dGRASP-Mediated Noncanonical Intigrin Secretion Is Required for Drosophila Epithelial Remodeling

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SUMMARY
Integral plasma membrane proteins are typically transported in the secretory pathway from the endoplasmic reticulum and the Golgi complex. Here we show that at specific stages of Drosophila development corresponding to morphological changes in epithelia, apposed basolateral membranes separate slightly, allowing new plasma membrane contacts with basal extracellular matrix. At these sites, newly synthesized integrin α subunits are deposited via a mechanism that appears to bypass the Golgi. We show that the Drosophila Golgi resident protein dGRASP localizes to these membrane domains and that, in the absence of dGRASP, the integrin subunit is retained intracellularly in both follicular and wing epithelia that are found disrupted. We propose that this dGRASP-mediated noncanonical secretion route allows for developmental regulation of integrin function upon epithelial remodeling. We speculate that this mechanism might be used during development as a means of targeting a specific subset of transmembrane proteins to the plasma membrane.

INTRODUCTION
The architecture of epithelial tissue in Drosophila depends on intercellular junctions at the apical and lateral plasma membrane (Knust and Bossinger, 2002; Pilot and Lecuit, 2005) and on adhesion. Adhesion with extracellular matrix components at the basal side is mediated by integrins, integral plasma membrane αβ heterodimers (Hynes, 1992), that in turn induce the formation of a focal adhesion site. The importance of integrin-mediated adhesion has been exemplified in the wing imaginal discs of Drosophila larvae, where αPS1|PS (PS1) and αPS2|PS (PS2) interact with the extracellular matrix between the basal sides of the two epithelial layers, thus maintaining the integrity of the developing wing (Bokel and Brown, 2002; Brower, 2003). Mutations in either of the genes encoding the integrin subunits (mew for αPS1 and inflated for αPS2) lead to striking wing phenotypes such as bubbles and blisters (Brower and Jaffe, 1989; Brabant and Brower, 1993; Wehrli et al., 1993).

During development, adhesion of the epithelia must be altered in order to allow modulation of organogenesis and morphogenesis. This is largely mediated by (de)phosphorylation of focal adhesion components (Cohen and Guan, 2005), but could also be modulated by the exocytosis of newly synthesized integrins. However, what triggers the deposition of integrins to the basal side and how they are transported remain largely unexplored.

Integral membrane proteins use the exocytic pathway for their transport and delivery to the plasma membrane. They are cotranslationally translocated into the lumen of the endoplasmic reticulum (ER), from which they exit at TER sites (or ER exit sites), in COPII-coated vesicles (Barlowe, 2002). Upon arrival at the Golgi apparatus, they are further modified by Golgi resident enzymes before being dispatched to their final destinations (Bonifacino and Glick, 2004). In Drosophila, the TER sites and the Golgi complexes are in very close proximity and form TER-Golgi units (Kondylis and Rabouille, 2003).

The myristoylated peripheral Golgi proteins GRASP65 and -55 were first discovered as factors required for the stacking of Golgi cisternae in vitro (Barr et al., 1997; Shorter et al., 1999). GRASP65 binds GM130 (Barr et al., 1998), and this complex has been implicated in vivo in the formation and maintenance of the structure of the Golgi (Kondylis et al., 2005; Putnaveedu et al., 2006; Marra et al., 2007). GRASP65 and -55 have also been shown to be involved in the Golgi G2/M checkpoint (Sutterlin et al., 2002; Preisinger et al., 2005; Feinstein and Linstedt, 2007; Rabouille and Kondylis, 2007), and GRASP65 has been proposed to regulate spindle dynamics (Sutterlin et al., 2005). The GRASP65/55 orthologs in Drosophila (dGRASP; Kondylis et al., 2005) and yeast (Grh1; Behnia et al., 2007) are largely dispensable for anterograde protein transport. However, GRASP55 has been shown to chaperone TGF-α to the plasma membrane (Kuo et al., 2000). Very recently, the GRASP65/55 homolog in Dictyostelium, GrhA, has been shown to mediate the unconventional secretion of AcbA, a protein that completely bypasses the ER-Golgi-plasma membrane exocytic pathway for its delivery to the extracellular space (Kinseth et al., 2007). This gives an unanticipated function for the GRASP protein family that, to date, has been strictly considered bona fide Golgi proteins.

To investigate further the role of these proteins in vivo, we studied dGRASP in the follicular epithelium covering the developing oocyte in the Drosophila egg chamber. At the stage 10A of development, the follicle cells are columnar and attached to one another by adherens junctions on the apical side. The basolateral septate junctions (equivalent to mammalian tight junctions) are still immature at this stage (Horne-Badovinac and Bilder, 2005). At stage 10B, the epithelial sheet migrates centripetally toward the anterior side of the oocyte, and it also has to accommodate the expanding volume of the oocyte. As a result of both mechanical stretching and movement, the follicle cells...
flattened. They transiently separate from each other at the basal site, thus exposing to the basal extracellular matrix portions of basolateral plasma membrane that were engaged in cell-cell contact (Figure 1C).

