AP-1 and clathrin are essential for secretory granule biogenesis in Drosophila

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ABSTRACT Regulated secretion of hormones, digestive enzymes, and other biologically active molecules requires the formation of secretory granules. Clathrin and the clathrin adaptor protein complex 1 (AP-1) are necessary for maturation of exocrine, endocrine, and neuroendocrine secretory granules. However, the initial steps of secretory granule biogenesis are only minimally understood. Powerful genetic approaches available in the fruit fly Drosophila melanogaster were used to investigate the molecular pathway for biogenesis of the mucin-containing “glue granules” that form within epithelial cells of the third-instar larval salivary gland. Clathrin and AP-1 colocalize at the trans-Golgi network (TGN) and clathrin recruitment requires AP-1. Furthermore, clathrin and AP-1 colocalize with secretory cargo at the TGN and on immature granules. Finally, loss of clathrin or AP-1 leads to a profound block in secretory granule formation. These findings establish a novel role for AP-1– and clathrin-dependent trafficking in the biogenesis of mucin-containing secretory granules.

INTRODUCTION Constitutive secretion of proteins and lipids from the trans-Golgi network (TGN) toward the cell surface is believed to operate in all cells. Constitutive secretion is characterized by the rapid deployment of newly synthesized cargo toward its final cellular destination. Specialized secretory cells such as endocrine, neuroendocrine, and exocrine cells contain an additional pathway termed the regulatedsecretory pathway. One hallmark of this pathway is the storage of regulatedsecretory proteins at high concentration in dense-core secretory granules that can be released in response to an external signal. How secreted proteins enter the regulated secretory pathway is a source of debate and may prove to be cargo and cell-type specific (Dikeakos and Reudelhuber, 2007). In the case of endocrine and neuroendocrine cells, sorting of secreted cargo is believed to be content driven, with selective aggregation of regulated secretory proteins at the TGN playing a major role in secretory granule biogenesis (Borgono\textit{vo} et al., 2006).

Little is known about the coat proteins that might be required on the cytoplasmic face to promote budding of lumenal regulated secretory cargo from the TGN. Initial studies in AtT20 pituitary cells noted that condensing secretory products accumulate in dilated regions of the TGN that are coated with clathrin (Tooze and Tooze, 1993). This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-01-0054) on April 13, 2011. J. B. and M. J. generated mosaic clones, performed immunostaining, and acquired fluorescence micrographs. J. T. analyzed Sgs transcripts. J. B. and J. R. prepared salivary glands for electron microscopy. J. B., H. C., S. L., and R. L. B. generated fluorescently tagged constructs and transgenic flies. S. L. and R. L. B. recombined AP-4\textsuperscript{Δ7a-6-1} onto an FRT chromosome and generated anti–AP-1y antibodies. P. A. L. and G. L. B. generated anti-LqfR antibodies. H. K. performed mosaic clone analysis and contributed to experimental design. J. B. and J. A. B. conceived of the experiments and cowrote the manuscript. All authors commented on the manuscript. Address correspondence to: Julie A. Brill (julie.brill@sickkids.ca).

Abbreviations used: AP, adaptor protein; AP-1, clathrin adaptor protein complex 1; AP-3, clathrin adaptor protein complex 3; AP-4, gene encoding Discosoma species red fluorescent protein; ER, endoplasmic reticulum; GFP, monomeric enhanced green fluorescent protein; LqfR, Liquid facets-Related (Discosoma EpsiniR); Lva, Lava lamp; mCherry, monomeric Cherry fluorescent protein; PBS, phosphate-buffered saline; RFP, monomeric red fluorescent protein; RT, reverse transcriptase; Sgs, salivary gland secretion; TEM, transmission electron microscopy; TGN, trans-Golgi network; UAS, upstream activating sequence; VFP, Venus fluorescent protein.
Glue granule biogenesis is developmentally regulated

To develop a system in which regulated secretion could be genetically manipulated, we characterized the process of glue granule formation and maturation in third-instar larval salivary glands using a fluorescently tagged glue protein (Sgs3-DsRed) expressed under control of its own promoter (Biyasheva et al., 2001; Costantino et al., 2008). Glue expression was first visible in the distal-most cells of the salivary gland and proceeded proximally over time (Figure 1, A–C), with the salivary gland increasing in size as glue production progressed. We defined the stages as 0 (no expression), 1 (small granules, expression in distal cells), and 2 (fully mature granules, expression in distal and proximal cells). Unlike glue protein, gamma-adaptin (AP-1γ), a large subunit of the AP-1 complex, was expressed in all cells of the salivary gland throughout third-instar larval development (Figure 1, A–C).

