gcggtattcggcttgat. This fragment was used as a template for the preparation of dGRASP double-stranded RNA (dsRNA) after purification from agarose gel with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany).

The dsRNAs corresponding to dGM130 and EGFP have been described in Kondylis and Rabouille (2003). dsRNA synthesis was performed using the MEGASCRIPT T7 and T3 transcription kit (Ambion, Austin, TX), according to manufacturer's protocol.

Construction of GFP-tagged dGRASP Proteins

The full-length, the N-ter and C-ter fragments of the dGRASP protein (amino acids 1–203 and 203–460, respectively) were amplified by PCR from dGRASP cDNA (gift from Henry Chang, West Lafayette, IN) using primers to introduce *Eco*RI and *Xho*I restriction sites. The PCR fragments were cloned into the pCR II-TOPO vector (Invitrogen) and sequenced. Correct clones were cut with *Eco*RI and *Xho*I and subcloned into the pRmeGFP vector, which contains an inducible metallothionein promoter (gift from Thomas Vaccari, EMBL, Heidelberg, Germany; Farkas *et al.*, 2004). dGRASP point mutation of glycine at position 2 (myristoylation site, G2A mutant) was made using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. All dGRASP fusion proteins contained the EGFP tag at their C-terminus.

Cell Cultures

Wild-type and Delta S2 cells were grown as described in Kondylis and Rabouille (2003).

Transient Transfection of S2 Cells

About 0.5×10^6 S2 cells were plated on glass coverslips in 3.5-cm wells and were transfected the next day with 0.5 μg plasmid DNA per well using the Effectine transfection reagent (Qiagen) according to the manufacturer's protocol. After 48 h, the synthesis of the GFP fusion proteins was induced by adding 1 mM $CuSO_4$ in the cell medium for 3 h at 27°C. The $CuSO_4$ was washed out and the cells were further incubated for 2 h in fresh medium before being fixed for immunofluorescence (IF) or immunoelectron microscopy. On overage, 50% transfection efficiency was achieved.

RNAi

The RNAi experiments were carried out in both cell lines in the same way as described in Kondylis and Rabouille (2003). Double depletion experiments were performed by adding simultaneously in the culture medium 30 μg of dsRNA corresponding to each of the two target genes.

Expression of dGRASP-GFP in Salivary Glands

UAS-dGRASP-GFP/TM6B,Tb flies (Bloomington *Drosophila* Stock Center, Bloomington, IN) containing the dGRASP-GFP transgene under a UAS promoter were crossed to a fly stock carrying a late third instar salivary gland GAL4 driver (gift from Pascal Therond, Nice, France). UAS-dGRASP-GFP/GAL4 late third instar larvae of F1 progeny were dissected and processed for immunoelectron microscopy. TM6B,Tb/GAL4 flies were used as control.

Antibodies

dGM130 was detected by MLO7 antibody (gift from Martin Lowe, Manchester, United Kingdom), which was raised against the N-terminal 73 amino acids of rat GM130, one of the highly conserved domains in dGM130 (Nakamura *et al.*, 1997; Kondylis *et al.*, 2001; Kondylis and Rabouille, 2003). dGRASP was detected using a rabbit polyclonal antibody raised against the mammalian recombinant GRASP65 (Shorter *et al.*, 1999), which was a kind gift from Francis Barr (Martinsried, Germany). dSec23p, d120 kDa, and C594.9B antibodies have been described previously (Kondylis and Rabouille, 2003) and were used to mark the tER sites, the Golgi apparatus, and the protein Delta, respectively. dCOG3 was detected with a cross-reacting antibody raised against the yeast homologue Sec34p (Van Rheenen *et al.*, 1999; Whyte and Munro, 2001). p24δ1 (p23) antibody has been characterized by Majoul *et al.* (1998). Finally, the anti-GFP rabbit polyclonal antibody and secondary antibodies conjugated to Alexa 488, 568, and 647 fluorophores were purchased by Molecular Probes (Eugene, OR).

Western Blotting

Samples of S2 cells were processed for Western blotting as described in Kondylis and Rabouille (2003).

(Immuno) Electron Microscopy

S2 cells were fixed and processed for electron microscopy as described in Kondylis and Rabouille (2003). To reduce the background labeling, all the blocking and washing steps during immunolabeling were carried out in 0.5% fish skin gelatin (Sigma, St. Louis, MO) and 0.1% bovine serum albumin-C (Aurion, Wageningen, the Netherlands) in phosphate-buffered saline (PBS).

Stereology/Quantitation of Immunolabeling

Stereological definitions are given in Kondylis et al. (2001) and Kondylis and Rabouille (2003). As control, Golgi stacks were considered all stacks having a cross-sectional diameter longer than 0.184 μm, a figure that represents 50% of the average cisternal length observed in mock-treated and mock-depleted cells (0.368 ± 0.047 μm). Although arbitrary, this criterion was used to

