Changing directions: clathrin-mediated transport between the Golgi and endosomes

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Summary

Clathrin-coated vesicles mediate transport between the trans-Golgi network (TGN) and endosomes. In recent years there has been tremendous progress in identifying factors involved in anterograde and retrograde transport steps. The well-characterised heterotetrameric clathrin adaptor complex AP-1 has long been thought to mediate anterograde transport from the TGN to endosomes. However, recent studies of AP-1-knockout mice implicate AP-1 in retrograde as well as anterograde transport. The recently identified Golgi-associated, γ-ear-containg, ARF-binding (GGA) proteins share functional similarities with tetrameric adaptor complexes and are essential for anterograde transport of mannose-6-phosphate receptors, the sorting receptors for soluble lysosomal enzymes. To date, it is not clear whether GGAs and AP-1 mediate transport in different directions, act in parallel pathways, or cooperate in the same transport steps. Recent data have shed light on the locations, functions and interactions of AP-1 and GGA proteins. These data provide support for the role of both in anterograde transport from the Golgi complex.

Key words: AP-1, GGA protein, Golgi, Endosome, TGN

Introduction

In eukaryotic cells, vesicles transport proteins and lipids between membrane-bound organelles. The protein coats on these vesicles recruit the appropriate cargo into the nascent vesicle, generate membrane curvature and allow vesicles to pinch off the donor membrane. Three different classes of coated vesicle have been identified so far (Kirchhausen, 2000b). COPI- and COPII-coated vesicles mediate transport between the endoplasmic reticulum (ER) and the Golgi, as well as within the Golgi (Antonny and Schekman, 2001; Nickel et al., 2002). Clathrin-coated vesicles transport proteins and lipids from the plasma membrane to endosomes and mediate transport between the TGN[†] and endosomes. The tetrameric clathrin adaptors AP-1 and AP-2 have been and continue to be studied in much detail, along with the more recently identified AP-3 and AP-4 complexes. The unique subcellular localization of each adaptor complex reflects its function in different post-Golgi and endocytotic transport steps (Boehm and Bonifacino, 2001). All four adaptor complexes consist of two large subunits (also termed adaptins) of ~100 kDa, one medium subunit of ~50 kDa, and one small subunit of ~20 kDa. One large subunit is an adaptor-specific subunit, called α, γ, δ and ε, in AP-1, AP-2, AP-3 and AP-4, respectively; the other is a generic subunit, designated β1, β2, β3 and β4, in AP-1, AP-2, AP-3 and AP-4, respectively. The smaller subunits are called μ and σ, and are distinguished by the number of the adaptor to which they belong, i.e. AP-1 contains the μ1 and σ1 subunits, whereas AP-2 contains the μ2 and σ2 subunits, etc. Most subunits have two or three isoforms, which are present in all cells, except μ1b, which is present in epithelial cells, and β3b and μ3b, which are expressed in neurons and neuroendocrine cells.

Although AP-2 has a well-established function in receptor-mediated endocytosis (Kirchhausen, 2002; Slepnev and DeCamilli, 2000; Takei and Haucke, 2001), many views about AP-1 function have had to be modified in recent years. It was long accepted that AP-1 functions in anterograde trafficking from the TGN to endosomes; however, the picture emerging now is that AP-1 functions in both anterograde and retrograde trafficking. Accessory factors that might be specific for retrograde transport have been identified. The recent discovery of GGA proteins – monomeric, adaptor-related proteins – has extended our understanding of how clathrin-coated vesicles form on the TGN. The domain organisation of adaptor complexes and their structure-function relationships have been discussed in detail elsewhere (Kirchhausen, 1999), as have AP-3 and AP-4 (Robinson and Bonifacino, 2001). Here, we discuss the recent progress in our understanding of AP-1 and GGA function and how they might interact.

AP-1: subunit organisation and accessory factors

The AP-1 complex consists of two adaptins, (γ and β1), a 47 kDa μ1 subunit and a 19 kDa σ1 subunit (Fig. 1A). At steady state, most AP-1 is localised to tubular, clathrin-coated

[†]Abbreviations: ARF: ADP-ribosylation factor; CD4: cluster of differentiation antigen 4; CD-MPR: cation-dependent mannose-6-phosphate receptor of 46 kDa; CI-MPR: cation-independent mannose-6-phosphate receptor of 300 kDa; CK2: Casein kinase 2; clathrin TD: terminal domain; CPY/S: carboxypeptidase Y/S; EEA1: early endosomal antigen 1; ER: endoplasmic reticulum; GAE: γ-ear homology; GAP: GTPase activating protein, GAT: GGA and TOM1; GGA: Golgi associated, γ ear-containing, ARF-binding proteins; Hrs: hepatocyte growth factor receptor substrate; LAMP-1 or -2: lysosomal associated membrane protein-1 or -2; LRP3: low density lipoprotein receptor-related protein 3; PACS-1: phosphofurin acidic cluster sorting protein 1; SNARE: SNAP receptor; STAM: signal transducing adaptor molecule; TGN: trans-Golgi network; TIP47: tail-interacting protein of 47 kDaTOM1: target of myb 1; TOML1: TOM-like protein 1; VHS: Vps27p, Hrs, STAM; Vps: vacuolar protein sorting.