Glue granules exhibit several hallmarks of regulated secretory granules, including post-Golgi maturation and storage in the cytoplasm until an external stimulus triggers release. Sgs3-DsRed expression was undetectable prior to mid–third-instar (compare Figure 1, D and E). Accordingly, at stage 0, glue granules were not detected by transmission electron microscopy (TEM) (Figure 1G). At the onset of glue production (stage 1), small electron-dense glue granules were visible in the cytoplasm with an average diameter of 1.0 μm (Figure 1, E and H). Occasional L-shaped granules were also visible (Figure 1H). Consistent with previous data suggesting growth by accretion (Farkas and Suakova, 1999). Between stages 1 and 2, glue granules increased in size (Figure 1, D–J) and electron-dense material became more prominent near the granule membrane (Figure 1I, arrowhead). During stage 2, fully mature glue granules reached an average diameter of 3.5 μm (Figure 1J). Following stage 2, glue granules fused with the apical membrane and glue cargo was secreted into the salivary gland lumen (Thomopoulos and Kastritis, 1979; Farkas and Sutakova, 1998; Biyasheva et al., 2001; unpublished data).

To investigate the origin of glue granules, we used electron microscopy to analyze salivary glands in which glue granule production was just initiating. At the beginning of glue granule synthesis (stage 1), Golgi units composed of clusters of vesicles and tubules were present in close proximity to the rough endoplasmic reticulum (Thomopoulos et al., 1992; Farkas and Sutakova, 1998; Kondylis et al., 2001; Figure 1K). Small glue granules were visible in the cytoplasm adjacent to the Golgi complex. We also observed coated vesicles in the vicinity of the Golgi and early glue granules (Figure 1L). These ranged from 60 to 90 nm in diameter and were bristle-like in appearance, reminiscent of clathrin coats. The presence of small coated vesicles near the Golgi and developing glue granules suggested that glue formation might proceed via the formation of coated intermediate vesicles and consequently require coat proteins such as clathrin and its adaptors.
Clathrin heavy chain and AP-1 localize to the trans-Golgi network

To identify coats that might function in granule biogenesis, we examined the subcellular distribution of clathrin heavy chain, as well as subunits of the clathrin adaptor protein complexes AP-1 and AP-3, which reside on intracellular organelles (note that Drosophila lacks AP-4; Boehm and Bonifacino, 2001). We first examined clathrin, AP-1, and AP-3 in salivary gland cells at stage 0, just prior to glue production. At this stage, Golgi bodies are easily visualized using antibodies directed against the golgin Lava lamp (Lva), which localizes to the cis-Golgi (Sisson et al., 2000) (Figure 2, A–D’’). Note that the cis-Golgi has a cup-shaped appearance. A monomeric red fluorescent protein fusion to clathrin heavy chain (RFP-Chc) predominantly localized to large puncta adjacent to the concave face of the cis-Golgi (Figure 2, A–A’’), consistent with a previous report showing localization of endogenous Chc to intracellular puncta in these cells (Wingen et al., 2009). Endogenous AP-1γ showed a similar distribution (Figure 2, B–B’’). A projection constructed from serial confocal sections revealed numerous Golgi units scattered throughout the cytoplasm (Figure 2C). There was a one-to-one correspondence between AP-1γ– and Lva-positive structures, with the cis-Golgi cups surrounding AP-1γ in a manner consistent with AP-1 localizing to the TGN (Figure 2, C–C’’’). Indeed AP-1γ and RFP-Chc colocalized with the trans-Golgi protein EpsinR (also called Liquid facets-Related or LqfR; Lee et al., 2009) (Supplemental Figure S1A). In contrast, AP-1 showed only minimal overlap with the recycling endosome regulator Rab11 (Buszczak et al., 2007; Lighthouse et al., 2008) (Supplemental Figure S1B). AP-1γ and RFP-Chc colocalized at the TGN (Figure 2, D–D’’’), although AP-1γ distribution appeared slightly more diffuse in salivary gland cells expressing RFP-Chc than in nonexpressing cells (compare Figure 2, B and D). Localization of AP-1 to the TGN is adaptor-protein specific, because a functional monomeric cherry fluorescent protein (mCherry) fusion to AP-3δ (called Garnet in Drosophila) showed no overlap with a Venus fluorescent protein (VFP) fusion to AP-1μ (called AP-47 in Drosophila) (Figure 2, E–E’’’), but rather colocalized with the late endosome marker Rab7 (unpublished data). Given the high degree of colocalization of clathrin and AP-1, we wondered whether AP-1 might be required to recruit clathrin to the TGN.

FIGURE 1: Glue granule biogenesis is developmentally regulated. (A–C’) Confocal micrographs of whole third-instar larval (L3) salivary glands expressing Sgs3-DsRed (red) and stained for AP-1γ (green), showing developmental timing of Sgs3-DsRed expression from stage 0 (no granules) through stage 1 (initiation of granule production) to stage 2 (fully mature granules or glands). AP-1γ is expressed in all cells of the salivary gland throughout development, whereas Sgs3-DsRed is first detected in distal (d) mid-L3 salivary gland cells (B, B’) and is expressed in more proximal (p) cells as development proceeds (C, C’). (D–F) Confocal micrographs of individual salivary gland cells showing developmental expression of Sgs3-DsRed. Sgs3-DsRed is not expressed in stage 0 (D). In stage 1, granules surround the nucleus (n) and appear uniformly small (E). In stage 2, granules are larger and occupy most of the cytoplasmic space (F). (G–I) Transmission electron micrographs (TEM) of L3 salivary glands stained using the Sgs3-DsRed marker. No granules were detected in stage 0 (G). Glue granule (Gr) maturation observed by TEM (H, I) parallels that seen by Sgs3-DsRed, validating this marker for following glue granule biogenesis (E, F). (J) Granules increase in size over time, from an average length of 1.0 μm ± 0.3 (n = 91) at stage 1 (red bar) to a maximum length of 3.5 μm ± 1.0 (n = 54) at stage 2 (green bar). (K, L) TEM of stage 1 salivary gland cells. Rough ER, transitional ER (tER), Golgi, and TGN (defined morphologically as in the work of Thomopoulos et al., 1992; Kondylis and Rabouille, 2009) are present near small glue granules (Gr) (K). Coated vesicles (CV) were also observed near glue granules (Gr) (L).
AP-1 recruits clathrin to the trans-Golgi network

