these differences is the high sequence diversity in the acidic loop of dIII among the calpains. μ-calpain has three more acidic residues in the dIII loop relative to m-calpain; human calpain 3 has no acidic residue in this loop. As the central domain, dIII may play a major role in the Ca^{2+}-mediated activation of calpain[6,8]. It is conceivable that changes in dIII affect the catalytic and structural integrity of the catalytic dII, as well as that of the other Ca^{2+}-binding motifs in calpain. Further studies are needed to understand the variability of calpain's Ca^{2+}-sensitivity.

Another major unresolved question concerns the intracellular Ca^{2+} level, which is generally 1 μM at most, even in stimulated cells, and never reaches the high concentration range at which the calpains become active. Thus, it may be that other biological molecules such as protein inhibitors (calpastatin[11] and Gas-2[12]) as well as phospholipids[13] are required to modulate the Ca^{2+}-sensitivity of calpains. Recently, Tompa *et al.*[14] reported that the isolated C2-like dIII of classic calpains binds Ca^{2+} and that its affinity to Ca^{2+} is enhanced by the presence of di- and triphosphoinositide-containing liposomes. Furthermore, Ca^{2+}-binding to calpain promotes several processes: (i) translocation of the enzyme to the plasma membrane[15] (ii) autolysis in both the large and small calpain subunits[16,17] (Fig. 1*a*), and (iii) dissociation of the two subunits into truncated fragments[1]. Together these processes contribute to the biological activity of calpain in the cell, adding a complexity to the mechanism underlying the Ca^{2+}-dependent activation of calpains. The next challenge for calpain structural biology would be the determination of a membrane-bound, fully Ca^{2+}-activated enzyme.

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Single-handed recognition of a sorting traffic motif by the GGA proteins

Tom Kirchhausen

Selective transport of cargo between membrane-bound organelles is vital for the well-being of cells. The crystal structure of a short peptide signal from the cytoplasmic tail of the mannose-6-phosphate receptor bound to the VHS domain of GGA proteins gives hints to how sorting works.

In most cells, it takes an hour or less to recycle lipids and many transmembrane proteins between various cellular membranes. Vesicles and tubulo-vesicular carriers shuttle these components, but remarkably, the compositions of the various membrane compartments remain distinct (Fig. 1). This feat depends in part on the controlled formation of the vesicles and tubulo-vesicular structures that bud from the donor membrane, their movement inside the cell, and their targeting and fusion with the acceptor membrane. Much like the zip code addressing system for letters and packages, sorting at the beginning of the process ensures correct selection of cargo molecules for transport and delivery. Sorting of transmembrane proteins involves recognition of short peptide signals in their cytoplasmic tails by special cytosolic proteins, which function as adaptors to link the cargo and the coat machinery responsible for membrane deformation and budding[1].

Cellular processes that depend on accurate membrane traffic range from endocytosis of hormones, nutrients and viruses to protease secretion, antigen presentation and membrane recycling during neurotransmission. The bi-directional traffic along the secretory and endocytic pathways intersect at the interface between the trans Golgi network (TGN, a tubulo-vesicular network abutting the distal side of the characteristic Golgi membrane stacks) and the endosomal/lysosomal compartment (a tubulo-vesicular network that is more dispersed throughout the cells). One class of cargo proteins in this bi-directional traffic comprises the mannose-6-phosphate receptors (MPRs), transmembrane proteins that are essential for normal lysosomal function in mammalian cells. For example, these receptors recognize the mannose-6-phosphate groups on lysosomal hydrolases and are involved in transporting these enzymes from the TGN to endosomes. After making such a delivery, the receptors cycle back to the TGN for another round of traffic.