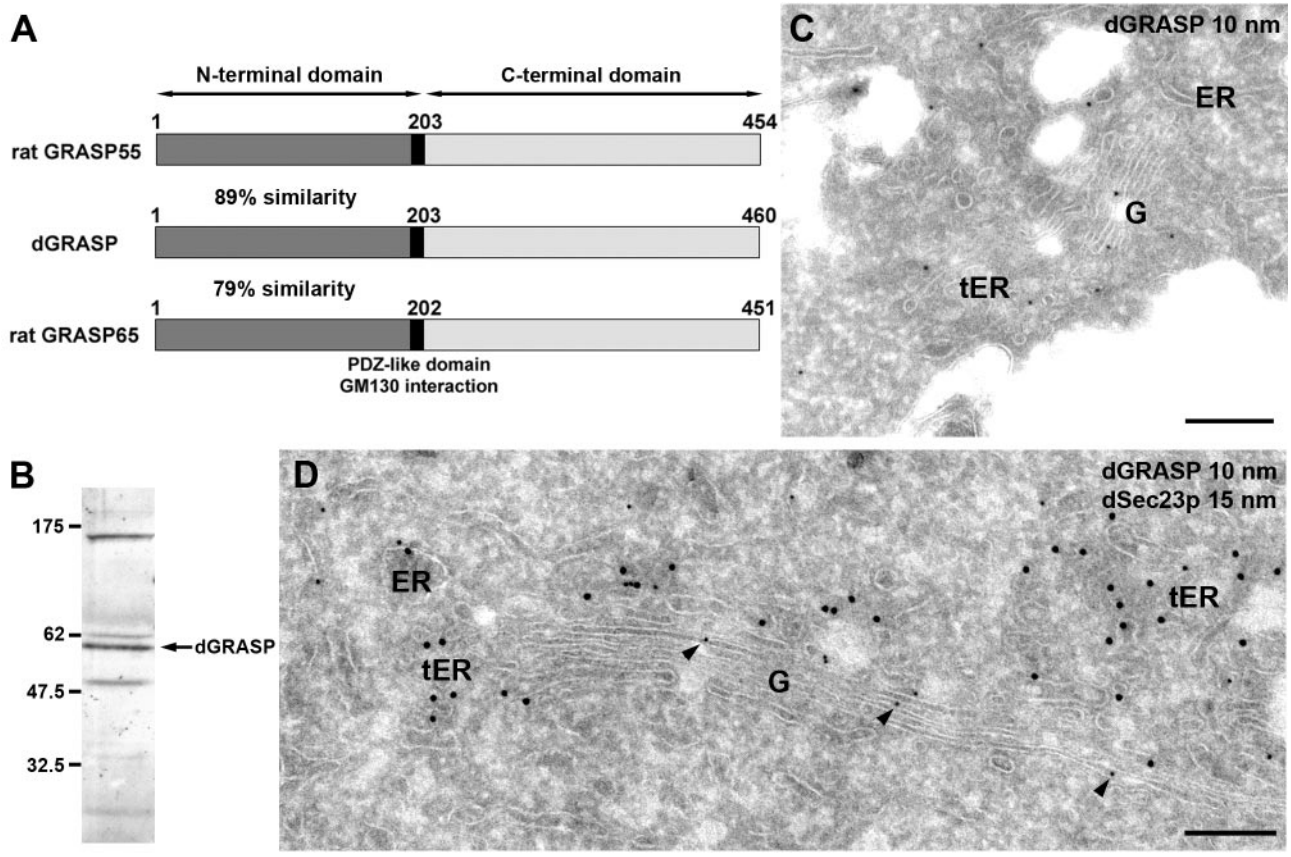

Figure 1. Localization of dGRASP in *Drosophila* cells. (A) Schematic comparison between rat GRASP65 and GRASP55, and dGRASP. The proteins were aligned using the EMBOSS-Align program and the domains of high homology are shaded in dark gray. (B) Western blot of a 2 million *Drosophila* S2 cell extract with an anti-GRASP65 antibody. (C and D) dGRASP localization was performed on cryosections of S2 cells (C) and salivary glands (D) fixed with 2% PFA and 0.2% GA and labeled with the anti-GRASP65 antibody (C) or double-labeled for GRASP65 (10 nm gold) and dSec23p (15 nm gold) (D). Arrowheads point to dGRASP localizing in the intercisternal space. G, Golgi stacks; ER, endoplasmic reticulum; tER, transitional ER. Bars, 200 nm.

illustrate the significantly increased number of cell profiles that exhibited short Golgi stacks in dGRASP/dGM130 double-depleted cells compared with the mock-treated cells.

The relative distribution and linear density of the gold labeling was estimated as described before (Rabouille, 1999; Kondylis and Rabouille, 2003).

Indirect Immunofluorescence

S2 cells grown on coverslips were fixed in 4% PFA in PBS and processed for IF as described in Kondylis and Rabouille (2003). Cells were viewed under a Leica TCS-NT (Jena, Germany) or a Zeiss LSM-510 confocal microscope (Wetzlar, Germany).

Fluorescence Recovery after Photobleaching Analysis

For the fluorescence recovery after photobleaching (FRAP) analysis of dGRASP-GFP, a confocal section of S2 cells was captured with a 63× objective (prebleach), and a square box around a selected fluorescent spot was bleached using 50 consecutive scans at full laser power. The fluorescence recovery was monitored by time-lapse imaging at low-intensity illumination and quantified with the Zeiss LSM software relative to the fluorescence intensity of nonbleached spots at the same section.

Delta Transport Assay/Quantitation

Delta transport assay has been described previously (Kondylis and Rabouille, 2003). Briefly, after each RNAi experiment on Delta S2 cells, 1 mM $CuSO_4$ was added in the culture medium for 60 min to induce the synthesis of Delta protein. Subsequently, the $CuSO_4$ was washed out and the transport of Delta through the Golgi apparatus to the plasma membrane was monitored for 90 min. This protocol was used to estimate the steady-state anterograde transport of Delta.

The quantification of Delta transport to the plasma membrane was performed as described in Kondylis and Rabouille (2003) on ~300 randomly selected cells from at least two different experiments using the Leica TCS-NT software.

H89 and BFA Treatment

In H89 experiments, the drug was added in the cell medium for 2 h at 27°C at a final concentration of 50 μM. Subsequently, Delta was induced with $CuSO_4$ for 60 min, and its transport to the plasma membrane was chased for 90 min at 27°C, always in the presence of the inhibitor. BFA treatment was performed as described previously (Kondylis and Rabouille, 2003).

RESULTS

dGRASP Localization

In a functional characterization of the *Drosophila* homologue of p115 (dp115), we have shown that the protein associates with the compartments of the early exocytic pathway (tER sites, Golgi apparatus, and to a smaller extent with ER membranes; Kondylis and Rabouille, 2003). To investigate whether the single GRASP homologue identified in *Drosophila* (dGRASP, CG7809) exhibits a similar distribution, we localized it by immunoelectron microscopy (IEM).

dGRASP consists of 460 amino acids and the N-terminus is highly homologous to both mammalian GRASPs, exhibiting 89% similarity to GRASP55 and 79% to GRASP65 (Figure 1A). Functionally important sites of the N-terminal portion of GRASP proteins conserved in dGRASP (N-ter, AA1–203) are the glycine residue at position 2 (myristoylation site

Table 1. Stereological analysis of dGRASP localization in *Drosophila* cells and tissues

		Relative distribution			Linear density (gold/ μm of membrane)		
	% of membrane-associated gold particles	Golgi	tER	ER	Golgi	tER	ER
Endogenous dGRASP[a]							
Salivary glands	77.1 ± 3.0	33.9 ± 2.5	40.0 ± 1.3	26.1 ± 1.7[b]	0.84	0.53	0.12
S2 cells	69.4 ± 6.8	46.7 ± 9.2	33.4 ± 3.9	19.9 ± 8.6			
dGRASP-GFP							
Salivary glands	74.7 ± 7.3	52.6 ± 8.9	30.7 ± 7.7	16.7 ± 6.8			
S2 cells	78.4 ± 6.9	53.6 ± 11.9	30.1 ± 10.2	16.3 ± 7.8			

[a] Data obtained from S2 cells and salivary glands with the anti-GRASP65 antibody.

[b] The increased percentage of gold labeling on the ER is due to its large surface section in salivary glands, making the background contribution higher than for the other smaller organelles.

mediating the binding to Golgi membranes), the cysteine at position 191 (possibly responsible for GRASP sensitivity to sulfhydryl modifying agent NEM; Barr *et al.*, 1997), and the two PDZ-like domains (Barr *et al.*, 1998). The second PDZ-like domain of GRASP65 is known to interact with GM130, and though it is also present in GRASP55, the latter does not seem to interact with GM130, in vivo (Shorter *et al.*, 1999). Recently, this conserved N-terminal part of GRASP65 was shown to mediate the formation of dimers and oligomers (Wang *et al.*, 2005).

The C-terminal half of the GRASP homologues is very diverse. In GRASP65, it is rich in proline residues and contains multiple serine/threonine phosphorylation sites, the phosphorylation of which regulates the oligomerization of the N-terminal half (Preisinger *et al.*, 2005; Wang *et al.*, 2005). Similarly, the C-terminal part of dGRASP (C-ter, AA 203–460) is rich in proline and alanine residues and contains five putative phosphorylation sites (T270, T301, S307, S411, T421; NetPhos v2.0 computer program, http://www.cbs.dtu.dk/services/NetPhos/).

The localization of dGRASP by IEM was performed using an antibody recognizing mammalian GRASP65 (see *Materials and Methods*). On Western blots, this antibody recognizes two strong bands (Figure 1B), one migrating around 60 kDa (predicted molecular weight for dGRASP) that disappears upon depletion of the protein by RNAi (see Figure 4A and 6A), and another around 160 kDa that is not depleted (see Figure 4A).

In S2 cells, dGRASP was found on the Golgi apparatus and small pleiomorphic structures (Figure 1C) that were confirmed to be tER sites by dSec23p labeling (unpublished data). On depletion of dGRASP by RNAi, the labeling density of the tER-Golgi area was reduced from 18.5 ± 1.9 gold particles/μm^2 to 3.3 ± 2.1, indicating that the labeling of these membranes was highly specific (see Supplementary Figure 1, A and B). In late 3rd instar salivary glands, the distribution of dGRASP was similar except for a small fraction of the labeling on ER membranes. The rest was equally distributed over the Golgi apparatus and the tER sites marked by dSec23p labeling (Table 1; Figure 1D). The linear density on the Golgi membranes was 58% higher than on the tER membranes (Table 1). The Golgi-associated dGRASP labeling was often found in the intercisternal space (arrowheads in Figure 1D), which is consistent with the proposed stacking role of GRASP proteins.

