GRAF1-dependent endocytosis

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Abstract

The role of endocytosis in controlling a multitude of cell biological events is well established. Molecular and mechanistic characterization of endocytosis has predominantly focused on CME (clathrin-mediated endocytosis), although many other endocytic pathways have been described. It was recently shown that the BAR (Bin/amphiphysin/Rvs) and Rho GAP (GTPase-activating protein) domain-containing protein GRAF1 (GTPase regulator associated with focal adhesion kinase-1) is found on prevalent, pleiomorphic endocytic membranes, and is essential for the major, clathrin-independent endocytic pathway that these membranes mediate. This pathway is characterized by its ability to internalize GPI (glycosylphosphatidylinositol)-anchored proteins, bacterial toxins and large amounts of extracellular fluid. These membrane carriers are highly dynamic and associated with the activity of the small G-protein Cdc42 (cell division cycle 42). In the present paper, we review the role of GRAF1 in this CLIC (clathrin-independent carrier)/GEEC (GPI-anchored protein-enriched early endocytic compartment) endocytic pathway and discuss the current understanding regarding how this multidomain protein functions at the interface between membrane sculpting, small G-protein signalling and endocytosis.

Introduction

Endocytosis describes the process by which transmembrane and membrane-associated proteins and their ligands, extracellular fluid with nutrients, and plasma membrane lipids, are internalized by a cell. In so doing, endocytosis regulates the concentration of lipids and proteins on the cell surface (and therefore cell–environment interactions), as well as creating novel internal vesicles (endosomes) from which signalling events can occur and which are subsequently trafficked to other regions of the cell to which they deliver their contents by fusing with other membrane-bound compartments. Cells are specialized for different functions, and endocytosis plays distinct roles in each cell type, being coupled with countless processes including neurotransmission, cell–cell and cell–matrix adhesion, cell morphology changes, growth factor signalling and mitosis. As well as regulating these essential cell physiological phenomena, endocytic pathways can also be hijacked by pathogens and toxins. Endocytosis necessarily requires the production of highly curved membranes from the flat plasma membrane, and cellular proteins mediate this membrane sculpting by a variety of mechanisms (see [1] for a review). The best-studied endocytic pathway is CME (clathrin-mediated endocytosis), where transmembrane receptors have cytosolic motifs (or bind proteins that function analogously) that recruit a large network of proteins that stimulate the endocytic process, resulting in the formation of a densely coated, highly curved CCV (clathrin-coated vesicle). However, it is also evident that many other internalized proteins, including GPI (glycosylphosphatidylinositol)-anchored proteins, use distinct, clathrin-independent endocytic pathways, whereas some exogenous proteins used as endocytic pathway probes, such as CTxB (cholera toxin B subunit), enter cells via various pathways. Many clathrin-independent endocytic pathways have been described (see [2,3] for extensive reviews of these pathways and their cell biological functions). These include a highly prevalent pathway involving pleiomorphic CLICs (clathrin-independent carriers) that deliver material to GEECs (GPI-anchored protein-enriched early endosomal compartments), the so-called CLIC/GEEC pathway that is the focus of the present review. It appears that clathrin-independent pathways have similar lipid requirements and function independently of rigid protein networks such as those found around CCVs. Instead, these pathways have reliance on particular protein kinases, small G-proteins and associated effector proteins, implying the involvement of a more dynamic protein assembly and endosomal generation process.

Small G-proteins and endocytic membrane carrier formation

Small G-proteins of the Rho, Arf (ADP-ribosylation factor) and Rab families are associated with membrane compartments via post-translational modifications and loading of GTP. They signal via a variety of effector molecules to

Key words: Bin/amphiphysin/Rvs domain (BAR domain), clathrin-independent endocytosis, glycosylphosphatidylinositol-linked protein (GPI-linked protein), GTPase regulator associated with focal adhesion kinase-1 (GRAF1), oligophrenin, small G-protein.

Abbreviations used: Arf, ADP-ribosylation factor; BAR, Bin/amphiphysin/Rvs; CCV, clathrin-coated vesicle; Cdc42, cell division cycle 42; CLIC, clathrin-independent carrier; CME, clathrin-mediated endocytosis; Cdc42, chelera toxin B subunit; FAK, focal adhesion kinase; GAP, GTPase-activating protein; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; GEEC, GPI-anchored protein-enriched early endosomal compartment; GEF, guanine nucleotide-exchange factor; GRAF, GTPase regulator associated with FAK-1; N-WASP, neuronal Wiskott–Aldrich syndrome protein; PH, pleckstrin homology; SH3, src homology 3; SNX9, sorting nexin 9.