How is this trafficking pattern maintained? Enter the GGAs (Golgi-localized γ-ear-containing ARF binding proteins)[2–5]. Unknown until barely two years ago, GGAs are ubiquitous cytosolic proteins of 613–721 amino acids that cycle between the cytosol and the TGN and link clathrin to membrane-bound ARF–GTP. The three mammalian GGAs — GGA1, GGA2 and GGA3 — are responsible for the accurate trafficking of MPRs and

sortilin (a multifunctional receptor that binds lipoprotein lipase, neurotensin and receptor-associated protein) from the TGN to endosomes. In yeast, the GGAs are similarly responsible for the trafficking of carboxypeptidase Y and proteinase A from the Golgi to the vacuole[6–10]. The mammalian GGAs recognize an acidic-cluster-dileucine signal of the form $(-)_{-1}(D)_0X_1X_2L_3L_4X_5X_6$ (the subscripted numbers indicate positions relative to the acidic Asp residue; '–' is a negatively charged residue and X is any amino acid), present in the cytosolic tail of MPRs and necessary for accurate trafficking of MPRs from the TGN to endosomes.

GGAs are linear chains of four functional domains. The N-terminal VHS domain is responsible for the highly specific recognition of the acidic-cluster-dileucine motif. It is followed by the GGAH/GAT domain, a region of conserved sequence that binds to ARF1 and its small GTPase relatives in the GTP-bound form. Next comes a variable region that in the case of GGA1 and GGA3 contains clathrin-box motifs recognized by the N-terminal domain of clathrin. Finally, the C-terminus contains the AGEH/GAE domain, a region homologous to the C-terminal ear domain of the γ-adaptin subunit of the TGN clathrin adaptor AP-1. AP-1 is a tetrameric complex that also binds clathrin and is responsible for trafficking MPRs and other membrane proteins from the endosomes back to the trans Golgi network.

How do GGAs specifically recognize the sorting signals of their cargo? As reported in a recent issue of *Nature*[11,12], the crystal structures of the VHS domains of GGA1 and GGA3 in complex with peptides containing the acidic-cluster-dileucine sorting sequences reveal detailed interactions between these components and provide insights into the specificity of the sorting process.

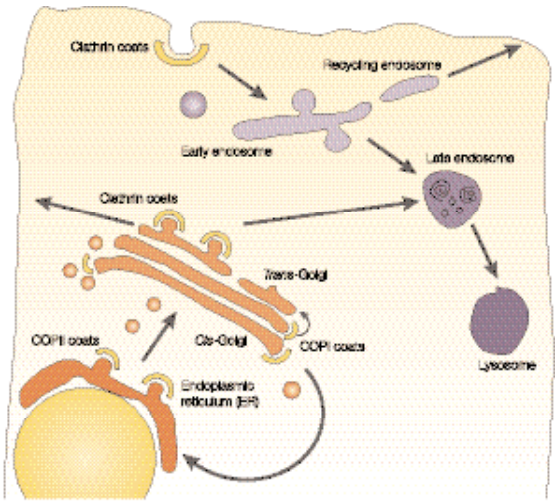

Fig. 1 The major routes that use vesicles and tubulo-vesicular carriers to traffic cargo molecules along the biosynthetic and endocytic pathways. The controlled formation of the vesicles and tubulo-vesicular carriers that bud from the donor membrane are key for the controlled trafficking. Sorting of the cargo molecules at the budding site requires their faithful recognition by cytosolic adaptors that act on given donor membranes. Figure reproduced with permission from ref. 19.

Sorting signal recognition

The VHS domains of GGA1 and GGA3 are composed of a right-handed super helix of eight α-helices (top insert of Fig. 2), which define convex and concave surfaces on the domain[11,12]. Although the new structures are very similar to the VHS domains of two other unrelated proteins, Hrs and Tom1, sequence comparison shows that residues along helix 6 are highly conserved among GGAs but not shared with other VHS domains. Moreover, residues in helices 1–5 and 7 are reasonably conserved among all known VHS domains, while high diversity is detected for residues in helix 8 that turn out to be important for the specific recognition of the acidic-cluster-dileucine motif.