We found that, during this event, dGRASP is no longer restricted to the TCR-Golgi units and instead is found anchored at the newly exposed portions of plasma membrane described above. There, dGRASP becomes essential for the specific deposition of the newly synthesized integrin δPS1 to the same location, which triggers the formation of a focal adhesion site, thus contributing to epithelial integrity at later stages. We found that the dgrasp mutants exhibit severe disorganization of both the follicular epithelium and elongating wing disc. Furthermore, we show that, at stage 10B but not earlier, the dGRASP-mediated δPS1 deposition in follicle cells is insensitive to Brefeldin A and independent of the t-SNARE (SNAP receptor) Syntaxin5, suggesting that it bypasses the Golgi.

Taken together, we have unraveled a developmentally regulated noncanonical secretion route that appears independent of the Golgi. One of the substrates of this pathway is δPS1, and dGRASP is a key component of the molecular machinery that outlines it. Our findings shed light on how integrin-mediated adhesion might be modulated during morphogenetic events. In a broader perspective, we propose that this secretory route might be used during development as a means of transporting a specific subset of transmembrane proteins crucial for development to the plasma membrane.

RESULTS

The Golgi Protein dGRASP Localizes to the Plasma Membrane in a Stage 10B Follicular Epithelium

To gain insight into the role of dGRASP in epithelium development, we used a follicular epithelium covering the developing Drosophila oocyte as a model to investigate its localization.

As expected from tissue-cultured cells and salivary glands (Kondylis et al., 2005), dGRASP localized to the multiple TCR-Golgi units of the follicle cells at stages 9-10A of oogenesis and exhibited a significant colocalization with the Golgi marker d120kd (Figure 1A), dGRASP-GFP expressed from a transgene (that rescues a dGRASP mutant phenotype; see below) was also found exclusively on the TCR-Golgi units of the follicle cells (Figure 1A').

At stage 10B, however, dGRASP localization changed drastically. dGRASP no longer significantly colocalized with Golgi markers (Figures 1B and 1B'). This change is concomitant with the flattening of the follicular epithelial cells, during which they expose a portion of basolateral plasma membrane to the basal extracellular matrix (see Introduction; Figures 1C–1C'). The zone at which three cells were in contact (zone of contact; ZOC) at the basolateral side at stage 10A expands and forms a funnel that, when viewed from the top, is visualized as a triangular gap, referred to as the open ZOC (arrowheads in Figures 1C' and 1C').

dGRASP localized to the open ZOC at stage 10B, as well as retained a faint labeling corresponding to TCR-Golgi units (Figure 1B). dGRASP-GFP and its binding partner dGM130 exhibited the same localization as the endogenous dGRASP. By immunoelectron microscopy, both were found to be specifically associated to the plasma membrane lining and protruding into the open ZOC (Figures 1D and 1E). The TCR-Golgi units were still labeled, albeit at a much reduced level (Figure 1D; inset in Figure 1E). This reveals an unexpected localization for dGRASP and dGM130 that until now were strictly considered as markers of the early exocytic pathway in many cellular systems (Barr et al., 1997; Shorter et al., 1999; Kondylis et al., 2005).

dgrasp RNA Localization Mediates the Unusual Localization of dGRASP

As dGRASP localized to the Golgi at stage 10A and earlier in oogenesis, it could be redistributed from this location to the open ZOC. Alternatively, it might be newly synthesized and localized via the targeting of its mRNA, which is a very powerful cell mechanism to ensure timely and restricted protein localization (St Johnston, 2005). To test the latter alternative, we visualized the dgrasp mRNA by fluorescence in situ hybridization (FISH) using an antisense probe. At stage 10A, the mRNA appeared as random cytoplasmic spots (Figure 2B). At stage 10B, it was found concentrated near the open ZOC that, when viewed from the top, forms a triangular pattern (Figures 2A and 2B). The sense probe failed to produce a pattern at any stage (not shown). Out of ~30 mRNAs tested, only a very small subset was found in the open ZOC in the same pattern (see below, and Figure S5 in the Supplemental Data available with this article online). To address whether the unusual localization of dGRASP could be mediated by local translation of its targeted mRNA, we double-labeled dgrasp transcript and dGRASP protein, and found that they colocalize (Figure 2C), strongly suggesting that dGRASP is translated from its local mRNA pool. How dGRASP is anchored at the plasma membrane remains to be further investigated. As dGRASP-GFP also localized to the open ZOC at stage 10B, we tested whether the mRNA encoding this transgene exhibited the same localization as the endogenous transcript. We performed FISH using an antisense gfp probe and found that it was localized in the same pattern as the endogenous dgrasp mRNA (Figure 2D). The transgene only comprises the dGRASP coding sequence tagged with GFP lacking the 5' or 3' sequences, strongly suggesting that the localization signal for dgrasp mRNA resides within the coding region, and not in the UTRs as is the case for many localized mRNAs in fly embryos and oocytes (St Johnston, 2005).