Because the tER localization of dGRASP has not been reported for the mammalian GRASPs, we set out to confirm it by expressing the full-length dGRASP C-terminally tagged with GFP in *Drosophila* salivary glands (Figure 2, A and B) and S2 cells (Figure 2, C–H). In both cell types, dGRASP was present on the Golgi stack (Figure 2, A–C and E) and peripheral Golgi vesicles (arrows in Figure 2, B–E), as well as on tER sites (arrowheads in Figure 2, B, D, and E). Both in S2 cells and salivary glands, ~53% of dGRASP-GFP was confined to the Golgi apparatus and 30% to the tER sites (Table 1). These results indicate that the tER localization observed with the anti-GRASP65 antibody was not due to unspecific labeling.

The dGRASP-GFP localization on both compartments was also exemplified by IF. dGRASP-GFP partially colocalizes with the Golgi marker d120 kDa (Figure 2F) and with dSec23p (Figure 2G), whereas some of the GFP signal did not seem to colocalize with any of the two other markers, likely corresponding to the peripheral vesicles associated with the stack (arrows in Figure 2, B, D, E, and H). As a control, GFP alone showed a cytosolic and nuclear distribution (Figure 3A).

We also examined the behavior of dGRASP-GFP by performing FRAP experiments in transfected S2 cells. In agreement with what has been reported for GRASP65-GFP (Ward *et al.*, 2001), the fluorescence intensity of dGRASP-GFP spots was fully recovered within 30–60 s after photobleaching (Figure 2I), suggesting that it is actively recruited from the cytosol.

The myristoylation of the glycine at position 2 mediates the Golgi localization of GRASP65 and GRASP55 (Barr *et al.*, 1997; Shorter *et al.*, 1999). To investigate whether dGRASP association with the membranes of the early exocytic pathway (tER sites and Golgi apparatus) was also mediated by the same mechanism, we mutated glycine 2 into an alanine (G2A-dGRASP). On transfection of S2 cells with G2A-dGRASP-GFP, the fluorescence was strongly cytosolic, independently of the transfection level (compare Figure 3, B and C).

The N-terminal half of dGRASP (N-ter-GFP) did localize to the tER-Golgi units (Figure 3D), although a weak cytosolic signal was observed, even at low expression level. The C-terminal half (C-ter-GFP), on the other hand, gave a strong cytoplasmic pattern indistinguishable from the G2A chimera.

All GFP constructs exhibited the expected molecular weight after analysis by Western blot using an anti-GFP antibody (Supplementary Figure 2A) and were recognized by the anti-GRASP65 antibody except for the C-ter-GFP (Supplementary Figure 2B).

Taken together, these results show that, similar to dp115, dGRASP is not restricted to the Golgi apparatus, but it is

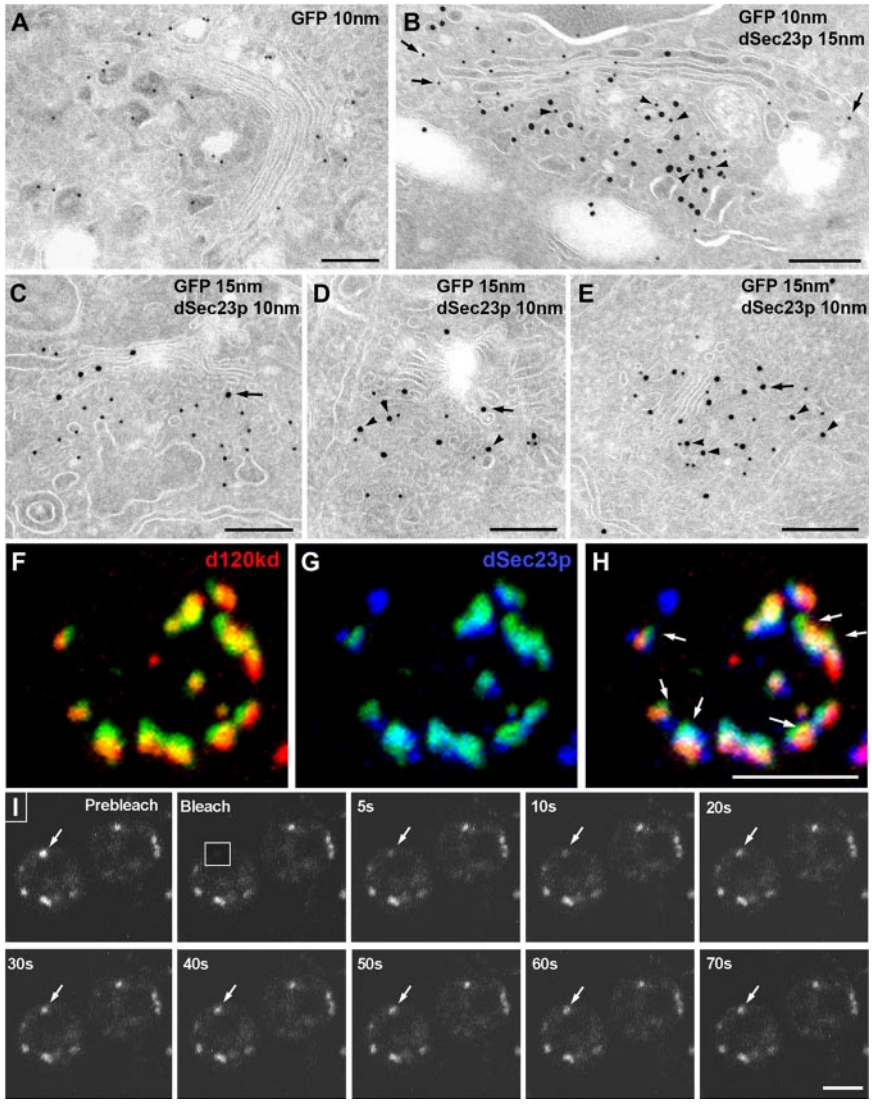

Figure 2. dGRASP-GFP localizes on Golgi stacks and tER sites in *Drosophila* cells. (A–E) Cryosections of salivary glands (A and B) and S2 cells (C–E) expressing the full-length dGRASP-GFP labeled with anti-GFP and dSec23p antibodies (the size of the gold particles associated with each antibody is mentioned at the top of each figure). dGRASP is found in the Golgi stacks, in peripheral Golgi vesicles (arrows) and in the tER sites (arrowheads). (F–H) IF localization of dGRASP-GFP with d120 kDa (F), dSec23p (G), and both (H). Note that full-length dGRASP-GFP colocalizes with both the tER sites and the Golgi stacks, but also shows a wider distribution representing the peripheral Golgi elements (white arrows in H). The projection of 20 confocal sections is presented. (I) Fluorescence recovery after photobleaching (FRAP) of dGRASP-GFP. S2 cells were transiently transfected with dGRASP-GFP, and a square area around a fluorescent spot (arrow in prebleach image) was photobleached. The recovery of fluorescence in the area was monitored and images at representative time points after bleaching are shown. Note the fast reappearance of fluorescence in the bleached dGRASP-GFP spot. One confocal section is presented. Bars, 200 nm (A–E); 5 μm (F–I).

also localized on the tER sites mostly through its myristoylation site.

Golgi Stack Architecture Is Disrupted when dGRASP Is Depleted Alone and in Combination with dGM130

The localization of dGRASP suggests that its function could be related to the organization of both the Golgi stacks and the tER sites.