To test whether AP-1 recruits clathrin to the TGN, we made use of a μ1-adaptin null allele, AP-47<sup>SHE-11</sup> (see Materials and Methods). To bypass late embryonic lethality caused by this allele, we generated mosaic clones in the salivary gland using FLP-FRT–based recombination (see Materials and Methods). Briefly, the wild-type chromosome carries a copy of green fluorescent protein (GFP) such that homozygous mutant cells are marked by the absence of GFP expression and heterozygous and wild-type cells are marked by one or two copies of GFP, respectively. AP-47<sup>SHE-11</sup> clones were generated during embryogenesis and analyzed in third-instar larval salivary glands at stage 0, just prior to glue production. To determine whether other AP-1 subunits can localize to the TGN in the absence of AP-47, we examined the distribution of AP-1γ and found that its punctate localization was entirely lost in AP-47<sup>SHE-11</sup> mutant cells (Figure 3, A–A'). Hence AP-47 is required for efficient recruitment or stability of AP-1γ, similar to what was previously observed in μ1-adaptin–deficient mouse embryonic fibroblasts (Meyer et al., 2000). Not all trafficking markers were affected by the loss of AP-47, as the early endosome marker Rab5 was unperturbed (Figure 3, B–B').

Strikingly, in AP-47<sup>SHE-11</sup> mutant cells, RFP-Chc localization to the Golgi was dramatically reduced (Figure 3, C–C''). The effect on RFP-Chc distribution was also observed in salivary gland cells in which expression of a double-stranded RNA was used to knock down expression of AP-1γ by RNA interference (RNAi) (Supplemental Figure S2). Most cells depleted of AP-1γ exhibited strong delocalization of RFP-Chc (compare Figure 3, D–D'', with Figure 3, E–E''), with only a few cells retaining weak RFP-Chc localizations.

To obtain images of salivary gland cells stained for AP-1γ (green), Lva (red), and DNA (stained with DAPI; blue) reveals numerous Golgi bodies scattered throughout the cytoplasm. A three-dimensional rotation of a single Golgi body shows AP-1γ (green) adjacent to the cup-shaped Lva-positive cis-Golgi (red) (C'–C''). Images were generated from Z stacks of 28 (C) or 5 (C'–C'') optical sections acquired at a distance of 0.3 μm (see Materials and Methods). (D–D') AP-1γ (green) and RFP-Chc (red) colocalize adjacent to Lva (blue). Colocalization of AP-1γ and RFP-Chc appears yellow in the merged image. (E–E') Spin-disk confocal images reveal that VFP-AP47 (green) does not colocalize with mCherry-AP3δ (red). Boxed region is shown at 2× higher magnification in the insets.
To determine whether glue protein colocalizes with clathrin and/or AP-1, we analyzed Sgs3-DsRed localization in cells that had just switched on glue protein expression. In early stage 1, only a subset of the most distal salivary gland cells had initiated glue production (Figure 1B and Figure 4, A–A’ and B–B’’). Sgs3-DsRed and AP-1γ partially colocalized adjacent to Lva, suggesting these proteins were at or near the TGN (Figure 4, A–A’). The association of glue-containing structures with AP-1 appeared to be transient, because some of the glue was also present in AP-1–negative puncta outside the Golgi (Figure 4, A–A’’, yellow arrows). Importantly, Sgs3-DsRed colocalized with GFP-Chc as well as AP-1γ (Figure 4, B–B’’).

Later in stage 1, both GFP-Chc (Figure 4, C–C’’) and AP-1γ (Figure 4, D–D’’) associated with a subset of granules. Indeed, time-lapse fluorescence microscopy of live cells expressing VFP–AP-47 revealed that AP-1 localizes to the limiting membrane of a portion of small Sgs3-DsRed–containing granules (Supplemental Video 1). Moreover, small granules could be seen moving rapidly in the cytoplasm. Notably, granules exhibiting directed movement were not associated with AP-1, suggesting this coat must be shed for transport to occur. At stage 2, when granule biogenesis is nearly complete, GFP-Chc–positive puncta could still be seen. However, it was difficult to discern whether GFP-Chc localized to the limiting membrane of granules or to the cytoplasm adjacent to the granules (Figure 4, E–E’’).