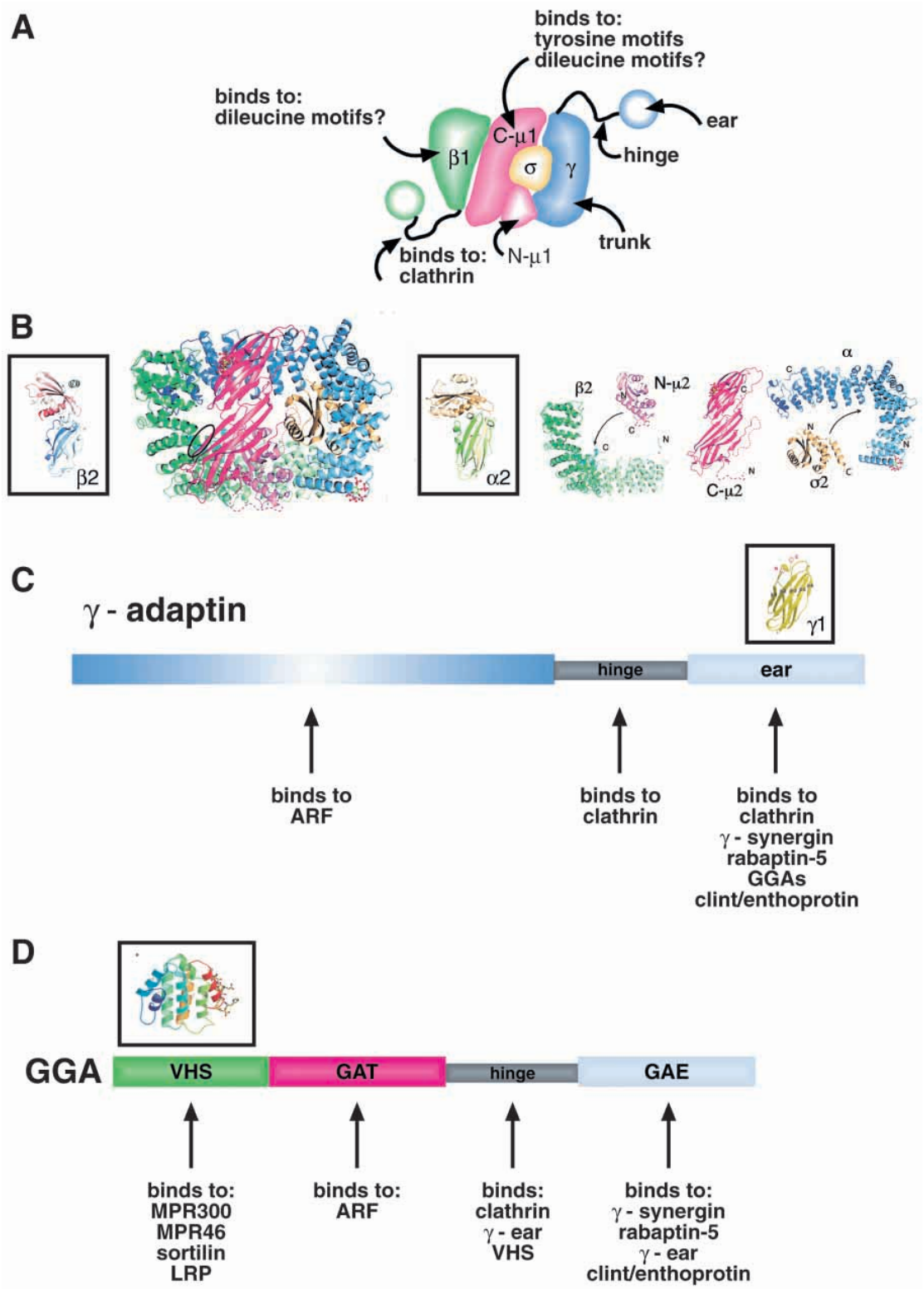

Fig. 1. Adaptor structure and interacting proteins. (A) Proposed AP-1 subunit organisation based on two hybrid studies and the structure of AP-2 and location of sites for interaction with binding partners. AP-2 is so far the only clathrin adaptor whose complete structure has been determined. (B) The 3D structure of the subunits of AP-2 (Collins et al., 2002) illustrates the compact nature of the large subunit body domains in association with the μ2 and σ2 chain. The appendages of the β2 and α subunits, determined independently (Owen et al., 2000; Traub et al., 1999) are shown in the left and right box, respectively for comparison with the γ1 appendage in panel C. (C) Regions of the γ1 subunit that interact with cytoplasmic proteins involved in coat recruitment. The 3D structure of the γ-ear/appendage domain is shown (Nogi et al., 2002). (D) The modular domain organisation of GGA and interacting proteins, illustrating the 3D structure of the VHS domain in association with the dileucine motif from MPR (Misra et al., 2002; Shiba et al., 2002).

structures on the TGN, and endocrine and exocrine cells have clathrin-coated immature secretory granules that contain AP-1 (Tooze, 1998). Recruitment of AP-1 to membranes is dependent on the small GTPase ARF. As in the case of COPI-coated vesicles (Nickel et al., 2002), activated ARF is first recruited to the membrane, then the AP-1 complex can bind and ARF interacts transiently with β-adaptin and γ-adaptin (Austin et al., 2000). Recruitment of both AP-1 and COPI to membranes is inhibited by the fungal metabolite brefeldin A (BFA), which disassembles the Golgi complex and blocks secretion by directly inhibiting some of the ARF exchange factors. However, unlike in COPI-coated vesicles, in clathrin-coated vesicles ARF is not a stoichiometric component.