The structures also show that the peptides containing the acidic-cluster-dileucine signals bind to the VHS domain in an extended conformation along a surface formed in the groove between helices 6 and 8. Binding of either peptide has little influence in the overall structure of the VHS domain. The structures of the cocrystals reveal that D_0 and L_3L_4 in the acidic-cluster-dileucine motif provide key contacts between the peptides and the VHS domain (Fig. 2). The side chain of D_0 forms a salt bridge with Lys 131 of GGA1 or Arg 130 of GGA3, while the side chains of L_3L_4 fit into two hydrophobic pockets in VHS. Residues –6 to –3 in the peptides are disordered and residues –2 to 0 bind near the N-terminus of helix 6 of VHS.

Why are these structural results interesting and important? They provide a molecular explanation for the specificity in the recognition of a subset of the acidic-cluster-dileucine motif. They show why the acidic-cluster-dileucine motif present in the MPRs is recognized by VHS in GGA1 and GGA3 but not by the VHS domain in other proteins, such as Hrs and TOM1 that have different functions. It also explains why not all acidic-cluster-dileucine motifs are sorted by GGAs (for example, in the cytosolic tails of TRP-1, LIMPII and tyrosinase, the acidic cluster is further upstream of the dileucine and does not interact with GGA1 and GGA3).

Common themes

The results in these two papers expand the notion that peptide-in-groove interactions are a widespread recognition mode for proteins involved in trafficking. For example, the clathrin N-terminal domain, a seven-bladed β-propeller, recognizes the pentapeptide LL(–)L(–) of the clathrin-box in β-adaptins, GGAs and other clathrin-binding proteins (bottom insert of Fig. 2)[13]. Tetrameric clathrin adaptors consisting of adaptin subunits recognize tyrosine-based sorting signals: the YppΦ (where p is a polar residue and Φ is a hydrophobic residue) endocytic motif in the cytosolic tail of various transmembrane proteins contacts an unpartnered β strand in μ2-adaptin of AP-2[14]. Through β-adaptins, adaptor proteins (APs) also recognize the dileucine motif of the form (–)XXXLL, but the structural details for recognition remain to be determined[15]. Other examples of extended peptide-to-surface interactions are found in the contacts between proteins containing tetratricopeptide repeats, such as those in the PEX5 peroxisomal receptor or in the Hop protein adaptor, and the relatively short recognition sequences in the peroxisomal targeting signal PTS1 or the C-terminus of Hsp70[16,17]. Another common theme in these systems is that the