AP-1 and clathrin are required for glue granule formation

To determine whether AP-1 is required for glue granule formation, we examined AP-47SHE-11 homozygous mutant cells in late-third-instar larvae, when glue granules are fully mature (stage 2). AP-47SHE-11 mutant cells either lacked detectible Sgs3-DsRed–containing glue granules (8 of 13 cells) (Figure 5, A–A’’) or accumulated small granules in the cytoplasm (5 of 13 cells) (Figure 5, B–B’’). This difference is likely due to variations in perdurance of AP-1γ protein in mutant cells. In addition, AP-47SHE-11 mutant cells also appeared smaller, suggesting that additional secretory proteins might play a role in glue granule biogenesis.

Newly synthesized glue proteins colocalize with AP-1 and clathrin at the TGN

Glue granule production is believed to follow the classical model of secretion, as outlined by Palade and coworkers, with secretory cargo being transported through the endoplasmic reticulum (ER) and Golgi prior to incorporation into secretory granules (Jamieson and Palade, 1967a, 1967b; Thomopoulos and Kastritis, 1979).

FIGURE 3: AP-1 is required to recruit clathrin to the trans-Golgi network. (A–C’’) Confocal micrographs of stage 0 salivary glands showing mutant clones (cells) marked by absence of GFP (green) and outlined in yellow. (A–A’’) AP-1γ (red) localization is lost in an AP-1γ (AP-47SHE-11) mutant cell. (B–B’’) Rab5-positive early endosomes (red) are unaffected in AP-47SHE-11 mutant cells. (C–C’’) RFP-Chc (red) becomes largely cytoplasmic in an AP-47SHE-11 mutant cell, whereas the distribution of the cis-Golgi marker Lva (blue) is unaltered. Note that Lva shows a gradient of signal intensity due to incomplete antibody penetration of the tissue. (D–D’’) Control salivary gland cells expressing the AB1-GAL4 driver alone show colocalization of AP-1γ (green) and RFP-Chc (red) adjacent to Lva (blue). (E–E’’) Salivary gland cells expressing both the AB1-GAL4 driver and a UAS-AP-1γ RNAi transgene are depleted of AP-1γ (green) and show cytosolic distribution of RFP-Chc (red), whereas Lva (blue) is largely unaffected. See also Supplemental Figure S2.
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FIGURE 4: Sgs3-DsRed colocalizes with AP-1 and clathrin at the trans-Golgi network. Confocal fluorescence micrographs of third-instar salivary glands at the onset (early stage 1) (A–B’’), stage 1 (C–D’’), and stage 2 (E–E’’) of glue production. (A–A’) Projections of a series of spinning-disk confocal images showing cells initiating Sgs3-DsRed (red) expression, stained with AP-1γ (green) and Lva (blue). In an early stage 1 cell, Sgs3-DsRed and AP-1γ partially colocalize (yellow) adjacent to the cis-Golgi marker Lva in a subset of Golgi bodies (A’). Boxed region is shown at 2× higher magnification in the insets. Note that a subset of the Sgs3-DsRed puncta does not colocalize with AP-1γ (yellow arrows). Images were generated from a Z stack of five optical sections acquired at a distance of 0.3 μm. (B–B’’) Sgs3-DsRed (red) partially colocalizes with both GFP-Chc (green) and AP-1γ (blue). Colocalization of Sgs3-DsRed with GFP-Chc and AP-1γ appears white in the merged image (B’’). (C–C’’) Low-magnification view of a portion of a salivary gland expressing GFP-Chc (green) and Sgs3-DsRed (red), showing a distal cell with a large number of stage 1 glue granules (C, boxed region; shown at higher magnification in C’–C’’). GFP-Chc partially coats a subset of Sgs3-DsRed–containing stage 1 glue granules (C’–C’’, yellow arrows). (D–D’’) Low-magnification view of a portion of a salivary gland stained for AP-1γ (green) and expressing Sgs3-DsRed (red) reveals numerous cells with stage 1 granules (D; boxed region is shown at higher magnification in D’–D’’). AP-1γ partially coats a subset of Sgs3-DsRed–containing granules (D’–D’’, yellow arrows). (E–E’’) Spinning-disk confocal micrographs of cells from a mature stage 2 salivary gland expressing GFP-Chc (green) and Sgs3-DsRed (red). GFP-Chc localizes near a broad range of Sgs3-DsRed–containing structures, including large stage 2 granules, as well as smaller vesicles (E’). Boxed regions 1–3 in E’ are shown at 2× higher magnification in the images on the right.

Salivary gland cells in which AP-1γ was knocked down using RNAi were morphologically similar to AP-47SHE-11 mutant cells; they either lacked detectible glue granules or accumulated very small granules (compare Figure 5, C and D). Because AP-1 is required to recruit clathrin to the TGN (Figure 3, C–C’’ and E–E’’), we asked whether clathrin is also required for glue granule formation. The effect of depletion clathrin heavy chain by RNAi was even more dramatic than for AP-1, resulting in a complete block in glue granule formation in most cells, with only rare cells exhibiting small granules (Figure 5G). Consistent with a dramatic depletion of glue granules, pupal cases were poorly adherent to the vial wall and could easily be removed with a small paintbrush. These effects were specific to loss of AP-1 and clathrin, as mutations in carmine (cm) and garnet (g50S), which encode the AP-3 subunits AP-3δ and AP-3μ, respectively, exhibited normal-sized glue granules (Figure 5, H and I).