The large adaptins (α, β, δ and ε) have similar domain organisations and consist of an N-terminal body/trunk domain, a variable hinge region and a C-terminal ear/appendage domain, which fulfill different functions (Fig. 1C). AP-1 binds to clathrin mainly through its clathrin-box motif in the hinge region of β1-adaptin (Gallusser and Kirchhausen, 1993). ter Haar et al. have recently obtained a crystal structure of the clathrin terminal domain (TD) complexed with a peptide representing the clathrin box motif of β3-adaptin, the β-subunit of AP-3 (ter Haar et al., 2000). The clathrin-box motif consensus sequence, L(L/I)(D/E/N)(L/F)(D/E), binds to the WD40 motif of the clathrin TD (Kirchhausen, 2000a). More recently, Doray and Kornfeld have shown that the hinge domain of γ-adaptin, and to a lesser extent the ear domain of γ-adaptin (γ-ear), can also bind to clathrin (Doray and Kornfeld, 2001). The body domain of γ-adaptin is responsible for correct membrane targeting of the AP-1 complex: chimeric adaptor complexes consisting of AP-1 and AP-2 adaptor complexes in which the body and hinge from γ-adaptin are linked to the ear from α-adaptin (α-ear) still target to the TGN (Robinson, 1993).

The γ-ear is thought to recruit additional regulatory factors to the site of vesicle formation (Fig. 1C). A two-hybrid screen has identified a novel factor that interacts with the γ-ear, γ-synergin (Page et al., 1999). This protein contains an Eps15-homology domain, and, by analogy with the well-known protein network assembled on the α-ear, is thought to recruit additional proteins. In GST-pulldown assays, the γ-ear interacts with several proteins, including rabaptin-5, although the function of rabaptin-5 in vesicle biogenesis remains elusive (Hirst et al., 2000). Furthermore, Wasiak et al., using a proteomic approach, have identified an additional ENTH-domain-containing protein, enthoprotin, which is enriched in clathrin-coated vesicles and binds AP-1 (Wasiak et al., 2002). Kalthoff et al. identified the same protein, called Clint, by screening the database for uncharacterized ENTH-containing proteins (Kalthoff et al., 2002). Nogi et al. and Kent et al. recently determined the crystal structure of the γ-ear (Fig. 1C), revealing that this domain has an immunoglobulin-like β-sandwich fold similar to that of the α-ear and β2-ear (Nogi et al., 2002; Kent et al., 2002). However, the γ-ear is about half the size of the β-ear (and the α-ear) and does not have a hydrophobic C-terminal platform domain (compare boxed ear structures in Fig. 1B and C) shown to be the binding site for accessory proteins in the AP-2 subunits (Owen et al., 2000; Traub et al., 1999). Rather, the binding of accessory proteins to the γ-ear is mediated by residues found on the surface of the γ-ear domain, although there are conflicting views on exactly which residues are important (Nogi et al., 2002; Kent et al., 2002).

The μ-subunit mediates cargo recognition and recruitment (Fig. 1A). It binds to tyrosine-related sorting motifs in the cytoplasmic tails of transmembrane proteins (Ohno et al., 1995; Owen and Evans, 1998). It is still a matter of debate which adaptor subunit binds to dileucine sorting motifs: two-hybrid studies and phage display identified the μ1 subunit as the interacting subunit of the AP-1 complex (Rodionov and Bakke, 1998; Storch and Braulke, 2001), but crosslinking studies identified the β1-body as the region that interacts with dileucine-motif containing-peptides (Rapoport et al., 1998). Note that these studies are usually complicated by the fact that most cargo proteins bind to several adaptor complexes and accordingly contain several adaptor-interaction motifs; thus it is difficult to study these interactions with native, full-length proteins.

Knockout mice lacking the γ1-adaptin or the μ1a-adaptin genes die early in embryonic development (Meyer et al., 2000; Zizioli et al., 1999), although μ1a knockouts survive for longer, presumably because the μ1b subunit can substitute for μ1a in early development. The μ1a-knockout animals die at day 13.5 of embryonic development and show evidence of haemorrhage into the ventricles and the spinal canal. Interestingly, no AP-1 subunits are found in γ1 knockouts at all. Because the mRNA levels of the other subunits are not reduced, the remaining subunits must be unstable and degraded rapidly. By contrast, embryonic fibroblasts from μ1a-knockout animals contain trimeric complexes consisting of β1-adaptin, γ-adaptin and σ-adaptin. However, these complexes appear to be nonfunctional since no membrane-associated γ-adaptin could be observed.

What is the exact function of the AP-1 complex?