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direct and co-ordinate events such as actin polymerization and membrane remodelling, thus acting as master regulators to facilitate processes such as membrane protrusion and invagination, cell movement and adhesion. Cycles of GTP loading (turning them ‘on’) and hydrolysis (turning them ‘off’) by these proteins are in turn regulated by GEFs (guanine-nucleotide-exchange factors) and GAPs (GTPase-activating proteins) (see [4,5] for reviews). Cycles of GTP hydrolysis seem to be important for the progression and maturation of individual cellular processes and function as checkpoints. It is striking that particular small G-proteins have been characterized as regulators of clathrin-independent endocytic pathways [2]. This implies that these endocytic pathways are likely to be closely coupled with other cellular processes controlled by small G-proteins and that endocytosis and these processes require close co-ordination. Membrane nanodomains enriched in certain lipids (including cholesterol) are not only known to be essential for clathrin-independent endocytic pathways but also for receptor clustering and Rho family small G-protein localization. Formation of such domains would therefore be an ideal way to sense the cellular milieu and to concomitantly transmit signals to stimulate an endocytic apparatus and one or more small G-protein (and their effector molecules). Both RhoA and Rac1 have been suggested to play a role in the uptake of the interleukin-2 receptor [6,7], whereas Cdc42 (cell division cycle 42) is necessary for the uptake of GPI-linked proteins, CTxB and the Helicobacter pylori VacA (vacuolating cytotoxic A) toxin via the CLIC/GEEC pathway [8–10] (see below). Proteins of the Arf family have the ability to induce and sense high membrane curvature in their GTP-bound state in addition to recruiting effector molecules [11,12]. Not surprisingly therefore, these proteins are found to play important roles in several membrane trafficking events in the cell, including endocytosis. Arf6 has been shown to be required for uptake of the MHC class I receptor via a tubular, clathrin-independent pathway [13], whereas Arf1 is involved in the distinct CLIC/GEEC pathway [14].

The multidomain GRAF [GTPase regulator associated with FAK-1 (focal adhesion kinase-1)] protein family

GRAF1 belongs to a subfamily of GAPs whose members share a high degree of sequence identity and similar domain composition and organization [with BAR (Bin/amphiphysin/Rvs), PH (pleckstrin homology), GAP and SH3 (Src homology 3) domains] (Figure 1A). Proteins of this family include GRAF1–GRAF3 and oligophrenin (which lacks the SH3 domain present in other family members). It is interesting to note that normal GRAF1 and oligophrenin function is required for health. Oligophrenin mutations are frequently found in patients suffering from X-linked mental retardation [15], whereas GRAF1 appears to act as a tumour suppressor in leucocytes, where deletions, truncations and mutations in both alleles have been found associated with acute myeloid leukaemia and myelodysplastic syndrome [16,17]. The G:C-rich promoter of GRAF1 is ordinarily unmethylated, but approx. 38% of biopsies of bone marrow from patients with these conditions exhibit GRAF1 promoter methylation, which is associated with reduced protein expression [18].