To test this hypothesis, S2 cells were efficiently depleted of dGRASP by RNAi, after a 72-h and up to a 120-h incubation with dsRNA (Figure 4A). When examined by EM, the majority of dGRASP-depleted cell profiles still exhibited Golgi stacks in their cytoplasm, occasionally of smaller diameter compared with mock-treated cells (Figure 4C, arrow). About 20%, though, contained vesiculated Golgi apparatus (unpublished data). Importantly, a small, but significant percentage of cells exhibited single cisternae embedded in clusters of vesicles and tubules (Figure 4B, arrowhead), a feature rarely observed in control cells or upon RNAi depletion of dp115 (unpublished data) or dGM130 (Figure 5B).

In an attempt to enhance this statistically significant, but nonpenetrant loss of Golgi stack architecture observed upon dGRASP depletion (Figure 5, A and B), we depleted S2 cells of dGRASP in combination with its potential interacting protein dGM130.

RNAi depletion of dGM130 in S2 cells was performed in a previous study, but did not lead to a significant morphological alteration of the early exocytic compartments (Kondylis and Rabouille, 2003). Both dGRASP and dGM130 were confirmed to be depleted at least 72 h after addition of the dsRNAs (Figure 6A). Furthermore, it was confirmed that each single depletion did not affect the expression of the other protein (unpublished data).

EM analysis of the double-depleted samples unraveled an extensive loss of Golgi stack architecture. After 72 h of double depletion, 47.9% of the cell profiles did not exhibit control Golgi stacks (vs. 17.8% for dGRASP depletion; Figure 6B), a figure that reached 75.8% at 120 h (Figure 5A). In the remaining cells exhibiting Golgi stacks, these stacks were shorter than in mock-treated cells and often contained only two cisternae (Figure 6C).

In agreement with our morphological observations, the percentage of Golgi membranes in total cisternae in dGRASP/dGM130 depleted cells was significantly reduced compared with the respective single depletions

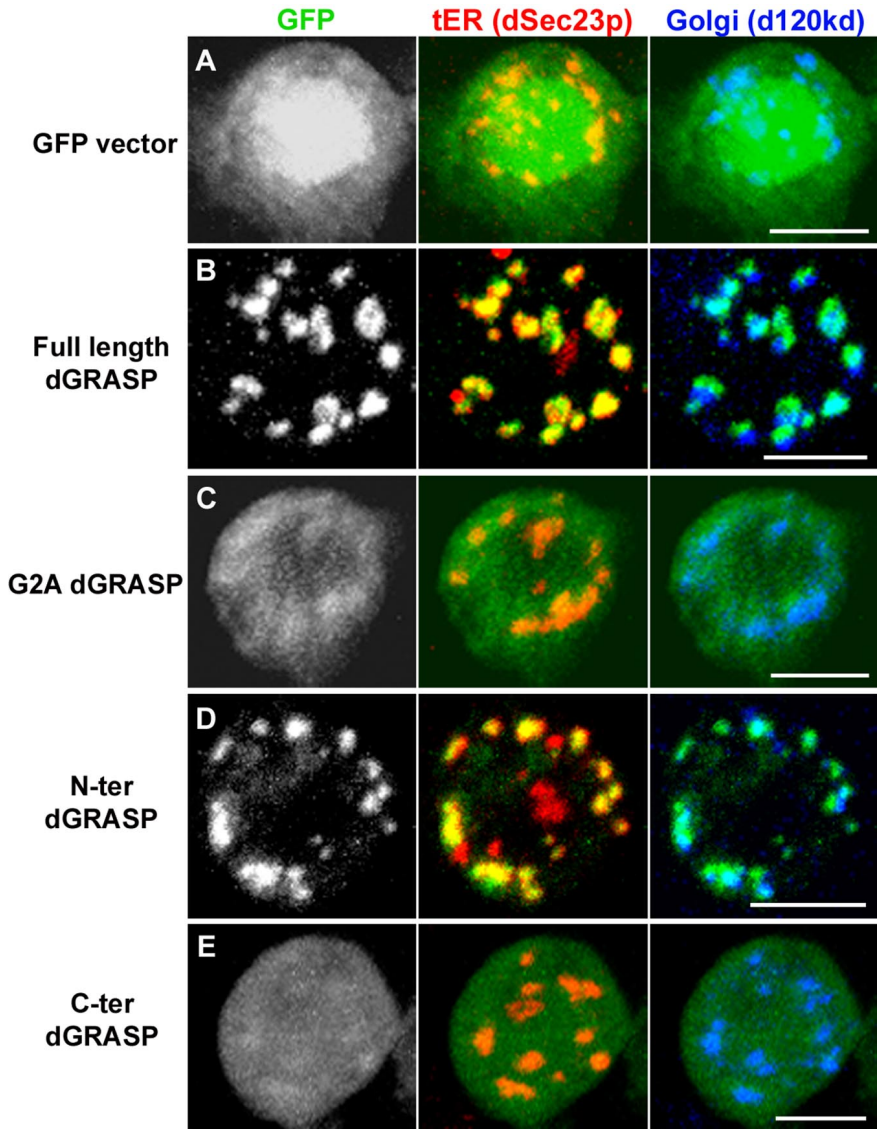

Figure 3. The tER and Golgi association of dGRASP is mediated by the same mechanism. S2 cells expressing GFP alone (A), full-length dGRASP-GFP (B), G2A-dGRASP-GFP (C), N-ter-GFP (D), and C-ter-GFP (E) were double-labeled with dSec23 (red, tER) and d120 kDa (blue, Golgi). Note that G2A-dGRASP-GFP and the C-ter-GFP exhibit a cytosolic distribution, whereas the N-ter-GFP is mostly associated with tER-Golgi units similar to the full-length dGRASP. Projections are presented. Bar, 5 μm.

(Figure 5B, black and light gray bars). In contrast, the percentage of membranes in single cisternae increased at least ninefold in dGRASP/dGM130-depleted cells when compared with control or dGM130-depleted cells, illustrating their significance (Figures 5B, light gray bars, and 6D). Overall, these results suggest a specific role for dGRASP in cisternal stacking in vivo, which is in line with the proposed role for GRASP proteins as stacking factors in mammalian cells.

Last, we confirmed that the fragmented Golgi stacks obtained upon the double depletion contained membranes with Golgi identity by labeling cryosections for Golgi markers, such as d120 kDa and dCOG3 (Van Rheenen *et al.*, 1999; Whyte and Munro, 2001; Figure 6, E–G).

tER Organization Is Unaffected upon Depletion of dGRASP

The structural integrity of the tER sites was examined by monitoring the distribution of dSec23p (Figure 7, A and D) together with the Golgi integral membrane protein, d120 kDa (Figure 7, B and E; Kondylis and Rabouille, 2003). Under normal conditions (mock-depleted cells), the tER sites appear by IF as ~20 focused spots throughout the cytoplasm (Figure 7A), closely associated to the Golgi apparatus (Figure 7C; Kondylis and Rabouille, 2003). In dGRASP/dGM130-depleted cells, d120 kDa distribution appeared more diffused compared with mock-depleted cells (Figure 7E), consistent with our observations at the EM level of a quantitative loss of Golgi stack structure and possibly a partial dispersion of the Golgi fragments. However, in 84.5 ± 1.1% of these cells, the dSec23p pattern appeared similar to the mock-depleted cells, showing that the tER organization was not affected by this double depletion (Figure 7D). A slight difference from control cells was that ~15% of dSec23p-positive spots were no longer in close proximity to a d120 kDa-positive structure (Figure 7F, arrows), possibly due to the Golgi disorganization and dispersion.