For a long time, AP-1-containing-clathrin-coated vesicles were thought to form at the TGN and transport mannose-6-phosphate receptors (MPRs), the sorting receptors for lysosomal hydrolases that contain mannose-6-phosphate residues (Hille-Rehfeld, 1995), to endosomes. Several observations supported this idea. Firstly, AP-1 colocalises with both MPRs on the TGN in clathrin-coated areas (Ahle et al., 1988; Klumperman et al., 1993). Secondly, the cation-independent MPR (CI-MPR) is found in membrane fractions that are highly enriched in clathrin-coated vesicles (Pauloin et al., 1999), and the cytoplasmic tails of MPRs interact with AP-1 in vitro (Dittié et al., 1999; Honing et al., 1997; Le Borgne et al., 1993). Manipulation of these tails results in the missorting of MPRs and lysosomal enzymes (Hille-Rehfeld, 1995). Finally, additional support for AP-1 acting at the TGN came when Honig et al. demonstrated that transport of LAMP-1 (lysosome-associated membrane protein 1) from the TGN to endosomes is abolished if an AP-1-interacting motif is mutated (Honing et al., 1996).

The first doubts that AP-1 indeed functions in anterograde transport appeared when Meyer et al. investigated the trafficking of MPRs in the μ1a-knockout mice (Meyer et al., 2000). If AP-1 mediates anterograde transport from the TGN to endosomes, one would expect that in AP-1 knockouts the MPRs would get stuck in the TGN. This is not the case, however: the MPRs exit the Golgi, get transported to the plasma membrane and are re-endocytosed from there,

accumulating in an early endosomal compartment that contains the early endosome marker EEA1. This indicated that AP-1 might mediate not anterograde, but retrograde, transport between endosomes and TGN. The observation that Shiga toxin[‡] co-localises with AP-1 on early/recycling endosomes during a 20°C block of retrograde transport and that toxin transport is inhibited by BFA supports this idea (Mallard et al., 1998). Recently this model gained further support when Bonafacino and co-workers suggested that the recently discovered GGA proteins mediate anterograde transport of MPRs and other transmembrane proteins that have acidic dileucine motifs (Puertollano et al., 2001a). However, it is not easy to reconcile the idea that AP-1 acts only in retrograde transport with its steady-state distribution concentrated at the TGN, and indeed AP-1 is involved in anterograde transport (see below).

GGAs

The GGAs (Golgi-associated, γ-ear-containing, ARF-binding proteins) comprise a group of proteins that are conserved from yeast to humans (Boman, 2001) and have been identified independently by several labs (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). In yeast, there are two different GGA proteins, whereas there are three isoforms in humans (GGA1 – GGA3), one isoform (GGA3) undergoing alternative splicing. The proteins are 60-75 kDa in size and share the same domain organisation (Fig. 1D): an N-terminal VHS domain (for Vps27p, Hrs, and STAM – the first three known proteins sharing this domain), a GAT (GGA and TOM1) domain, a variable hinge domain and a GAE (γ-ear-homology) domain at the C-terminus. Like the AP-1 γ-ear, the GAE domain of the GGAs binds to γ-synergin and rabaptin-5 (Hirst et al., 2000).

GGA function in *S. cerevisiae*

Yeast lacking GGA2 or both GGA proteins exhibit defects in protein sorting to the vacuole different from a class E phenotype (Costaguta et al., 2001; Hirst et al., 2001; Mullins and Bonifacino, 2001). In class E mutants, transport from the endosome to both the TGN and vacuole is inhibited, which produces enlarged endosomal structures that are proteolytically active (the class E compartment). In GGA mutants the vacuoles appear fragmented, and the yeast SNARE protein Pep12p, which is involved in transport from the TGN to late endosomes, is misrouted (Black and Pelham, 2000). These yeast cells show defects in α-factor processing, presumably because the enzyme that processes α-factor, Kex2p, is missorted from the TGN to the vacuole. The lysosomal enzyme CPY is missorted such that ~50% of the enzyme is secreted, and undergoes aberrant processing. However, Vps10p, the sorting receptor for CPY, is not missorted to the vacuole and is able to bind CPY in the TGN, which indicates the retrieval pathway is functional. Furthermore, the VHS domain (which binds ligands containing dileucine motifs) is needed for GGA function, and it cannot be substituted by the VHS domain of Vps27p (a homologue of Hrs[§], whose mutation produces a vacuolar protein sorting defect) (Hirst et al., 2001). The GAE domain is not essential for GGA function, although yeast expressing a truncated GGA protein, where the GAE domain is missing, show a weak impairment of CPY sorting and α-factor processing. This domain can be substituted by the γ-ear (Hirst et al., 2001).

The effect of GGA mutants on CPY sorting and α-factor processing resembles the phenotype of yeast expressing a temperature-sensitive mutant of clathrin under non-permissive temperature (Deloche et al., 2001) and thus was the first indication that GGAs might interact with clathrin. Indeed, GST pulldown and co-immunoprecipitation experiments have shown that clathrin and GGAs proteins interact and that a triple knockout of clathrin and the GGA proteins is either synthetically lethal or aggravates the phenotype, depending on the yeast background that is used (Costaguta et al., 2001; Hirst et al., 2001). Taken together, these results suggest that clathrin and GGAs act together in anterograde transport from the TGN to the vacuole.

The only adaptor complex that interacts with clathrin in yeast is AP-1. Disruption of any of the AP-1 subunits, which results in an absence of heterotetrameric complexes, gives no phenotype with respect to CPY sorting and α-factor processing; however, simultaneous knockout of AP-1 and GGA exacerbates the phenotype of GGA knockouts, enhancing the effect on α-factor processing more than the effect on CPY transport (Costaguta et al., 2001; Hirst et al., 2001). This result suggests that AP-1 and GGA proteins cooperate in anterograde transport from the TGN to the vacuole.