To rule out an indirect effect of AP-1 on glue granule biogenesis, we tested whether glue gene transcripts were expressed at normal levels. Glue granule cargo consists of at least eight different proteins, all of which are highly expressed in the salivary gland during the third-instar larval stage. Semiquantitative reverse transcriptase–coupled PCR (RT-PCR) confirmed that AP-1γ transcripts were depleted in salivary glands in which AP-1γ had been knocked down by RNAi, whereas transcript abundance of a control gene, α-tubulin84B, was unaffected (Supplemental Figure S2, A and B). Interestingly, depletion of AP-1 had a moderate effect on glue gene expression. Levels of glue gene transcripts, including Sgs3-DsRed, were reduced by ~50% in AP-1γ knockdown glands as compared with controls. Importantly, glue gene transcription was still robustly initiated in salivary glands depleted of AP-1γ.
Glue protein accumulates in aberrant vacuolated organelles in AP-1γ-depleted cells

To further define the fate of Sgs3-DsRed in AP-1γ-depleted cells, we assessed the localization of Sgs3-DsRed relative to the Golgi marker Lva. In wild-type stage 2 salivary gland cells, Lva-labeled Golgi bodies appeared scattered throughout the cytoplasm and were not tightly associated with mature granules containing Sgs3-DsRed (Figure 6, A–A”). In contrast, in AP-1γ-depleted salivary gland cells, glue protein showed an increased association with Lva-positive Golgi bodies (Figure 6, B–B”), and these appeared swollen relative to those in control cells (compare Figure 6, A and B). Indeed, the Golgi in AP-1γ knockdown cells more closely resembled those of cells prior to onset of glue production (compare Figure 2, A’ and B”, with Figure 6B). Notably, a similar increase in TGN volume was observed when post-Golgi secretion was blocked by incubation of kidney epithelial cells at moderately low temperatures (20°C) (Griffiths et al., 1989; Ladinsky et al., 2002). Sgs3-DsRed was also visible in post-Golgi structures most likely corresponding to abnormal immature granules (Figure 6, B’ and B”).

To determine the morphology of the Sgs3-DsRed–positive organelles in AP-1γ-depleted cells, we examined salivary glands by TEM. Numerous enlarged, vacuolated structures containing fibrillar cargo were visible throughout the cytoplasm (compare Figure 6, C and D). Notably, these structures did not resemble early glue granules observed in wild-type cells, clearly indicating an essential role for AP-1 in the formation of normal secretory granules.

**DISCUSSION**

We provide compelling evidence of a previously unknown function for clathrin and AP-1 in the formation of mucin-type secretory granules. We show that clathrin and AP-1 localize to the TGN prior to synthesis of secretory cargo, colocalize with newly synthesized secretory cargo, and are required for secretory granule formation. Hence AP-1 and clathrin play a crucial role in early stages of secretory granule formation in salivary gland cells. Consistent with this idea, clathrin becomes delocalized upon AP-1 depletion, indicating that other adaptors cannot recruit clathrin in the absence of AP-1 at this stage of salivary gland development.

Our results suggest that formation of mucin-containing glue granules and Weibel-Palade bodies might be similar. Weibel-Palade bodies have an unusual cigar-shaped appearance and it was proposed that AP-1 and clathrin might participate in their formation at the TGN by allowing luminal cargo to properly fold and aggregate or by preventing premature scission (Lui-Roberts et al., 2005; Metcalf et al., 2008). Indeed, depletion of AP-1 in endothelial cells results in the formation of small, round von Willebrand factor–containing organelles lacking other...
yet-unidentified transmembrane receptor might mediate this interaction.

A distinct possibility is that AP-1 might be required to maintain a steady-state distribution of proteins that shuttle between the TGN and endosomes such that they are available at the TGN during granule formation. For instance, the protein convertase furin recycles between the TGN and endosomes and is required to process numerous secreted proteins such as von Willebrand factor (Creemers et al., 1993). Importantly, furin is no longer concentrated at the TGN in μ1A-deficient fibroblasts (Fölsch et al., 2001). Thus failure to recycle transmembrane enzymes that play a crucial role in processing secreted cargo could also contribute to defective granule formation.

Reduced levels of AP-1 resulted in intermediate-sized granules, suggesting AP-1 might have an additional role during glue granule maturation. The development of Drosophila glue granules is characterized by an overall increase in size and decrease in number, consistent with homotypic fusion of smaller granules over time (Farkas and Suakova, 1999). Whether small and large granules are equally capable of fusing and whether fusion events are temporally regulated is not known. AP-1 might regulate glue granule maturation by sorting or retrieving membrane proteins required for homotypic fusion and eventual exocytosis. Additionally, AP-1 might function directly on maturing granules to remove missorted proteins, such as lysosomal hydrolases, similar to what has been reported for other types of secretory granules (Dittle et al., 1996, 1997, 1999; Klumperman et al., 1998). In support of this view, live imaging revealed a dynamic association of AP-1 with immature granules. Further studies are needed to resolve whether AP-1 functions in the addition and/or removal of proteins from maturing glue granules.