When examined by IEM, the tER sites in dGRASP/dGM130-depleted cells were also found indistinguishable from the mock-depleted S2 cells in terms of size and appearance (Figure 7E).

These results suggest that, although localizing to the tER sites, dGRASP does not appear to play a role in their organization, contrary to what was observed for dp115.

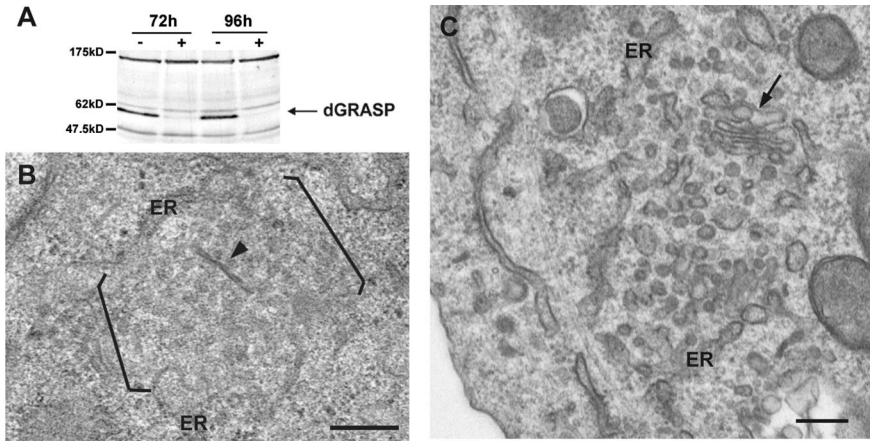

Figure 4. Effect of dGRASP depletion on the Golgi stack architecture. (A) A total extract from 2 million S2 cells incubated with (+) or without (−) ds dGRASP for 72–96 h was analyzed by Western blotting using the anti-GRASP65 antibody. The band appearing at 60 kDa (predicted molecular weight for dGRASP) was the only one to be specifically depleted in dsRNA-treated cells. (B and C) The effect of dGRASP depletion on Golgi stack architecture was assessed on S2 cells incubated with ds dGRASP for 96 h and processed for conventional EM. Golgi areas are marked between brackets, and the Golgi stack by an arrow. Note the presence of single cisternae among clusters of vesicles and tubules at the Golgi area (arrowhead in B). ER, endoplasmic reticulum. Bars, 200 nm

Effect of dGRASP Depletion in Anterograde Protein Transport

The depletion of dGRASP alone or in combination with dGM130 suggested a role in Golgi biogenesis and/or maintenance. This is not due to a disorganization of the tER sites but could be due to an inhibition of intracellular transport through the exocytic pathway, as it is the case upon dSed5p depletion (Kondylis and Rabouille, 2003).

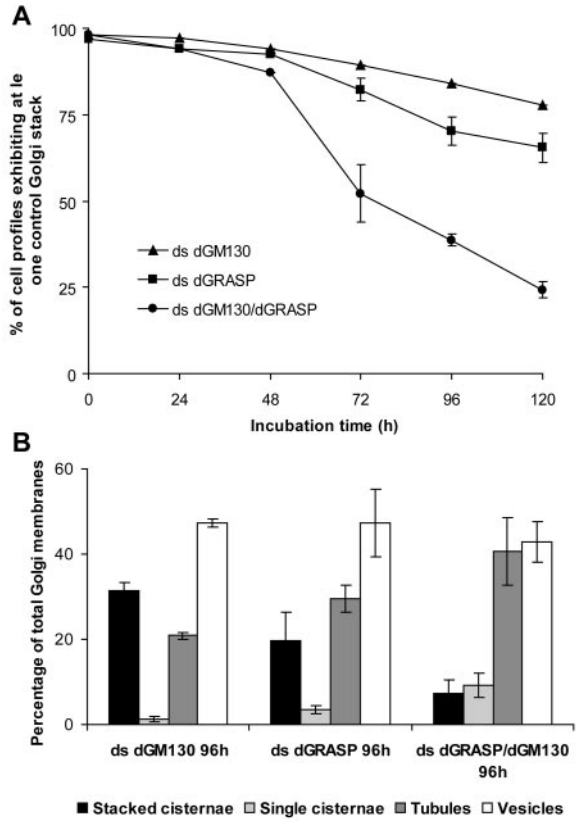

Figure 5. Quantitative analysis of dGRASP and dGRASP/dGM130 depletion on Golgi morphology. (A) The percentage of S2 cell profiles exhibiting at least one control Golgi stack in their cytoplasm was scored on ultrathin epon sections from cells incubated with ds dGM130 (triangles), ds dGRASP (squares), or ds dGRASP and ds dGM130 combined (circles) for 24–120 h. (B) Random EM pictures of dGM130-, dGRASP-, and dGRASP/dGM130-depleted cells for 96 h were used to estimate the percentage of Golgi membrane in stacked cisternae (black bars), single cisternae (light gray bars), tubules (dark gray bars), and vesicular profiles (white bars). Error bars, SD.

To measure the efficiency of anterograde transport, we used Delta S2 cells (S2 cells transfected with the transmembrane plasma membrane protein; Delta; Klueg *et al.*, 1998) in a transport assay that we have established previously (Kondylis and Rabouille, 2003; see *Materials and Methods*).

We have shown that conditions that completely block intracellular transport in mammalian cells equally block the arrival of Delta to the plasma membrane in our system. Both BFA treatment and depletion of dSed5p led to a 90% reduction in the anterograde transport of Delta (Kondylis and Rabouille, 2003).

As an additional positive control, we also examined the transport competence of H89-treated cells. H89 is an isoquinolinesulfonamide shown to inhibit Sar1 recruitment on the ER membranes (Aridor and Balch, 2000), thus leading to a cytosolic redistribution of COPII coat subunits in mammalian cells (Lee and Linstedt, 2000). As expected, the efficiency of anterograde transport of Delta was reduced by 80% when compared with mock-treated cells (Figure 8C), reflecting the intracellular retention of the protein in more than 70% of the cells (Figure 8, A and B; The effect of H89 and BFA on the distribution of the tER and Golgi markers is shown in Supplementary Figure 3).

The transport competence of dGRASP/dGM130 depleted Delta S2 cells was tested. Similar to what has been previously reported for dp115- or dGM130-depleted cells (Kondylis and Rabouille, 2003), the arrival of Delta at the plasma membrane was only reduced by 7.1% when compared with mock-treated cells (Figure 8A; compare black and dotted bars in Figure 8C). Not surprisingly, Delta transport was also not significantly affected in cells depleted of dGRASP alone (Figure 8C, gray bars). The distribution of mock-treated and depleted cells in the four categories of plasma membrane intensity was comparable (Figure 8B), showing that the transport efficiency measured upon depletion was not due to a small fraction of the cells exhibiting an exaggerated transport of Delta to the plasma membrane. This is further supported by the fact that cell growth was not affected upon any of these depletions.