Several possible models could explain how AP-1 and GGA proteins might interact in yeast. One possibility is that they act in parallel clathrin-dependent pathways. In this case, two populations of clathrin-coated vesicles would bud from the TGN, one population that contains AP-1, and prefers Kex2p as cargo, and one that contains GGA proteins and prefers Vps10p as cargo, although it remains to be determined how Vps10p is recruited into clathrin-coated vesicles, since the cytoplasmic domain of Vps10p is dispensable for clathrin-dependent transport (Deloche et al., 2001). Such vesicles could have different destinations: AP-1-coated vesicles would be targeted to early endosomes, whereas GGA containing vesicles would travel to late endosomes (Black and Pelham, 2000). The model that they form distinct vesicles is consistent with the notion that GGA proteins and AP-1 show only limited co-localisation in mammalian cells (Dell'Angelica et al., 2000; Hirst et al., 2000). Another possibility is that GGA proteins and AP-1 are present in the same coated vesicles but recruit different types of cargo. This would be consistent with the finding that AP-1 and GGA proteins co-immunoprecipitate in yeast (Costaguta et al., 2001).

GGA function in mammalian cells

In mammalian cells, the GGA proteins localise to the TGN, partially colocalising with clathrin and AP-1 (Doray et al., 2002b); however, they do not co-purify with clathrin-coated vesicles (Hirst et al., 2000). All GGA proteins bind to activated ARF (Boman et al., 2000). ARF is responsible for their

[‡]Shiga toxin is a bacterial toxin from *Shigella dysenteriae*. Cell biologists use the B-subunit of the toxin as a tool to follow retrograde transport from the plasma membrane, where it becomes endocytosed and transported through endosomes and the Golgi apparatus to the ER.

[§]Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) is a substrate for activated tyrosine kinase receptors that is involved in endosomal membrane trafficking.

recruitment to trans-Golgi membranes, and accordingly, they are redistributed when cells are treated with BFA. The hinge region is the most variable domain amongst the GGA proteins, however, all hinge domains contain a clathrin-binding motif (Puertollano et al., 2001a; Zhu et al., 2001). GGA proteins bind to clathrin in vitro and colocalise with clathrin in coated areas of the TGN (Hirst et al., 2001).

Although the structures of the VHS domain of TOM1 (target of myb1) and Hrs have been determined (Mao et al., 2000; Misra et al., 2000), the function of this domain remained unknown for some time. Recently, it was shown that the VHS domain of the GGA proteins binds directly to the acidic dileucine motifs of CI-MPR and the cation-dependent (CD)-MPR (Puertollano et al., 2001a; Zhu et al., 2001), sortilin¶ (Nielsen et al., 2001) and LRP3 (Takatsu et al., 2001). The determination of the crystal structure of the complex demonstrated that, like the VHS domains of TOM1 and Hrs, that of the GGA proteins forms a right-handed superhelix consisting of eight α helices (Fig. 1D). The acidic dileucine motif binds to GGA in an extended conformation by electrostatic and hydrophobic interaction with helix 6 and helix 8 (Misra et al., 2002; Shiba et al., 2002). However, it remains to be determined whether other VHS domains bind at all to acidic dileucine motifs.

The mammalian GGA proteins are essential for the anterograde transport of MPRs from the TGN to the endosome, which is consistent with the observed interaction between the GGA VHS domain and the dileucine motif of MPRs. Remarkably, proteins that have similar dileucine sorting motifs, such as tyrosinase, LAMP-2 and the transferrin-receptor [for the full list of analysed proteins, see Puertollano et al. (Puertollano et al., 2001a)], do not bind to GGA proteins. Furthermore, the GGA VHS domain can not be substituted by VHS domains from other proteins, such as STAM1, Hrs, TOM and TOML1. This indicates a high degree of selectivity for the interaction of VHS domains with particular dileucine motifs. Site-directed mutagenesis of the MPRs revealed that the acidic cluster N-terminal of the dileucine-motif is essential for GGA binding. Furthermore, Misra et al. demonstrated that the dileucine motif must be located at the C-terminus of the protein and that there must be a spacing of two residues between the two leucine residues and the C-terminus for optimal binding (Misra et al., 2002). Thus, it came as a surprise when Dennes et al. demonstrated that the cytoplasmic tail of Vps10p binds to mammalian GGA proteins, because the dileucine motif is localised to the middle portion of the cytoplasmic tail (Dennes et al., 2002). While Dennes et al. did not use mutagenesis of the dileucine motif to demonstrate that the internal dileucine motif is required, they were able to show a chimeric protein consisting of the lumenal and transmembrane domains of CI-MPR and the cytoplasmic tail of Vps10p is sorted like wild-type CI-MPR and, in MPR-deficient cells, can rescue missorting of soluble lysosomal hydrolases with the same efficiency as wild-type CI-MPR.