On the basis of the small size of mutant cells, AP-1 likely participates in additional trafficking pathways. In mammalian cells, AP-1A is ubiquitously expressed and required for trafficking between TGN and endosomes, whereas AP-1B is present only in polarized epithelial cells and is required for basolateral sorting from recycling endosomes (Fölsch et al., 1999, 2001; Cancino et al., 2007; Gravotta et al., 2007; Deborde et al., 2008). The sole AP-1 complex in Drosophila might mediate both functions in a single cell type. Interestingly, depletion of AP-1α in salivary glands after granule formation caused the basolateral protein Discs large to redistribute to the apical surface (Peng et al., 2009), suggesting that AP-1 is required for basolateral targeting of proteins in this tissue. However, an independent analysis of AP-1α null cells in the dorsal thoracic epithelium failed to reveal a similar polarity defect (Benhra et al., 2011). This discrepancy might be due to cell type–specific requirements for AP-1 or to differences in RNAi versus mutant clones.

The observation that the abundance of Sgs3-DsRed protein and several Sgs mRNAs is reduced upon AP-1 knockdown suggests the existence of a negative-feedback loop, whereby a block in

Weibel-Palade body markers. Our data demonstrate that the requirement for clathrin and AP-1 is not restricted to one specific type of granule. Depletion of clathrin or AP-1 in Drosophila salivary glands resulted in the accumulation of glue protein both at the TGN and in small organelles of aberrant morphology. This finding extends the role of AP-1 and clathrin to the formation of granules containing mucoprotein cargo and suggests a broader requirement for this coat complex in granule production.

How might AP-1 participate in glue granule formation? One possibility is that AP-1 and clathrin are directly involved in packaging glue granule cargo at the TGN. In mammalian cells, several transmembrane proteins are targeted to regulated secretory granules, including peptidyl-α-amidating monoxygenase, mucin, and phogrin (Bell-Parikh et al., 2001; Wasmeier et al., 2002; Boulatnikov and De Lisle, 2004; Dikeakos and Reudelhuber, 2007). Indeed, phogrin has been shown to bind to AP-1 and AP-2 through well-conserved tyrosine and dileucine sorting motifs present in its cytosolic tail (Tori et al., 2005; Wasmeier et al., 2005). How AP-1, a cytosolic coat protein, might interact with luminal glue proteins in salivary cells remains to be determined. Because none of the known granule proteins contains a predicted transmembrane domain, a

FIGURE 6: Glue protein accumulates at the trans-Golgi network and in aberrant vacuolated organelles in AP-1γ-depleted cells. (A–B′) Confocal fluorescence micrographs of late-third-instar (stage 2) salivary glands expressing Sgs3-DsRed (red) and stained for the cis-Golgi marker Lva (green). Lva is distributed throughout the cytoplasm but is not associated with mature Sgs3-DsRed–containing granules in control cells expressing AB1-GAL4 alone (A–A′; boxed regions 1–3 in A′ are shown at 2× higher magnification in the images on the right). In cells expressing both the AB1-GAL4 and a UAS-AP-1γ RNAi transgene, Sgs3-DsRed is associated with Lva-containing Golgi bodies and can also be seen in larger organelles (B–B′; boxed regions 1–3 in B′ are shown at 2× higher magnification in the images on the right). (C and D) TEM of stage 1 salivary glands. Rough ER, mitochondria (m), nascent granules (Gr), and coated vesicles (CV) are visible in a wild-type cell (C). AP-1γ-depleted cells expressing both the AB1-GAL4 and a UAS-AP-1γ RNAi transgene exhibit rough ER and mitochondria (m), as well as a large number of aberrant vacuolated organelles (D, white arrow). Micrograph is from a single experiment.

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antegrade secretory trafficking results in down-regulation of secretory genes. A block in secretion at the TGN could potentially induce the unfolded protein response, analogous to what happens upon depletion of the Arf1 GEF GBF1 (Citterio et al., 2008). However, GBF1 functions early in the secretory pathway, and knockdown of two Arf-GEFs that act on the TGN did not elicit a similar response (Citterio et al., 2008). Alternatively, a block in antegrade trafficking might repress transcriptional activation of secretory genes by Drosophila CrebA and Forkhead (Fkh) by some as-yet-unknown mechanism (Abrams and Andrew, 2005; Abrams et al., 2006).

In addition to the AP-1 complex, the Drosophila genome encodes two other Golgi-localized clathrin adaptor proteins, EpsinR/ LqfR and Golgi-localized, γ-ear-containing, ADP-ribosylation factor–binding (GGA) protein (Drosophila has only one GGA) (Hirst et al., 2009; Lee et al., 2009; Kametaka et al., 2010). LqfR partially colocalizes with AP-1 at the TGN in salivary gland cells and lqfR mutants exhibit small salivary glands, suggesting defects in granule biogenesis (Lee et al., 2009). It will be interesting to determine whether LqfR and GGA participate in glue granule biogenesis, especially since these clathrin adaptors might facilitate sorting of other types of cargo. For example, EpsinR has been shown to bind SNARE proteins and could function to provide vesicle identity to nascent glue-containing granules (Miller et al., 2007; Chidambaram et al., 2008). SNAP-24 was previously identified as a glue granule–specific SNARE, although whether this SNARE mediates homotypic fusion of granules or functions during exocytosis of granules at the plasma membrane is unclear (Niemyer and Schwarz, 2000). Given the apparent similarities between glue granule and Weibel-Palade body biogenesis, as well as the high degree of conservation of TGN sorting machinery in Drosophila, our findings suggest that Drosophila salivary glands are of great utility to further elucidate the mechanisms of biogenesis of regulated secretory granules.