However, to confirm that Delta did not use an alternative pathway and passed through the Golgi clusters generated upon dGRASP/dGM130 depletion, we immunolocalized Delta in cryosections of depleted cells. Golgi clusters (positive for dCOG3 and adjacent to dSec23p) were strongly

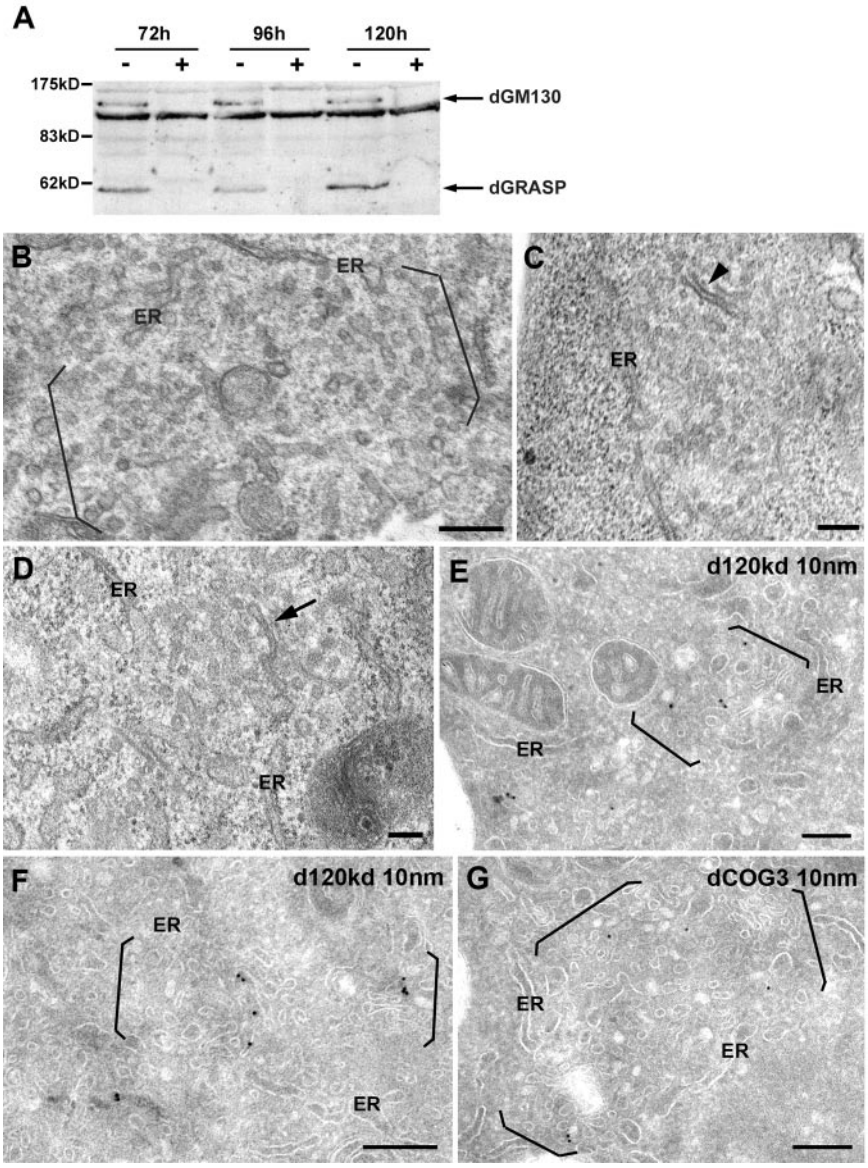

Figure 6. Effect of dGRASP/dGM130 double depletion on the Golgi architecture. (A) A total extract from 2 million S2 cells were incubated with (+) or without (−) dsRNAs corresponding to dGM130 and dGRASP for 72 and 120 h was analyzed by Western blotting using MLO7 and anti-GRASP65 antibodies. (B–D) Conventional EM of S2 cells incubated in the presence of ds dGRASP and ds dGM130 combined for 96 h. Vesiculated Golgi areas (between brackets in B), short 2 cisterna stacks (arrowhead in C), and single cisternae (arrow in D) were common features of the double-depleted cells. (E–G) Immunolocalization of d120 kDa (E and F) and dCOG3 (G) in cryosections of dGRASP/dGM130-depleted S2 cells. Golgi areas are shown in brackets. ER, endoplasmic reticulum. Bars, 200 nm.

positive for this cargo protein (Figure 8, D and E). Moreover, in depleted cells exhibiting both Golgi clusters and small Golgi stacks, Delta was equally distributed on both organelles (Supplementary Figure 4). Last, Delta could also be chased out of the Golgi clusters, because after 90 min of chase, tER-Golgi units were depleted of Delta, which was now found exclusively at the plasma membrane and in endosomes (unpublished data). These results suggest that Golgi clusters are as competent for anterograde transport as Golgi stacks.

p24δ1 Localization upon dGRASP Depletion

We also investigated the potential role of dGRASP in retention of p24 proteins. Mammalian GRASPs have been shown to interact with p24 family of cargo receptors that cycle between the early exocytic compartments (Dominguez *et al.*, 1998; Blum *et al.*, 1999; Barr *et al.*, 2001). p24β1 interacts directly with GRASP65 through its two extreme C-terminal valines, mutations of which lead to the deposition of the protein at the plasma membrane (Barr *et al.*, 2001). p24δ1 forms a stoichiometric complex with p24β1 and other p24 proteins, by virtue of which it is also found associated with GRASP55 and to a smaller extent with GRASP65 in protein binding studies (Barr *et al.*, 2001).

We analyzed the distribution of the *Drosophila* homologue of p24δ1 that shares 69% homology with its rat counterpart including the RYFKAKKLIE extreme C-terminus. We reasoned that if dGRASP mediates the retention of the *Drosophila* p24 family members, directly or indirectly, its depletion might lead to the escape of at least some of them, including p24δ1, to the plasma membrane.

In *Drosophila* S2 cells, this protein is localized mostly at tER sites overlapping with dSec23p (Supplementary Figure 5, A and B, arrowheads), as shown by IEM, at the same location as dGRASP, and colocalizes slightly with the Golgi marker d120 kDa by IF (Supplementary Figure 5C). However, this distribution did not change in cells depleted of dGRASP/dGM130 combined. p24δ1 was not found in the Golgi apparatus and/or the plasma membrane (Supplementary Figure 5D), suggesting that whatever the fate of p24β1

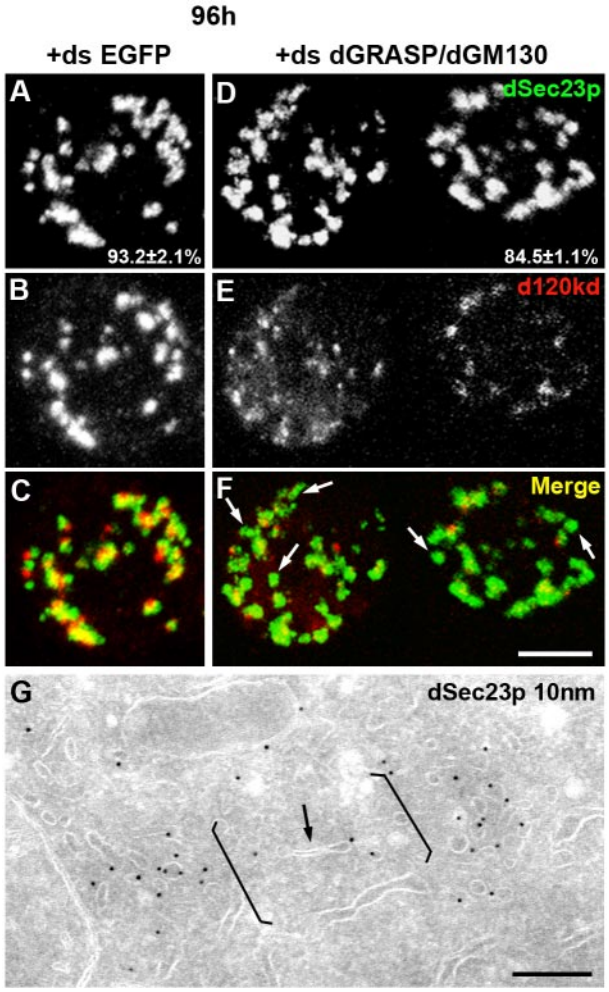

Figure 7. Effect of dGRASP/dGM130 double depletion on the organization of the tER sites. (A–F) Mock (+ds EGFP, A–C) and dGM130/dGRASP depleted (D–G) S2 cells for 96 h were labeled for dSec23p (A and D, green) and d120 kDa (B and E, red). In the merge pictures (C and F), the overlapping areas are presented in yellow. Note the similar dSec23p pattern in mock- and double-depleted cells. The white arrows point to dSec23p-positive structures that are not located close to a d120 kDa-positive Golgi membrane. (G) Depleted cells were labeled for dSec23p for IEM. Note the Golgi cluster (between brackets) containing a single cisterna (black arrow) surrounded by two tER sites. Bars, 5 μm (A–F) and 200 nm (G).

in the depleted cells, p24δ1 was efficiently retained and recycled by other means, such as the KXKXX sequence that binds COPI coat subunits (Dominguez *et al.*, 1998).