Mutation in dgrasp Leads to Defects in Epithelium Integrity

In order to understand the role of dGRASP at the open ZOC, we created mutant alleles of the dgrasp gene by mobilizing a P element inserted 176 bp upstream of the start codon (Supplemental Experimental Procedures; Figure S1A). We isolated a semiviable stock (dgrasp100.2) that resulted in approximately 10% homozygous survivors (Table S1A). The gene lesion was mapped which showed that the first exon and part of the first intron of the dgrasp gene are missing, corresponding to the loss of about 375 bp (Figures S1B and S1C). The levels of both the dGRASP transcript and protein were strongly reduced in dgrasp100.2 (Figures S1C and S1D) and failed to exhibit this specific location.

We found that the stage 11-12 dgrasp100.2 follicular epithelium was disrupted to several degrees, including misshaped cells,
with occasionally loss of cell-cell contact leading to a severe disruption (Figures 3A and 3B) but without loss of polarity (not shown). This phenotype was totally rescued by the transgenic expression of dGRASP-GFP in dgrasp100.2 (Table S1B). Taken together, these results suggest a role for dGRASP in epithelial integrity and adhesion.

Integrin αPS1 Is Mislocalized in the dgrasp Epithelium

Epithelial disorganization might be a result of the mislocalization or absence of the proteins involved in the formation of junctions or in adhesion. We tested whether the transmembrane proteins of the adherens junctions, DE-Cadherin (Tepass et al., 1996) and Echinoid (Lecuit, 2005; Laplante and Nilson, 2006), and of the tricellular septate junctions, Gliotactin (Schulte et al., 2006), were correctly deposited in dgrasp100.2. We found that they were localized in a manner indistinguishable from wild-type (Figures 3A and 3B), consistent with the fact that dGRASP depletion in Drosophila S2 cells does not inhibit anterograde transport of a reporter transmembrane protein (Kondylis et al., 2005).

We then investigated the change in the localization of a number of other adhesion proteins, including integrins (see Introduction). Because αPS2 is not expressed in the wild-type follicular...
tissue (our unpublished results; Fernandez-Minan et al., 2007), we focused on αPS1. During stages 9-10A, αPS1 remained intracellular in the follicle cells with a pool transported to the basolateral plasma membrane (Figure 3C). At stage 10B, however, αPS1 localization changed drastically and became very similar to that of dGRASP at the same stage; αPS1 was found concentrated at the open ZOC (Figure 3C) together with βPS (Figure 3D). Integrins are known to be part, and to induce the formation, of focal adhesion sites. Indeed, we could also localize other landmarks of focal adhesion sites, F-actin, the LIM protein PINCH (particularly interesting cysteine-rich protein; Clark et al., 2003), ILK (Integrin-linked kinase; Zervas et al., 2001), and Lamelin (not shown), that were found enriched at the same location, at the same stage (Figure 3D). This suggests that, as the cells flatten, the open ZOC becomes a basal focal adhesion (Clark et al., 2003).

Strikingly, in the dgrasp100.2 mutant follicular epithelium, αPS1 failed to be deposited to the open ZOC, as in wild-type tissue (Figure 4A). Instead, we found it was mostly intracellular. This phenotype was confirmed using other allelic combinations of dgrasp (Figure 4A) and was rescued by the transgenic expression of dGRASP-GFP in the dgrasp100.2 follicular epithelium (Figure 4C). We also generated an FRT-dgrasp100.2 recombinant chromosome to make mosaic follicular epithelia (Dang and Perrimon, 1992) and monitored αPS1 deposition in homozygous dgrasp100.2 clones. We found that within the clones, αPS1 was absent from the open ZOC, whereas it was perfectly deposited in the surrounding heterozygous cells (Figure 4B). Surprisingly, βPS localization to the open ZOC was not affected by the absence of dGRASP (Figures 4D and 4E). This suggests that dGRASP is required for the specific transport of αPS1 to the plasma membrane of the open ZOC where, together with βPS, it induces the formation of a basal focal adhesion at stage 10B that acts in building a strong adhesion with the extracellular matrix at stage 11-12 (Figure S1E). Accordingly, the follicular epithelium defects observed in dgrasp100.2 were very similar to those in mew, the αPS1 mutant (Figure S2A).