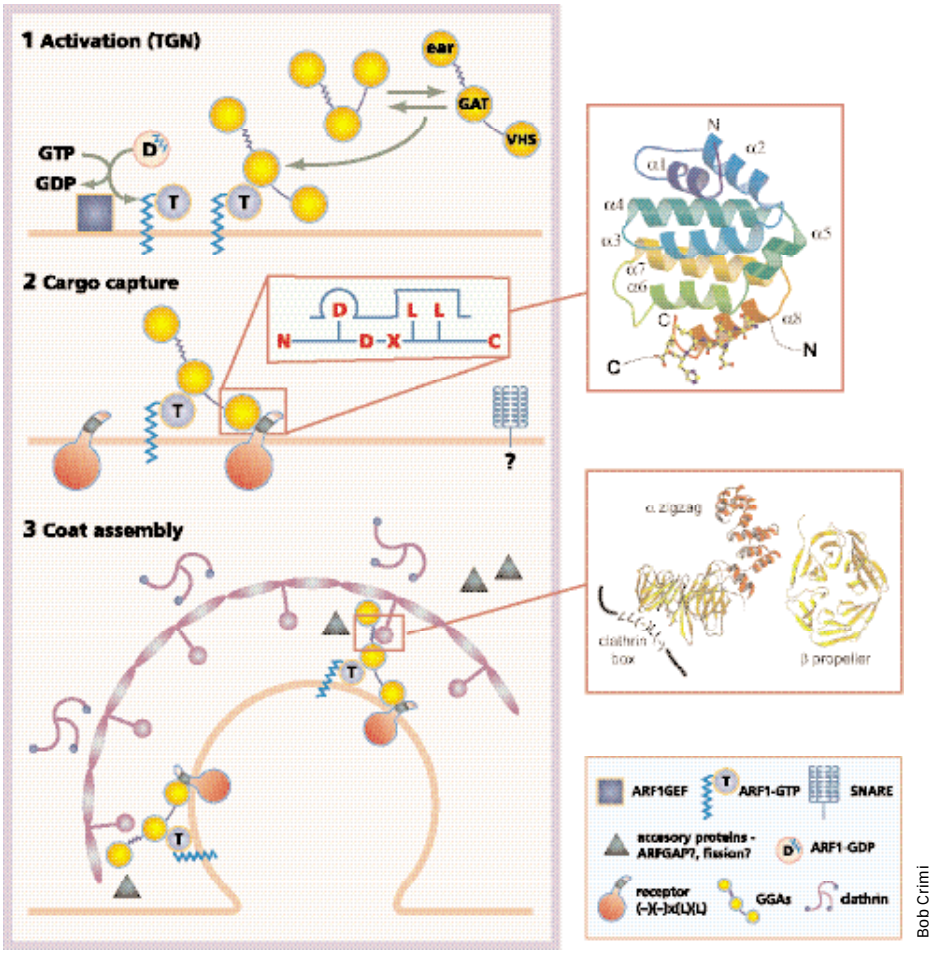

Fig. 2 GGAs as adaptors that link coat formation with cargo sorting. At the trans Golgi network, association with ARF1 bound to GTP initiates GGA recruitment (activation). The cytosolic tails of transmembrane proteins such as the mannose-6-phosphate receptor and sortilin have a short peptide motif containing an acidic-cluster-dileucine sequence that is specifically recognized by the VHS N-terminal domain of GGA1 and GGA3 (cargo capture). It is not clear whether binding of GGAs to ARF1–GTP induces a conformational change in the GGAs to facilitate cargo recognition. Coat assembly proceeds by recruitment of clathrin. The insets show views according to the current understanding at atomic level of the interaction between clathrin and its adaptors and between GGAs and cargo. In both cases, the motifs are presumed to be disordered until they are recognized by their receptors.

recognition sequences are usually in regions that appear to be disordered but acquire order upon binding to their receptors.

What is so special about the GGAs? Clathrin adaptors AP-1, AP-2 and AP-3 are the first proteins shown to have a role in cargo sorting, linking clathrin to membrane-bound proteins[18]. These adaptors are heterotetrameric complexes, and each one of their subunits contributes in different ways to the various roles of APs. The GGAs are interesting because they perform similar functions to the tetrameric APs, but they do so while providing all (or most) adaptor roles within a single polypeptide chain. Both classes of adaptors cycle between the cytosol, where they are presumably inactive, and their target membrane, where they are involved in cargo selection and linkage of clathrin to the membrane. How exactly this process works is still a matter of intense study, although for GGAs (in the TGN) and AP-1 (in the endosomal membranes), presence of the ARF family of G proteins is required for the membrane recruitment of GGAs and AP-1.

How do GGAs and APs coordinate the sorting process? Both GGAs and AP-1 bind other proteins by their γ-ear domains, but the significance of these interactions in the sorting mechanism remains to be deciphered. A common feature to both classes of adaptors, however, is that many of the interactions with their effectors occur through contacts of relatively low affinity, presumably reflecting the need to engage and disengage in rapid cycles (a few seconds or less). The order of events for cargo selection and coat formation (Fig. 2) is not clear, and GGAs may be recruited to the TGN membrane by binding to membrane-bound ARF–GTP. An increase in GGA concentration on the cytosolic side of the TGN membrane may restrict their three-dimensional movement, thereby facilitating their association with available acid-cluster-dileucine motifs of their cargo, such as MPR and sortilin that are already in the TGN. Clathrin may then be recruited by the membrane bound GGAs, with further associations stabilized through clathrin–clathrin contacts (leading to assembly of the clathrin coat) as well as incorporation of further GGAs, ARF and cargo (Fig. 2). One or more of the accessory proteins interacting with the C-terminal ear domain of GGAs could be involved in the recruitment of other proteins required for membrane fission and traffic.