When the N-terminal portion of a GGA protein that has the VHS and GAT domains, but lacks the clathrin-binding hinge domain and the GAE domain is expressed in mammalian cells, both MPRs accumulate in the TGN and clathrin is no longer detected on TGN membranes (Puertollano et al., 2001a). AP-1 localisation is unaltered if the expression of the GGA N-terminus is kept at moderate levels. These data suggest that GGA proteins mediate clathrin-dependent anterograde transport of MPRs from the TGN to endosomes, a function long attributed to AP-1. Time-lapse microscopy showing vesicles containing fluorescently labelled CD-MPR and GGA1 budding from the TGN provides further support for this hypothesis (Puertollano et al., 2001a). Given the work of Meyer et al. on MPR trafficking in AP-1-deficient mice (Meyer et al., 2000), clathrin-coated vesicles containing AP-1 might thus mediate retrograde trafficking from endosomes to the TGN, whereas clathrin-coated vesicles containing GGA proteins could mediate anterograde trafficking.

So far, however, in mammalian cells there is no evidence that GGA proteins are a stable component of clathrin-coated vesicles; instead they redistribute very quickly to the cytoplasm under conditions where AP-1 stays on the membrane (Hirst et al., 2001). This could be a preparation artefact, but it could also indicate that GGA proteins are not necessarily packaged into vesicles but rather help recruit coat components and cargo into a budding vesicle. New exciting data support this hypothesis, extending the data that demonstrate cooperation between AP-1 and GGA proteins in yeast (Costaguta et al., 2001), Doray et al. show that the GGA hinge region binds to the γ-ear of AP-1. This indicates that AP-1 and GGA proteins might interact and cooperate in the same sorting step (Doray et al., 2002b). In immunoelectron microscopic studies using cells stably transfected with GGA2, the authors demonstrate co-localisation of GGA2 and AP-1 on coated buds of the TGN. Moreover, mutant MPR that does not bind GGA proteins fails to enter AP-1-coated vesicles. Waguri et al. provide further support for AP-1-mediated transport of CI-MPR from the TGN in their recent study of fluorescently labeled CI-MPR and AP-1 in living cells. Their images show AP-1 and CI-MPR in tubules forming and detaching from the TGN and moving out towards the periphery of the cells (Waguri et al., 2003).

Additional circumstantial evidence suggests that GGA proteins regulate coat assembly rather than form a stoichiometric component of clathrin-coated vesicles. The GGA GAT domain is necessary and sufficient to target GGA proteins to the TGN. This domain binds ARFs and inhibits GAP-mediated GTPase activity of ARF, presumably because GGA proteins and GAPs compete for binding to the switch 2 domain in ARF (Puertollano et al., 2001b). GGA proteins might therefore provide a proof-reading mechanism by controlling the kinetics of ARF-mediated GTP hydrolysis, allowing activated ARF to be transiently stabilised on the membranes and thus recruit AP-1 and clathrin. In the absence of coat proteins, ARF would hydrolyse GTP quickly and recirculate into the cytoplasm. Indeed, when the GGA GAT domain is expressed at high levels, AP-1 redistributes to the cytosol, presumably because the GGA hinge (the AP-1 binding domain) is missing (Puertollano et al., 2001b). Although these data are consistent with the model that GGA proteins help recruit AP-1, an alternative explanation for this phenomenon is that the high GGA protein levels lock all ARF proteins onto the membrane. This would make it impossible for AP-1 to be recruited through simple competition for ARF-binding sites, although this is unlikely since AP-1 binds to the switch 1 domain (Austin et al., 2000). Such a model is, however,

¶Sortilin is considered the mammalian orthologue of Vps10p. However, the cytoplasmic tails do not share homology and the function of sortilin remains unknown to date.

consistent with the fact that AP-1 is redistributed only at very high GGA expression levels. It should be possible to distinguish between these two models by the following experiments. If GGA proteins and AP-1 compete for ARF-binding sites, then simultaneous overexpression of ARF should compensate for the GGA effect, and AP-1 should be recruited to the membrane. If GGA proteins help recruit AP-1, then overexpression of full-length GGA proteins should enhance recruitment of AP-1 to membranes.

Is retrograde transport AP-1 dependent?

Although the discovery of GGA proteins has expanded our understanding of how vesicles are generated at the TGN, equal progress towards elucidating the mechanism of retrograde transport from endosomes to the TGN has been made. Reversible modification of transmembrane receptors appears to determine their direction of transport by recruiting accessory proteins. It seems likely that, at least in some cases, AP-1 is also involved in these retrograde transport steps.

The protein TIP47 binds specifically to both CI-MPRs and CD-MPRs and is essential for their retrograde trafficking (Diaz and Pfeffer, 1998). TIP47 is a Rab9 effector, and Rab9 recruits TIP47 to endosomal membranes, where it then interacts with MPRs (Carroll et al., 2001). Both MPRs have cytoplasmic palmitoylation sites, which might affect TIP47 binding. So far, clathrin and AP-1 appear not to participate in this transport step.