This dGRASP-dependent transport of αPS1 to the plasma membrane is also observed in other epithelia. Out of the surviving homozygous dgrasp100.2 individuals, 35% exhibited severe wing problems, sometimes displaying severe adhesion problems exemplified in collapsed wings (not shown), and large blisters (Figure S2B). The majority of pupal wing imaginal discs were also severely compromised (not shown). The expression of dGRASP-GFP in the mutant background totally rescued the wing phenotype (Table S1B).

As integrins have been shown to play a crucial role in wing development (see Introduction), we tested their localization in
dgrasp$^{100.2}$ pupal wing discs. We found that the typical wild-type punctate localization of αPS1 (Bokel and Brown, 2002) (Figure S2C) and αPS2 (not shown) was replaced by a less intense and unfocused pattern in the mutant (Figure S2C'), suggesting that αPS1 was indeed mislocalized and also intracellularly retained. Accordingly, dgrasp wings showed similar blisters as in mew wings (Figure S2B). The analysis in dgrasp$^{100.2}$ clones in mosaic pupal wing discs revealed a similar pattern. αPS1 was not deposited to the plasma membrane in the absence of dGRASP (Figure S2C').

Taken together, these results show that dGRASP mediates the specific transport of αPS1 and αPS2, but not βPS, to the basal focal adhesion sites of epithelial cells at specific stages of epithelial morphological changes. Their transport at earlier stages of development does not depend on dGRASP. This adhesion is crucial for epithelial integrity as exemplified by its disruption in the dgrasp wing and follicular epithelium.

**The Pool of αPS1 Deposited at the Open ZOC Is Newly Synthesized**

Because integrins are deposited at the plasma membrane at earlier stages of development (Figure 3C), we set out to investigate whether they accumulate at the open ZOC by diffusion in the plane of the membrane. We first localized αPS1 mRNA by FISH and found that the expression of αPS1 was greatly increased at stage 10B when compared to 10A (Figure 5A) and that it was clearly enriched at the basal site (Figure 5A, inset), suggesting that it could sustain de novo synthesis of the protein.

**Evidence that αPS1 Bypasses the Golgi**

From the ER, transmembrane proteins are typically transported to the Golgi complex where they are processed and dispatched to their final destinations (see Introduction). However, because we found that cis-Golgi resident proteins were relocated to the plasma membrane, we set out to investigate the role of the Golgi in the transport of αPS1 to the open ZOC. We first visualized the Golgi complex using an antibody to the peripheral Golgi protein dGMAP (Kondylis et al., 2007) in stage 10A and 10B wild-type and dgrasp$^{100.2}$ follicular epithelia. The Golgi did not accumulate near the open ZOC in wild-type, and we found that the size, distribution, and morphology of the Golgi were equivalent in both epithelia (Figures S3A–S3C), suggesting that the dGRASP requirement of the αPS1 transport is not related to Golgi organization and positioning.

Next, we tested the effect of the drug Brefeldin A (BFA) that inhibits transport from the ER to the Golgi (Kondylis and Rabouille, 2003; Kondylis et al., 2005, 2007). We found that in the presence of BFA, αPS1 was still transported to the open ZOC at stage 10B, whereas Neurexin IV, another transmembrane protein of the cell surface expressed at the same stage and the same place (Schneider et al., 2006; Figure 6A), was unable to reach its location (Figure 6A'), suggesting that the deposition of αPS1 does not involve the Golgi.

Furthermore, we found that the deposition of αPS1 to the open ZOC at stage 10B was inhibited by the protein synthesis inhibitor puromycine (Figure 5B), corroborating that the αPS1 is newly synthesized from its pool of upregulated transcripts. However, unlike dGRASP, integrins are transmembrane proteins, and their translation needs to be coupled to translocation into the ER that we visualized using an anti-KDEL antibody. We found that at stage 10B, the ER was concentrated very near the open ZOC (Figure 5C).

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To further test this, we monitored αPS1 transport to the plasma membrane in the absence of Syntaxin5, the Golgi t-SNARE essential for the movement of newly synthesized proteins to and through the Golgi in all eukaryotes (Pelham, 2001), including Drosophila (Xu et al., 2002; Kondylis and Rabouille, 2003). We used a homozygous null dsyntaxin5 allele (AR113; Xu et al., 2002) to make mosaic follicular epithelia (Dang and Perrimon, 1992) in which αPS1 could be monitored in homozygous dsyntaxin5 clones.