In a variant of this model, we might imagine that the interaction of membrane-bound ARF–GTP with GGAs induces a conformational change in GGAs, which facilitates specific recognition between the sorting signal and the VHS domain of GGAs, as well as that between the clathrin box of GGAs and the N-terminal domain of clathrin. In this case, ARF–GTP would be the 'activator' of GGAs . In either case, the challenge now is to figure out how the GGA proteins manage to coordinate all these interactions that seem to occur simultaneously in time and space, to do it in such a way that no error in the recruitment to the correct membrane is made, and to ensure that proper sorting and budding is achieved. The linear domain organization of GGAs provides a wonderful opportunity to help tease apart the process from a structural point of view.

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Does Dengue virus fuse using β-barrels?

Dengue virus is transmitted to humans by mosquitoes, and there are more than 50 million infections each year. Typical symptoms include severe joint pain and fever, but serious complications such as hemorrhagic fever and Dengue shock syndrome sometimes occur following infection. Fortunately, less than 0.1% of Dengue infections are fatal, but it is still a major concern for health officials around the world.

The structure of Dengue virus at 24 Å resolution is now available and has been analyzed in the context of previous crystallographic work on one viral protein (Kuhn, R.J. *et al. Cell* 108, 717–725; 2002). Kuhn and coworkers embarked on this study to understand how the virus is organized and to obtain clues about how it enters host cells. While much is known about how some viruses (such as HIV) fuse to target membranes, the mechanism of Dengue virus fusion is thought to be quite different.

Kuhn and colleagues used cryoelectron microscopy to solve the structure of one Dengue strain at neutral pH, the pH at which viral particles do not fuse to membranes. The structure that emerges resembles an onion, with five distinct sheets visible in the electron density — two outer protein shells (dark and light blue), a lipid bilayer (green), a nucleocapsid shell (orange), and finally, the RNA genome (red) packaged in the center of the virus. They focus attention on the structure of the outermost shell, the layer that first contacts target membranes.

E glycoprotein is the viral component that mediates fusion to membranes. The crystallographic structure of an E glycoprotein from a related virus is known, and it contains a large amount of β-structure. Kuhn and colleagues fit this atomic structure into the electron density of the outermost shell of Dengue virus, revealing a closely packed array of E glycoproteins. They propose that, upon exposure to low pH, parts of the E glycoproteins change conformation and form closed β-barrels.

In other proteins, such as porins, β-barrels allow membrane spanning. By the same token, β-barrels on the surface of Dengue virus might be capable of inserting into target membranes, thereby promoting viral fusion. This proposed mechanism is distinct from the fusion system used by HIV, which relies instead upon a spring-like α-helical coiled coil.

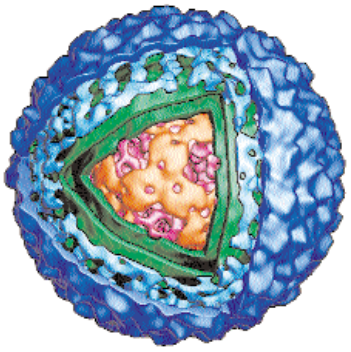

Figure kindly provided by Wei Zhang

Dengue virus is spreading, and there is no vaccine. It used to be confined primarily to Southeast Asia but is now common in South and Central America. Moreover, within the last six months, outbreaks have occurred in both Brazil and Hawaii. Now more is learned about the potentially novel fusion apparatus of this virus, perhaps new drugs to hamper it could be developed — just as fusion inhibitors that target the coiled coil mechanism of HIV are now being tested.

Tracy Smith