DISCUSSION

S2 Cells as an Adequate System to Study the Exocytic Pathway

The exocytic pathway in *Drosophila* S2 cells is organized as ~20 tER-Golgi units (Kondylis and Rabouille, 2003), located throughout the cytoplasm, made of a Golgi apparatus in very close proximity to one tER site, similar to what have been described in *Pichia* (Rossanese *et al.*, 1999; Mogelsvang *et al.*, 2003). The *Drosophila* Golgi stacks exhibit the same basic features as the mammalian ones (e.g., the polarity, the number of cisternae per stack), though their cross-sectional diameter is significantly smaller (with an average of 368 nm in S2 cells). The tER sites, marked by dSec23p, also exhibit similar morphological features, though they appear significantly larger than in mammalian cells.

Anterograde transport of cargo takes place similarly in mammalian and *Drosophila* tissue culture cells, both being sensitive to BFA, H89 and the depletion of syntaxin 5/dSed5p (Lippincott-Schwartz *et al.*, 1989; Dascher *et al.*, 1994; Lee and Linstedt, 2000; Kondylis and Rabouille, 2003; this study). Moreover, both drugs have similar effects on the Golgi resident proteins and the tER site organization.

Drosophila S2 cells are therefore an adequate biological system to investigate issues related to membrane traffic and organelle architecture. The simplified but comparable organization of their exocytic pathway provides us with the possibility to examine the molecular mechanisms underlying both its structure and functions.

dGRASP Localization and Functions in the Exocytic Pathway

In the Golgi Apparatus Because dp115, dGRASP localizes both to the tER sites and the Golgi apparatus. dGRASP localizes throughout the Golgi stack and peripheral Golgi elements, similar to the mammalian GRASPs (Shorter *et al.*, 1999). It was also observed between cisternae, and when S2 cells were depleted of this protein, a small but significant percentage exhibited a single cisterna phenotype, which is consistent with the in vivo and in vitro role of GRASP65 and GRASP55 in cisternal stacking (Barr *et al.*, 1997; Shorter *et al.*, 1999; Wang *et al.*, 2003).

In addition to single cisternae, the depletion of dGRASP also led to the conversion of Golgi stacks into clusters of vesicles and tubules. This could be due to the fact that single cisternae are relatively unstable and break down easily. Alternatively, dGRASP could be involved in the formation of Golgi cisternae. Independently of the mechanism, this phenotype was strengthened by the double depletion of dGRASP together with dGM130. The stronger phenotype observed when the two proteins are depleted together than when either one is depleted alone could be interpreted as a genetic interaction. Because in mammalian cells GM130 interacts biochemically with GRASP65 (Barr *et al.*, 1998), we hypothesize that the observed genetic interaction in S2 cells could also reflect a biochemical one, but this would need to be confirmed.

In the tER Sites Although our RNAi results in S2 cells have confirmed a role of dGRASP in Golgi architecture, this function is unlikely to be the only one in the exocytic pathway. First, a GRASP-like homologue is present in the genome of *Encephalitozoon cuniculi*, a protist containing the smallest eukaryotic genome sequenced to date, which does not exhibit a typical stacked Golgi apparatus (Franzen and Müller, 1999; Katinka *et al.*, 2001). Second, dGRASP also localized on the tER sites. The myristoylation of the glycine at position 2 is required for this localization (as well as for the Golgi), because its mutation to an alanine abolishes most of its membrane association. This does not exclude that other dGRASP domains could be also necessary. dGRASP myristoylation is only mediating the association with tER and Golgi membranes and additional, yet unidentified, proteins and/or lipids are likely to be the determinants for this localization.

Despite its localization on tER membranes, dGRASP depletion (alone or in combination with dGM130) did not lead to any disorganization of the tER sites, as assessed by IF or