In the absence of dSyntaxin5, we confirmed that the Golgi was disorganized (Figure S3D), in agreement with results obtained in S2 cells depleted of this protein (Kondylis and Rabouille, 2003). Furthermore, at stage 9-10A, transport of αPS1 (Figure S3E) and DE-Cadherin (Figure S3E) to the plasma membrane was strongly inhibited in the clones lacking dSyntaxin5, indicating that, at this stage, both proteins utilized the conventional exocytic pathway. At stage 10B, however, αPS1 was deposited to the open ZOC in the dsyntaxin5 clones (Figure 6B, arrow; Figure S4). To rule out that αPS1 was enriched in an adjacent ER rather than the plasma membrane, we performed the labeling without permeabilization, and confirmed that αPS1 was deposited to the open ZOC throughout the entire follicular epithelium, whether dSyntaxin5 was present or not (Figures 6B’ and 6B”, arrows; Figure S4), indicating that αPS1 could bypass the Golgi.

However, a possibility remained that at this specific stage of development, the general anterograde transport to and through the Golgi used another SNARE instead of dSyntaxin5. We therefore tested whether two other transmembrane proteins expressed at stage 10B and localized to the open ZOC, βPS and Neurexin IV, are transported efficiently in dsyntaxin5 clones. The deposition of both markers was strongly inhibited in the clones (Figures 6C and 6D; Figure S4), indicating that both use dSyntaxin5-mediated transport to and through the Golgi, even at stage 10B. Taken together, this strongly indicates that at stage 10B, αPS1 bypasses the Golgi and achieves its specific deposition to the open ZOC, where dGRASP is also localized, via a noncanonical secretion pathway.

Other Molecular Partners in This Noncanonical Secretion Pathway

In order to identify other molecular partners involved in this dGRASP pathway, we visualized ~35 proteins of the exocytic pathway and/or their mRNAs, and scored them for their localization in a pattern overlapping that of the dGRASP transcript and protein at stage 10B. dnsf1 and dsnap mRNAs (Figures S5A and S5B) were positive and dSNAP (Ordway et al., 1994) was found to display a pattern identical to dGRASP (Figure S5B’).
dgos28 mRNA and dGos28 (Figures S5C and S5C'), the encoded v-SNARE specific for ER-to-Golgi transport (Nagahama et al., 1996; Pelham, 2001), were also found at the open ZOC (Figure S5C). However, dgm130 mRNA was not, suggesting that dGM130 is recruited to the open ZOC by protein-protein interaction. We conclude that only a specific but reduced subset of the proteins of the exocytic pathway is localized to the open ZOC, either through the localization of their mRNAs or by protein-protein interactions.

**DISCUSSION**

**dGRASP-Mediated Golgi Bypass of Integrin αPS1**

We have identified a developmentally regulated noncanonical dGRASP-dependent and dSyntaxin5-independent secretion route that displays several characteristics: first, it is specifically built in epithelia that undergo rearrangement, such as the elongating discs and the flattening follicle cells. There, it is used by the transmembrane integrin subunit αPS1 for its transport and deposition at the open ZOC, the basolateral portion of the plasma membrane that was engaged in cell-cell contact and, after the morphological changes, is now facing the extracellular matrix. This deposition elicits the building of a focal adhesion (Figure 7, step 4) that helps maintain epithelium integrity at stage 11 onward (Figure 7, step 5). In the absence of dGRASP, the specific deposition of αPS1 is dramatically impaired and the resulting epithelium is severely disrupted in a similar fashion as in a hypomorphic mew. Second, this pathway is insensitive to BFA and the absence of the SNARE dSyntaxin5, suggesting that it bypasses the Golgi.

We propose that the building of this pathway starts with the upregulation of a subset of mRNAs encoding proteins of the Golgi, dGRASP and dGos28 (Figure 7, step 1). These mRNAs are targeted to the open ZOC, where they elicit the de novo synthesis of the corresponding proteins that are found anchored at the plasma membrane lining the open ZOC in the follicular epithelium (Figure 7, step 2). This RNA pattern was also observed with a handful of other transcripts. Remaining to be answered is what triggers the upregulation and localization of the dgrasp mRNA and the other transcripts to the open ZOC in response to epithelial morphological changes and how they are moved and anchored there. As mechanical tension and integrin binding have already been shown to induce the recruitment of mRNAs to focal adhesions (Chicurel et al., 1998), integrins themselves could be the sensor for the mechanical stretching during disc elongation and the centripetal movement of the follicle cells.