MATERIALS AND METHODS
Fly stocks and genetics crosses
Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). Visible markers and balance chromosomes are as described (Lindsley and Zimm, 1992). AP-475HE-11, a deletion of amino acids 146–158 followed by a frameshift in the gene encoding the AP-1 subunit mu-adaptin, was isolated in a screen for modifiers of Presenilin-dependent Notch phenotypes (Mahoney et al., 2006). AP-475HE-11 was recombined onto P(neoFRT)82B to generate mosaic clones and to remove the second site Psn 142 mutation (Xu and Rubin, 1993). P[GawB]AB1-GAL4 and P(wr, Sgs3-GFP) were obtained from the Bloomington Drosophila stock center. The Rab11-GFP protein trap line (CA07717), which carries a piggyBack transposon encoding EGFP inserted in frame with endogenous Rab11 coding sequences, was obtained from A. Spradling (Buszczak et al., 2007; Lighthouse et al., 2008). Transgenic fly stocks carrying P(wr, UAS-mRFP-Chc), P(wr, UAS-EGFP-Chc), P(wr, attub-mCherry-AP38), or P(wr, attub-AP38-mEGFP) (see later discussion) were generated by injection of Drosophila embryos using standard techniques. P(wr, Sgs3-DsRed) flies were obtained from A. Andres (Costantino et al., 2008). P(wr, UAS-AP47::VFP) flies were described previously (Benhra et al., 2011). RNAi stocks expressing double-stranded RNAs under control of GAL4 upstream activating sequences (UASs) (Vienna Drosophila RNAi Center, Vienna, Austria; Dietzl et al., 2007) were as follows: #3275 (AP17), #24017 (AP1j), and #23666 (Chc). To examine salivary glands depleted of AP-1 or clathrin heavy chain, a stock carrying Sgs3-DsRed, AB1-GAL4 was crossed to flies carrying the corresponding UAS-RNAi insertion.

Mosaic clones were generated using the FLP-FRT system (Golic and Lindquist, 1989) by crossing y, w118, P(70FLP)3F; P(neoFRT)82B, P(Ubi-GFP)D83/TM6B, Hu, Tb to flies containing Sgs3-DsRed; P(neoFRT)82B, AP-475HE-11/TM6B, Hu, Tb. To examine clathrin in AP-475HE-11 clones, the heat shock FLP stock was crossed to UAS-mRFP-Chc + / + ; P(neoFRT)82B, AP-475HE-11, AB1-GAL4/TM6B, Hu, Tb. To generate mosaic clones in the salivary gland, adult flies were allowed to lay embryos in a vial during a 60-min collection window. Flies were then removed and embryos were aged for 2.5 h at room temperature. Embryos were then heat-shocked for 90 min at 37°C by placing the vial in a water bath. Embryos were then incubated at 25°C to allow for further development. Salivary gland cells (~100 per lobe) are specified early in embryonic development and differentiate without dividing (Campos-Ortega and Hartenstein, 1985). Consequently, mosaic clones generated in early embryos typically give rise to single-cell mutant clones.

Molecular biology
For Chc constructs, mRFP and EGFP were inserted in frame at the N-terminus of Drosophila Chc. For EGFP-Chc, an EcoRI-NotI fragment containing EGFP (without the stop codon) was first cloned into pUAST. pOT2-Chc, a plasmid containing the entire Chc ORF, was obtained from the Drosophila Genomics Research Center (Bloomington, IN). This plasmid contained a point mutation in Chc, which was repaired using the QuikChange Site-Directed Mutagenesis Kit (Strategene, Cedar Creek, TX). QuikChange was also used to introduce a NotI site before the ATG and a KpnI site after the stop codon of Chc. After being verified by sequencing, the Chc ORF was cloned as a NotI-KpnI fragment into pUAST-EGFP; resulting in pUAST-EGFP-Chc (GFP-Chc). pUAST-mRFP-Chc (RFP-Chc) was made in a similar manner. To generate mCherry-AP38 and AP38-mEGFP (AP38-GFP), we used pCaSpeR-tub::mCherry, which contains a N-terminal mCherry (gift of R. Tsien; Shaner et al., 2004) followed by an XbaI site, or pCaSpeR-tub::GFP, which contains a C-terminal mEGFP (gift of E. Snapp; Zacharias et al., 2002) preceded by a KpnI site. Both vectors are derived from a version of pCaSpeR4 (Pirrotta, 1988) containing the α-Tub84B promoter (gift of S. Eaton; Marois et al., 2006). Restriction sites were introduced into a full-length garen (RE06749) cDNA (Berkeley Drosophila Genome Project; obtained from the Canadian Drosophila Microarray Center, Mississauga, Canada) by PCR and orientation after subcloning was confirmed by DNA sequencing (The Center for Applied Genomics, SickKids, Toronto). AP38-GFP was fully functional in that it rescued the eye pigmentation defect of gamma mutant flies (J. B. and J. A. B., unpublished data).