Phosphorylation of transmembrane proteins also determines their direction of transport (Breuer et al., 1997; Jones et al., 1995; Méresse and Hoflack, 1993; Pitcher et al., 1999). Proteins such as furin** contain phosphorylation sites that are not part of a dileucine motif and thus act independently as sorting motifs. At steady state, furin is localised to the TGN, from where it recycles to and from endosomes (Jones et al., 1995). Phosphorylation of furin enhances recruitment of AP-1 to membranes, and mutation of the phosphorylation sites results in missorting of the protein (Dittié et al., 1997). PACS-1, a ubiquitous cytosolic protein identified in a two-hybrid screen for proteins that bind to the phosphorylated cytoplasmic tail of furin (Wan, 1998), facilitates retrograde transport from endosomes to the TGN in a phosphorylation-dependent manner. It can also bind to CI-MPR and importantly AP-1. Thus, a trimeric complex consisting of a cargo protein bound to AP-1 forms and is stabilised by PACS-1 (Crump et al., 2001). Further evidence for its involvement in retrograde transport comes from antisense experiments demonstrating that furin accumulates in endosomes in the absence of PACS-1. AP-1 thus probably mediates retrograde trafficking in cooperation with PACS-1. In such a model, phosphorylation of furin and CI-MPR would occur on endosomes and recruit PACS-1 and AP-1.

PACS-1-dependent and TIP47-dependent retrograde transport mechanisms need not to be mutually exclusive for two reasons. First, TIP47 seems to recognise specifically MPRs; thus other transmembrane proteins may be recognised by other proteins that regulate retrograde transport. Second, retrograde transport from different endosomal compartments may be regulated by different proteins: TIP47-mediated transport originates from late endosomal compartments, whereas PACS-1-mediated transport may start on early endosomes.

**Furin, the mammalian Kex2p orthologue, is a transmembrane endoprotease that catalyses the maturation of some secretory proteins, bacterial toxins and viral envelope proteins.

How does phosphorylation influence transport between trans-Golgi network and endosomes?

The PACS-1 experiments demonstrate that the phosphorylation of cytoplasmic tails leads to recruitment of additional factors that facilitate transport, but increasing evidence indicates that the phosphorylation of acidic dileucine motifs facilitates transport directly by increasing the affinity of cytoplasmic tails for adaptor complexes or GGA proteins. For example, binding of AP-1 and AP-2 to the T-cell co-receptor CD4 is enhanced when serine residues neighboring the dileucine motif of CD4 become phosphorylated. Pitcher et al. demonstrated by surface plasmon resonance studies that the affinity of CD4 peptides for AP-1 and AP-2 is enhanced up to 700-fold when CD4 is phosphorylated (Pitcher et al., 1999). Since these experiments were performed with synthetic peptides and purified adaptors, the enhanced affinity is unlikely to be mediated by additional proteins. Although the authors demonstrated that endocytosis of CD4 is facilitated by phosphorylation, it remains unknown what effect phosphorylation has on CD4 trafficking in the biosynthetic pathway.

Kato et al. have recently shown a direct increase in affinity of CI-MPR for GGA proteins when CI-MPR is phosphorylated: binding of the phosphorylated MPR was increased ~3 fold compared with the non-phosphorylated protein (Kato et al., 2002). The determination of the crystal structure of the complex confirmed that the increase is caused by electrostatic interaction of phosphoserine at position 2485 upstream of the dileucine motif of CI-MPR with Lys86 and Arg88 of GGA3. The kinase that performs these phosphorylation reactions is casein kinase 2 (CK2), a heterotetrameric protein (Pinna, 2002), that co-purifies with adaptor complexes (Doray et al., 2002b; Méresse et al., 1990). GGA1 and GGA3 have very recently been identified as CK2 substrates (Doray et al., 2002a). The GGA hinge region contains an acidic dileucine motif very similar to that in CI-MPR. When the GGA hinge becomes phosphorylated, the neighboring VHS domain binds to this motif, and the molecule undergoes intramolecular autoinhibition such that it cannot bind MPRs.

These two opposing effects of CK2 on GGA1/3-MPR interaction during coat recruitment seem paradoxical at first, and at the moment we can only speculate on how CK2 regulates the interaction between GGA1/3 and MPRs in vivo. One possibility is that one, or several, as-yet-unknown phosphatases act as a “timer” to ensure cargo recruitment into budding vesicles. In this scenario, non-phosphorylated GGA proteins would initially bind to phosphorylated MPRs, thus facilitating clathrin recruitment and allowing the vesicle to bud off the donor membrane. The activation of a phosphatase that dephosphorylates MPRs and the continued action of CK2 would then produce phosphorylated GGA proteins and non-phosphorylated MPRs, triggering the dissociation of the complex and possibly uncoating of the vesicle.