Concomitant with the targeting of dgrasp transcripts, αPS1 mRNA is also upregulated and basally concentrated. We propose (Figure 7, step 3) that at stage 10B, the ER cisternae that reside near the open ZOC are actively involved in the local synthesis of αPS1. After synthesis in the ER membrane, a
yet-ununknown cargo receptor likely provides a very efficient exit for the newly synthesized αPS1 and prevents its diffusion through the entire ER membrane, similar to Gurken in the oocyte (Herpers and Rabouille, 2004). From these αPS1-enriched ER cisternae, carriers would form, although their nature remains elusive. Although Sar1 localization has not been addressed, none of the COPII subunits (Barlowe, 2002) we tested were concentrated near the open ZOC.

These ER-derived carriers bypass the Golgi and specifically fuse with the plasma membrane outlining the open ZOC. This membrane domain has become, at stage 10B, an acceptor compartment of an unexpectedly mixed nature, comprising plasma membrane resident proteins as well as cis-Golgi proteins dGRASP, dGM130, and dGos28 that are specifically localized there at this stage. These proteins could form a platform to which the αPS1-enriched ER-derived carriers fuse through the formation of a SNARE complex involving dGos28, and other SNAREs that have yet to be identified. dGRASP/dGM130 is likely to be involved in their tethering (Puthenveedu et al., 2006) through oligomerization of dGRASP (Wang et al., 2005) and promote the formation of this unusual complex. The fusion would involve the activity of the ATPase dNSF1 and its cofactor dSNAP, meaning that this system is clearly different from the Golgi-independent deposition of the transmembrane protein Ist2 from Saccharomyces cerevisiae that is Sec18/NSF independent (Juschke et al., 2005).

In addition to Ist2, a few proteins have already been shown to traffic directly from the ER to the plasma membrane, such as the cystic fibrosis transductance regulator in BTK cells (Yoo et al., 2002) and the simian rotavirus RRV in Caco-2 cells (Jourdan et al., 1997). Further research might reveal whether a pathway equivalent to the dGRASP-mediated pathway is involved in Golgi bypass in these cases.

GRASP-Mediated Unconventional Secretion

A number of mammalian factors, including Galectin, Interleukin, and Fibroblast growth factor 2, have been shown to be secreted in an unconventional manner that completely bypasses the exocytic pathway (Nickel, 2005). Very recently, the Dictyostelium GRASP homolog GrhA has been shown to be involved in the unconventional secretion of the polypeptide AcbA that is predicted to harbor no signal peptide in its coding sequence (Kinseth et al., 2007; Levi and Glick, 2007).

It is striking that both dGRASP and GrhA mediate unconventional secretion routes yet the pathways appear to be different. Unlike AcbA in Dictyostelium, αPS1 is translocated into the ER lumen using its signal peptide. In follicle cells earlier than stage 10B, αPS1 transport requires dSyntaxin5, suggesting that it travels via the typical ER-Golgi-plasma membrane transport route. There is no evidence suggesting that the signal peptide

Figure 6. Integrin αPS1 Deposition at the Open ZOC Is Insensitive to Brefeldin A and Does Not Require dSyntaxin5

(A and A′) IF localization of αPS1 (green) and Neurexin IV (Nrx; red) in an untreated (A) and BFA-treated (A′) stage 10B follicular epithelium. Note that, after BFA treatment, αPS1 is transported to the open ZOC, whereas Nrx is retained intracellularly.

(B and B′) IF localization of αPS1 (red) in stage 10B dsyntaxin5 homozygous clones (marked by a white dashed line and the absence of nuclear GFP, green). In (B′), the labeling was performed without cell permeabilization. Arrows indicate the localization of αPS1 to the open ZOC within the clones.

(C and C′) IF localization of βPS (red [C]) and Neurexin IV (Nrx; red [C′]) in non-permeabilized stage 10B dsyntaxin5 homozygous clones. Note that in the absence of dSyntaxin5, they are not transported.

Scale bars represent 5 μm.
Figure 7. The dGRASP-Mediated Noncanonical Deposition of αPS1 Leads to the Formation of a Focal Adhesion during Epithelium Rearrangement

Schematic representation of the different steps leading to the formation of a focal adhesion. At stage 10B, the stretching and migration of the columnar follicle cells lead to the separation of their basolateral plasma membrane at the basal side, thus creating the open ZOC (step 1). This induces the upregulation and targeting of dgrasp and dgos28 mRNAs in close proximity to the open ZOC. This in turn leads to the anchoring of dGRASP and dGos28 at the open ZOC plasma membrane, dGM130 is recruited by interaction with dGRASP (step 2). αPS1 mRNA is upregulated and concentrated at the basal side of the follicle cells. dGRASP localization leads to the transport of this subunit from the endoplasmic reticulum (ER) to the open ZOC in a Golgi-independent manner (step 3). βPS is deposited to the open ZOC by the typical exocytic pathway and independently of dGRASP. The integrin heterodimer recruits F-actin, PINCH, and Integrin-linked kinase, resulting in the formation of a focal adhesion site (step 4). Adhesion to the extracellular matrix is possible in the flattening cells and stabilizes the stage 11 epithelium. It is also maintained by cell-cell junctions, including the septate junction that has matured and membrane protrusions the cells send toward one another (step 5 and inset).
might be omitted at stage 10B and, importantly, no evidence of transmembrane proteins transported to the plasma membrane by the unconventional secretion pathway that AcbA is proposed to use.