RNA isolation and RT-PCR of salivary glands
To obtain salivary glands for RNA extraction, larvae were generated by crossing w, Sgs3-DsRed; AB1-Gal4 virgin females to UAS-AP17 RNAi/TM6B, Hu, Tb males. AP1-1-depleted salivary glands were dissected from non-Tb larvae, and salivary glands from Tb siblings were used as controls. Ten pairs of salivary glands for each genotype were dissected and pooled in a microfuge tube containing 0.7% NaCl buffer; 600 µl of TRIzol (Invitrogen, Carlsbad, CA) was added, and tubes were centrifuged at 14,000 rpm for 1 min. Following addition of 120 µl of chloroform, the mixture was shaken vigorously by hand, incubated at room temperature for 3 min, and centrifuged at 14,000 rpm for 15 min at 4°C. The upper aqueous phase was transferred to a new microfuge tube, to which 0.7 volumes of isopropanol were added. RNA was left to precipitate overnight at ~20°C, pelleted by centrifugation at 14,000 rpm for 30 min at 4°C, washed with 500 µl cold 70% ethanol, and dried by centrifugation in a SpeedVac (Thermo Scientific, Waltham, MA) for
5 min. The dry pellet was resuspended in 15 μl of diethylpyrocarbo nate water and incubated for 15 min at 37°C.

cDNA libraries were generated from 1.7 μg of total salivary gland RNA using random decamers and the RETRO-SCRIPT reverse tran scription kit (Ambion, Austin, TX) following manufacturer’s recom mendations for the two-step procedure. The linear range of PCR amplification was determined empirically, and PCR products of sali vary gland cDNA were evaluated after 20 cycles. PCR primers for each transcript were designed to span one or more introns.

**Fluorescence microscopy and imaging**

Salivary glands from third-instar larvae were dissected in phosphate buffered saline (PBS) (pH 7.4) and were either mounted and imaged directly in dissection buffer or fixed for 20 min on ice in PLP (4% paraformaldehyde, 0.01M sodium meta-periodate, 0.075 lysine, 0.035 phosphate buffer, pH 7.4). Fixed salivary glands were then washed once in PBS (pH 7.4) and permeabilized in PBST (PBS + 0.1% Triton X-100). Primary antibody incubation was performed overnight at 4°C in PBST with 5% normal goat serum. Salivary glands were mounted in PPD (0.1x PBS, 90% glycerol, 1 mg/ml p-phenyle nediouamide). Antibodies were used as follows: 1:1000 rabbit anti-Lva (gift of O. Papoulas and J. Sisson; Sisson et al., 2000), 1:500 mouse anti-GFP monoclonal 36E (Invitrogen/Molecular Probes, Eugene, OR); 1:50 rabbit anti-Rab5 (gift of M. González-Gaitán; Wucherpfennig et al., 2003), 1:500 mouse anti-AP-1γ (Benhra et al., 2011), and 1:100 rabbit anti-LqfR (generated against a truncated protein lacking the Epsin N-terminal homology (ENTH) domain and shown to specifically recognize LqfR in immunoblotting and immuno fluorescence experiments; P.A.L., G.L.B., J. B., and J.A.B., unpublished data). Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa-488, Alexa-568, or Alexa-633 were purchased from Molecular Probes (Invitrogen) and used as recommended by the manufacturer.

Fluorescence micrographs were acquired on a Zeiss LSM510 inverted laser scanning confocal microscope equipped with LSM objectives (20x, FLUAR NA 0.75; 40x, Plan-APOCHROMA NA 1.3; 63x, Plan-APOCHROMAT NA 1.4; or 100x, Plan-APOCHROMAT NA 1.4) and LSM510 software, or on a Quorum spinning-disc confocal microscope equipped with an SD 63x LCI Plan-NEOFLUAR 1.3 DIC Imm Kor (water) objective and Velocity acquisition software (Sick- Kids Imaging Facility). Spinning-disc confocal images were deconvolved using the Iterative Restoration function of Velocity 4. Three-dimensional reconstructions were created using the three-dimensional opacity renderings of Velocity 4. All images were further processed for brightness and contrast levels using Adobe Photoshop CS2.

**Electron microscopy**

Salivary gland samples were prepared for TEM as previously de scribed (Bazinet and Rollins, 2003). Salivary gland samples were staged using Sgs3-DsRed, which is expressed from endogenous promoter–enhancer elements, properly sorted into glue granules and secreted into the lumen in response to ec dysone (Biya sheva et al., 2001). The presence of Sgs3-DsRed did not noticeably alter the appearance of mature granules by electron microscopy (unpub lished data). Sections were viewed with a JEOL JTE 141011 micro scope (SickKids/Mt. Sinai Advanced Center for Bioimaging). Images were obtained using AmtV542 acquisition software, and brightness and contrast were adjusted using Adobe Photoshop CS2.

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