If GGA proteins cooperate with AP-1 during coat

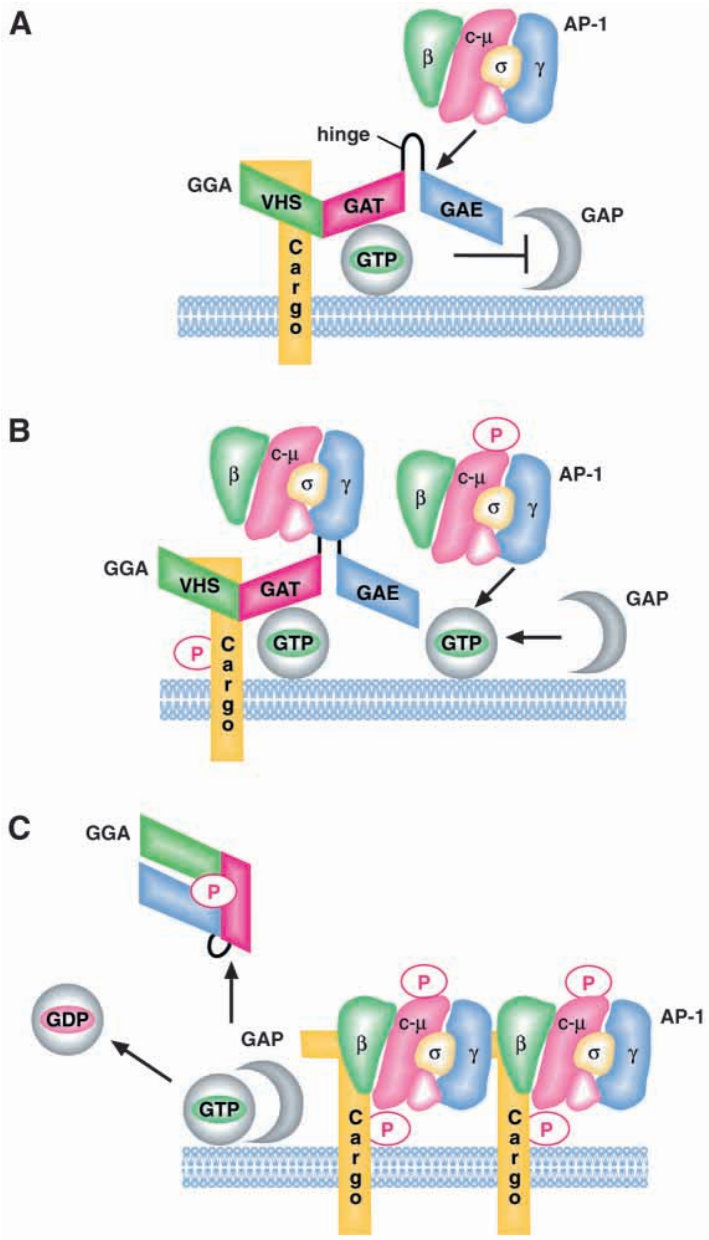

Fig. 2. Hypothetical model for the cooperation of GGA proteins and AP-1 during clathrin coat recruitment on the TGN. (A) Association of the GGA GAT domain with ARF-GTP causes an inhibition of the ARF-GAP and allows enhanced recruitment of AP-1. (B) During coat recruitment AP-1 interacts with both ARF-GTP and GGA. Phosphorylation of cargo by CK2 increases the interaction of the VHS domain of the GGAs, as well as AP-1 with cargo. (C) Subsequent phosphorylation of the GGA proteins allows dissociation of GGAs and activation of GAP activity. Budding of AP-1-coated vesicles then proceeds from the TGN. Please note, for simplicity, clathrin, the β-ears and γ-ears were omitted from the figure. Recently, it has been reported that μ1 and μ2 are phosphorylated by an adaptor-associated kinase called AAK1 (Ricotta et al., 2002). GAK (cyclin G-associated protein kinase) also has been shown to phopshorylate μ1 and μ2 (Umeda et al., 2000). Although it has been demonstrated that phosphorylation of μ2 by AAK1 fulfils a regulatory function in receptor-mediated endocytosis (Olusanya et al., 2001; Ricotta et al., 2002), the physiological significance of μ1 phosphorylation remains to be elucidated.

recruitment, however, then the phosphorylation of GGA proteins would explain why they are not part of clathrin-coated vesicles. The model shown in Fig. 2, which extends the model proposed by Kornfeld and co-workers (Zhu et al., 2001), is consistent with the hypothesis that GGA proteins help to recruit cargo into AP-1-containing clathrin-coated vesicles (Fig. 2). First, activated ARF recruits GGA proteins to TGN membranes. GGA inhibits the ARF GAP; thus ARF stays on the membrane for longer and increases the probability of recruiting AP-1 to this site. Meanwhile, GGA proteins recruit cargo proteins and clathrin. Together with AP-1, CK2 or a CK2-like enzyme is then recruited. CK2 phosphorylates the cargo protein as well as GGA proteins, subsequently causing the GGA proteins to dissociate and the cargo protein to bind AP-1. Budding then proceeds. As before, the action of one or more phosphatases is indispensable in this scenario.

Conclusions

It seems the main players for clathrin coat recruitment are now established: It will be exciting to learn how exactly they interact to generate a clathrin-coated vesicle. AP-1 appears to be involved in both anterograde and retrograde transport in mammalian cells. Indeed, Huang et al. have demonstrated by live-cell imaging that vesicles containing AP-1 can travel in both anterograde and retrograde directions (Huang et al., 2001). The picture emerging is that different accessory proteins are recruited to the sites of vesicle biogenesis, depending on the subcellular location. It remains to be determined how receptors "recognise" their current location, but reversible modifications such as phosphorylation or palmitoylation are likely to play a part.

Our understanding of GGA function has grown tremendously over the past two years. Although at first AP-1 and GGA proteins appeared to function at different transport steps in mammalian cells, it seems more likely now that in fact they cooperate. GGA proteins might even regulate anterograde, AP-1-mediated transport. Genetic manipulation of GGA proteins in multicellular organisms and the development of in vitro recruitment and budding assays for GGA proteins and AP-1 should clarify how they interact during clathrin coat recruitment.

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