AcbA has been postulated to be captured from the cytosol and stored in endosomes prior to release from this compartment to the extracellular medium (Levi and Glick, 2007). On the other hand, zPS1 could be made de novo and stored in an endosomal compartment (perhaps similar to Glu4 in adipocytes) localized near the open ZOC before being specifically recycled to the adjacent plasma membrane. Alternatively, the integrin could be stored in endosomes upon internalization from the plasma membrane, although the inhibition of integrin deposition to the open ZOC by protein synthesis inhibitors argues against this.

Both trafficking events could be insensitive to BFA or to the loss of dSyntax5 function, and the tethering and fusion of the recycling vesicles, or even a whole endosome, could require dGRASP and the other proteins found near the open ZOC, in a similar fashion as the exocytic carriers proposed above. GrhA could have an equivalent role. However, the recycling to the plasma membrane of the mammalian integrins (Caswell and Norman, 2006), like Glu4 (Kessler et al., 2000), has been shown to depend to a great extent on Rab11, and if this small GTPase is involved in zPS1 recycling, we would expect to find it concentrated near the open ZOC. This is not the case (Figure S5E). The identification of zPS1-positive carriers in follicle cells will shed light on the mechanism involved in its deposition.

Integrin zPS1: One Substrate of This Pathway

In the Drosophila wing epithelium, zPS1 and zPS2 are substrates of the dGRASP-mediated pathway and the dgrasp wings exhibit blisterers. However, when compared to the mew and inflated phenotype (not shown), dgrasp wings are smaller and rounder. This could be a result of the additive effect of stopping the transport of both zPS1 and zPS2. dGRASP itself could be involved in wing development, perhaps with a role in cell-cycle control (see Introduction). Other unidentified proteins involved in growth could use the same dGRASP-dependent pathway. It is also possible that the z subunits of integrin are involved in disc elongation, as they have been proposed to be in follicle cells (Bateman et al., 2001).

The intriguing question is why in the follicular epithelium, zPS1 uses an alternative pathway at stage 10B. The Golgi houses glycosylases and glycosyltransferases allowing the processing and building of complex oligosaccharides that are often required for the biological activity of glycoproteins. The Golgi bypass of zPS1 suggests that the oligosaccharide modifications carried out in this compartment are not necessary for zPS1 function at the open ZOC. Because the lack of a series of Golgi glycosylases enhances the adhesion activity of integrins (Guo et al., 2003; Chen et al., 2006), the Golgi bypass might indeed enhance or modulate integrin adhesion properties at this specific time of oocyte development. zPS is not a substrate of this noncanonical pathway. This is surprising, because z and β integrin subunits have been shown to oligomerize early in the secretory pathway (Ho and Springer, 1983), probably leading to their increased stability and efficient transport. Our results suggest that the subunits are also able to travel on their own, perhaps by binding to other proteins.

The dGRASP-Mediated Pathway in Other Developmental Processes

We have shown that the integrin subunits zPS1 and zPS2 are not properly deposited in two different dgrasp mutant epithelia. The mechanism we have unraveled here could therefore also be used in other tissue remodeling events throughout Drosophila development involving adhesion. In this context, the basal adhesion of follicle cells shares many similarities with dorsal closure in embryos (Clark et al., 2003). The secretory process we describe could also apply here, and perhaps more generally in embryogenesis.

This also gives an additional molecular handle to adhesion at the basal site that is crucially involved in the maintenance of epithelium integrity. Adhesion can be modulated by the phosphorylation of focal adhesion components leading to a change in integrin adhesive properties (Cohen and Guan, 2005). In the follicular epithelium, the receptor tyrosine phosphatase Dlar genetically interacts with JIPS with which it colocalizes in basal tri-cellular junctions in stage 7-8 (Bateman et al., 2001). There, it is involved in F-actin organization that ultimately stabilizes the epithelium. Here we show that adhesion can also be modulated at a pretranslational level by the transport (albeit noncanonical) and targeting of newly synthesized integrins to future adhesion sites.

Taken together, we propose that the GRASP-mediated secretory route might be used during development as a means of targeting a specific subset of transmembrane proteins crucial for development to the plasma membrane.