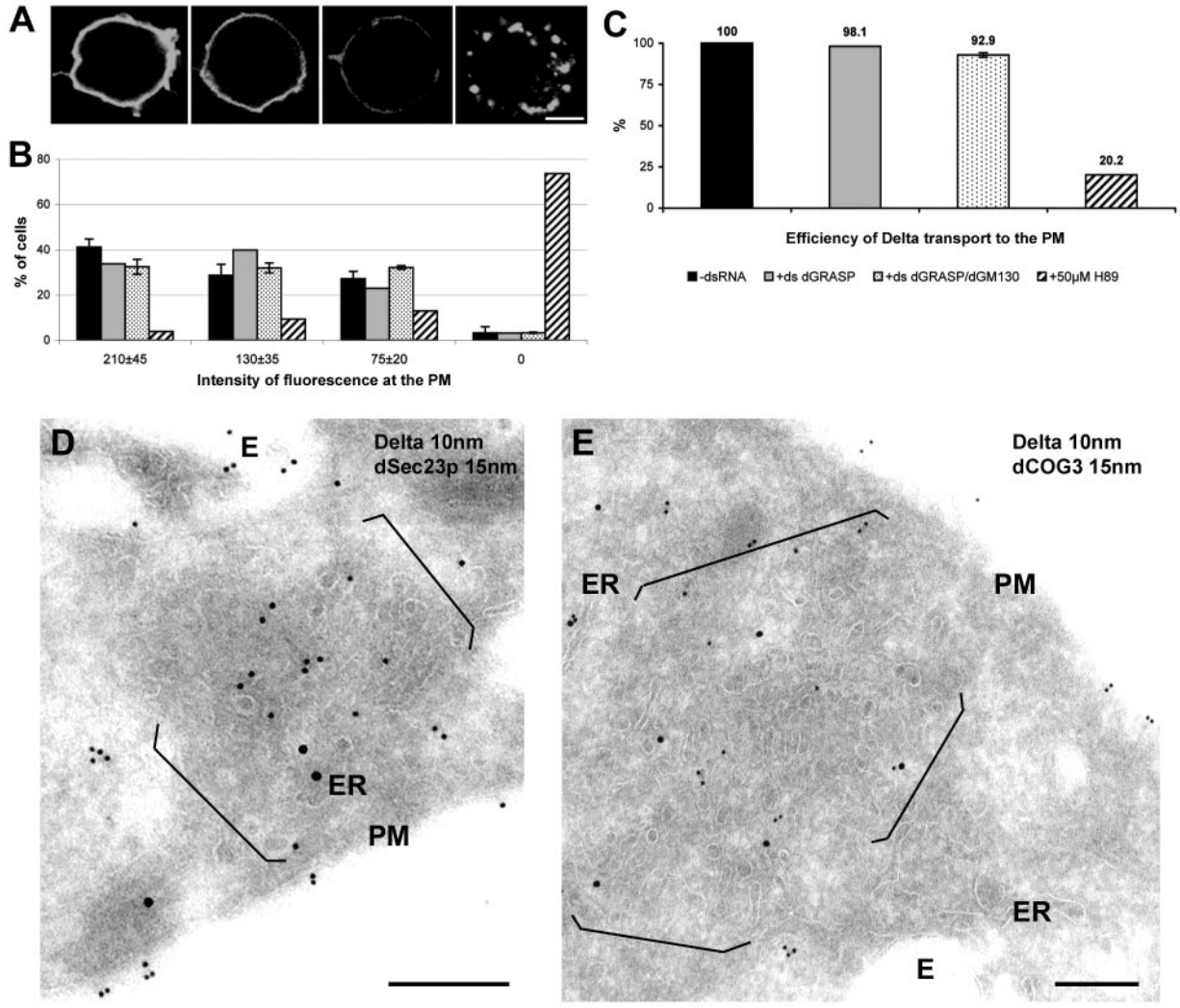

Figure 8. Effect of dGRASP and dGRASP/dGM130 depletion in anterograde transport of Delta to the plasma membrane. Delta S2 cells were incubated with the indicated dsRNAs for 96 h or with 50 μM H89 for 2 h, and Delta synthesis was induced for 60 min using $CuSO_4$ followed by a chase of 90 min. H89 was present throughout the induction and chase periods. (A) Examples of the three labeling categories of Delta labeling at the plasma membrane (three left pictures) or exclusive intracellular labeling (right picture). (B) The percentage of cells exhibiting each of the three different intensities of plasma membrane or intracellular staining was scored. The fluorescence intensity for each of the four categories is mentioned in *x*-axis. Results are expressed as a percentage of total number of cells examined. (C) The efficiency of anterograde transport of Delta to the plasma membrane was estimated as the percentage of the total fluorescence intensity after each treatment over the total fluorescence intensity in mock-treated cells, which was set as 100%. The error bars in B and C represent the SD. Note that Delta labeling in H89-treated cells was retained mostly intracellular. (D and E) Cryosections of dGRASP/dGM130-depleted cells for 96 h and induced with CuSO4 for 90 min were double-labeled for Delta (10 nm) and dSec23p (D) or dCOG3 (E; 15 nm). Note that Delta is transported through the Golgi clusters (between brackets). ER, endoplasmic reticulum; PM, plasma membrane; E, endosomes. Bars, 5 μm (A) and 200 nm (D and E).

IEM, contrary to what has been reported for dp115 depletion (Kondylis and Rabouille, 2003).

dGRASP could give the membranes exiting the tER sites their Golgi identity, as suggested by the self-organizing nucleation mechanism proposed to explain the Golgi stack formation (Kasap *et al.*, 2004; see *Introduction*). Except for dp115, dGRASP is the only example thus far of a Golgi matrix protein being localized at the tER sites under normal steady state conditions, whereas in mammalian cells this localization has been only exemplified under conditions of ER exit block. This suggests that dGRASP could cycle between tER sites and Golgi stack faster or more than its mammalian homologues. dGRASP could be first recruited to the Golgi stack and then cycles very rapidly back to the tER sites or first recruited to the tER sites and delivered to the Golgi apparatus upon anterograde transport. The latter possibility seems perhaps unlikely, because newly synthesized GRASP65 was found associated with Golgi membranes upon an ER exit block (Yoshimura *et al.*, 2001). As a final possibility, dGRASP could be dynamically recruited from the cytosol to both the tER and Golgi membranes, as suggested by the rapid recovery of fluorescence in our FRAP experiment, although this result cannot formally exclude a rapid transport between both compartments.

Effect of dGRASP Depletion in Anterograde Transport

The double depletion of dGM130/dGRASP did not lead to a significant inhibition of anterograde transport, at least at

steady state. Mammalian GRASP65 (and GM130) have been implicated in anterograde transport by capturing cargo-containing carriers emanating from the intermediate compartment to the *cis*-Golgi (Marra *et al.,* 2001). Nevertheless, in *Drosophila* S2 cells, the role of dGRASP could be nonessential because of the short distance between the tER sites and the Golgi stack.

This result shows that, as for dp115 (Kondylis and Rabouille, 2003), Golgi stack structure is not required for efficient anterograde transport, at least in *Drosophila*. Of course, there are many indications in the literature for this, ranging from the secretion in budding yeast that lacks Golgi stacks to those of lower eukaryotes, such as *E. cuniculi* (see above), that have no obvious Golgi stacks but contain genes encoding proteins of Golgi budding and fusion machinery, as well as some matrix proteins. Our data, however, shows that cells that normally have a stacked Golgi apparatus do not need it for transporting the bulk of proteins and so puts into sharp focus the real relationship between Golgi structure and its supposed primary function. Functional Golgi clusters have also been described in vivo in *Drosophila* (Kondylis *et al.,* 2001; unpublished data). Moreover, recently, the depletion of p125 that affects the organization of the tER sites, and ultimately the Golgi structure, does not inhibit forward transport of VSV-G (Shimoi *et al.,* 2005).

That the depletion of dGRASP did not affect anterograde transport of Delta to a significant extent does not mean that the transport of specific proteins might not be affected. GRASP65 has been reported to act as a chaperone for the transport of the TGF alpha proteins to the plasma membrane in human cells, and conversely to be part of a retention mechanism for the p24 family members to the *cis*-Golgi. We have not investigated the transport of TGF-alpha proteins (Gurken, Spitz, and Keren) in S2 cells. However, we have investigated the retention of a member of the p24 family, p24δ1, but found no change in its distribution upon dGRASP depletion.

Taken together, these results indicate that dGRASP is dynamically localized to the early exocytic pathway (tER sites and Golgi apparatus) but has a role in the acquisition of Golgi stack morphology without affecting anterograde transport. Its presence at the tER sites is intriguing because it is not involved in their organization. This suggests that dGRASP could be recruited at the tER sites providing Golgi identity to the exiting membranes and promoting the formation of Golgi stacks either in a transient way or as part of a Golgi matrix, however dynamic (Glick, 2002).

Other Possible Functions of dGRASP

In addition to the above mentioned roles, dGRASP could participate in signaling. The C-terminal half of dGRASP exhibits several phosphorylation sites, similar to GRASP65 and GRASP55 (see *Results*). Very recently, GRASP65 was shown to be phosphorylated by ERK on serine 277 in interphase and this phosphorylation step is strongly enhanced by the addition of serum or EGF in the medium, suggesting that GRASP65 may play a role in growth factor signal transduction (Yoshimura *et al.,* 2005). This is in line with the increasing number of signaling proteins reported to localize on the membranes of the early exocytic pathway, including the small GTPase upstream of ERK, the activated H-Ras, on the Golgi apparatus (Chiu *et al.,* 2002). This raises the question of their anchoring mechanism at these membranes. One may speculate that GRASP65 and dGRASP could act as scaffolds/receptors for these signaling proteins. Such a role has been shown for GM130, which binds and regulates the function of YSK1, a kinase of the STE family implicated in polarized secretion during wound healing (Preisinger *et al.,* 2004).

GRASP65 is also heavily phosphorylated at its C-terminal part during mitosis (Wang *et al.,* 2003, 2005). Interference with this C-terminal has led to a block in the entry of mammalian cells into mitosis (Sutterlin *et al.,* 2002) or a delay in mitotic progression (Preisinger *et al.,* 2005), and our preliminary data from transient transfection of dGRASP C-terminus in S2 cells suggest that a similar role could apply to dGRASP. Analysis of dGRASP *Drosophila* mutants at different development stages is currently underway and will be useful to elucidate this issue.

Note added in proof. During the revision of this article, a study investigating the effect of GRASP65 depletion on mammalian cells has been published (Sutterlin, C., Polishchuk, R., Pecot, M., and Malhotra, V. [2005]. Mol. Biol. Cell *16*, 3211–3222), which reaches similar conclusions with our study concerning the effect on Golgi architecture and anterograde transport.

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