EXPERIMENTAL PROCEDURES

Drosophila Stocks and Crosses

All the Drosophila melanogaster stocks came from the Bloomington Drosophila Stock Center (https://drcg.cgb.indiana.edu/), except for the ILK-GFP stock (gift from Nick Brown; Zervas et al., 2001). The flies were raised on standard cornmeal-agar at 25°C. The wild-type strains were Oregon R (OreR). The generation of the stocks is presented in the Supplemental Data.

Antisense Probes, Antibodies, and Reagents

The antisense probes to detect mRNAs were made with a DIG RNA labeling kit (SP6/T7, Roche) using the following cDNA clones. For dgrasp, a partial cDNA as described in Kondylis et al. (2005); for gfp, the EGFp cDNA (Clontech) cloned in the PBSII KS- vector; for digos28, the clone RE64493; for dsnip, LP04493; for draf1, the clone dN20 (Ordway et al., 1994); and for αPS1, GH14509 (DGC gold). Sense probes were made using the T3 promoter. The following antibodies were used: mouse monoclonal anti-αPS1 (DK 1A4, 1/100), αPS2 (CF.2CF, 1/100), and JIPS (CF.6G11, 1/100) (Developmental Studies Hybridoma Bank, DSHB); mouse monoclonal anti-d120kd (Calbiochem, 1/100; Kondylis and Rabouille, 2003); rat monoclonal anti-DE-Cadherin (DSHB, DCAD2, 1/200); rabbit polyclonal serum anti-Echinoid, (gift from A.P. Jarman, 1/5000; Rawlins et al., 2003); mouse monoclonal anti-Gliotactin (1F61D4; gift from V. Auld, 1/50; Auld et al., 1995); mouse monoclonal anti-α-β-Spectrin (DSHB, 3A9, 1/20); rabbit polyclonal anti-dGMI30 (gift from S. Munro, 1/100); rabbit polyclonal anti-dGRASP65 (PBA30 and -31; gift from F. Barr, 1/100 for immunofluorescence; IF; Barr et al., 1997); rabbit polyclonal anti-GFP (Molecular Probes, 1/200 for IEM); mouse monoclonal anti-KDEL (Calbiochem, 10C3, 1/200 for IF); rabbit polyclonal anti-rat Gos28 (gift from T. Soliner, 1/500 for IF and 1/20 for IEM; Nagahama et al., 1996); rabbit polyclonal affinity-purified serum anti-PINCH (B83/245; gift from M. Beckerle, 1/500; Clark et al., 2003); rabbit polyclonal anti-dGMAP (gift from P. Theron, 1/300; Kondylis et al., 2007); rabbit polyclonal anti-dSNAP (gift from Leo Palancock; Babcock et al., 2004). The secondary antibodies conjugated to Alexa 488 and 568 (Invitrogen, Molecular Probes) were used at 1/500. Phalloidin-TRITC was used at 1/10,000 to mark the cortex and 1/1000 to show the
enrichment at the open ZOC. ILK was visualized using ILK-GFP-expressing stock.

**Puromycin and BFA Treatment**

Ovaries were dissected and placed in Schneider’s insect medium supplemented with 80 μM puromycin for 90 min or 20 μM Brefeldin A for 2 hr at 27°C and with solvent at the appropriate concentration as negative controls. After the drug treatments, the ovaries were fixed for 15 min with 4% paraformaldehyde (PFA) in PBS. Detection of αPS1 and Neurexin IV was performed by standard IF protocol.

**Immunofluorescence and RNA Fluorescence In Situ Hybridization**

Ovaries were fixed in 4% PFA (Polysciences) in PBS for 15 min, followed by several washes with PBT (PBS + 0.3% Triton X-100) for IF or PTW (PBS + 0.1% Tween 20) for FISH. They were processed for IF as described in Herpers and Rabouille (2004) and FISH as described previously in Dunne et al. (2002) using the tyramide detection TSA Cyanine 3 kit (Perkin Elmer). After mounting in Vectashield containing DAPI (Vector), imaging was performed on a Leica TCS-NT or Zeiss LSM-510 confocal microscope using a 63 oil lens and a zoom of 2.

For experiments where the ovaries were not permeabilized, they were fixed in 4% PFA in PBS for only 5–7 min (as fixative can make cells permeable), followed by the normal IF procedure omitting the detergent. Top views were captured by imaging the follicular epithelium at 3–5 μm below the basal plasma membrane.

**Immunoelectron Microscopy**

Wild-type egg chambers were fixed, processed, sectioned along the long axis, and labeled as described in Herpers and Rabouille (2004). Images were captured on a Jeol EX1200 electron microscope.

**Supplemental Data**

Supplemental Data include five figures, one table, and Supplemental Experimental Procedures are available on this article at http://www.cell.com/cgi/content/full/14/2/171/DC1/.

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