Proteins of the GRAF family are able to stimulate the GTPase activity of proteins of the Rho family of small G-proteins. This activity has been most extensively studied in GRAF1 where the crystal structure of the GAP domain has been solved [19,20] (see Figure 1B for a structural model of GRAF1). In vitro studies demonstrated that the GAP domain of GRAF1 is more active against Cdc42 and RhoA than Rac1, and in vivo the overexpression of GRAF1 resulted in cell morphology changes that mimic those seen when RhoA activity is inhibited [20–22]. BAR modules, which form through BAR domain dimerization to produce a highly curved membrane-interacting module, are often found in conjunction with other lipid-binding regions. The N-terminal BAR and PH domains of proteins in the GRAF family localize these proteins to lipid membranes and work together to generate and sense membrane curvature in vitro [23], suggesting that they act similarly in this respect to other BAR-domain-containing proteins (see Figure 1A, inset). The BAR and PH domains of GRAF family proteins appear to work in concert since the individual domains, or proteins harbouring specific point mutants in either domain, are severely impaired in their abilities to bind membranes and to localize appropriately in cells. This type of co-operation has been shown for similar proteins with adjacent membrane-binding domains [24], and bestows on GRAF family members the ability to detect the coincidence in cells of a highly curved membrane and the presence of the plasma membrane-enriched phosphoinositide PtdIns(4,5)P2. Interestingly, it has been shown that the GAP domains of GRAF1 and oligophrenin are autoinhibited by their BAR and PH domains. This inhibition is due to a direct interaction between these protein regions and is independent of membrane binding of the BAR and PH domains [25]. GRAF1 was originally identified as an interactor of FAK, which binds GRAF1’s SH3 domain [21]. Later, it was shown that this domain also, and more strongly, interacts with the membrane scission protein dynamin with which GRAF1 forms a tight complex, suggesting that these proteins may function together in membrane sculpting processes in vivo [23]. In summary, by virtue of their domain structures, GRAF family members are perfectly designed to work at the interface between small G-protein regulation and plasma membrane remodelling.

**Highly dynamic GRAF1-positive tubulovesicular membranes are required for a high-volume constitutive endocytic pathway**

The localization of endogenous GRAF1 in fibroblasts is striking. GRAF1 is found on pleiomorphic tubular and punctate membrane structures that are easily obliterated by cold fixation (probably hampering their discovery until recently) [23].
Figure 1 | Role of the multidomain protein GRAF1

(A) Schematic representation of the domain structure in GRAF1 with the function of each domain indicated in text. ‘PIP2’ and ‘curvature’ indicate PtdIns(4,5)P2-containing and curved membranes respectively. The inset shows the extensive membrane tubular network generated in a cell overexpressing the membrane-remodelling region of GRAF1 as a GFP–BAR+PH protein. (B) Structural model of dimeric GRAF1 with the crystallized domains depicted in ribbon representation with their corresponding molecular modelling database (mmdb) numbers. Domains whose structures remain unsolved (BAR and PH) are depicted as cross-hatched replicas patterned from the crystal structures of SNX9 (BAR) and ARAP (Arf GAP with Rho GAP, ankyrin repeats and PH domains) (PH). (C) Model of the endocytic uptake of fluid, GPI-linked proteins and CTxB via a GRAF1-mediated pathway. Receptors and toxins are clustered in nanodomains of the plasma membrane that are recognized by the small G-proteins Arf1 and Cdc42 inside the cell. Subsequently, GRAF1 is recruited to the membrane bud, which results in the formation of a membrane tubule that is eventually released from the plasma membrane via a scission reaction.

These structures have been shown to co-localize with a large amount of endocytosed dextran and yet be devoid of known markers for CME events, suggesting that they are involved in clathrin-independent endocytosis. Indeed, depletion of GRAF1 resulted in a major reduction in fluid-phase uptake in unstimulated HeLa cells to an extent similar to that produced by blocking CME. GRAF1-positive tubules contained endocytosed material after short incubation times, indicating that they are of an early endocytic nature. Overexpressed wild-type GRAF1 showed a similar localization pattern and allowed the visualization of these structures in living cells, where they were shown to be highly dynamic membranes and also capable of internalizing CTxB and a model GPI-linked protein [GFP (green fluorescent protein)–GPI]. In contrast, a truncated version of GRAF1 (comprising the lipid-binding BAR and PH domains but lacking the C-terminal GAP and SH3 domains) localized to much more static tubules and puncta and inhibited the uptake of both CTxB and GFP–GPI. The surface label of these markers co-localized with truncated GRAF1 for long periods, suggesting that this protein functions as a dominant-negative mutant by stabilizing static, non-functional carrier intermediates at the cell surface.

Role of GRAF1 in the CLIC/GEEC endocytic pathway

While these studies of GRAF1 highlighted the nature and extent of a highly prevalent, pleiomorphic, clathrin-independent endocytic pathway using fluorescence
microscopy approaches, electron microscopy approaches had previously identified the CLIC/GEEC endocytic pathway to be a major source of fluid-phase uptake [8]. These CLICs are morphologically distinct from both CME intermediates and caveolae in that they have a tubular and ring-like appearance. Molecules such as GPI-linked receptors and CTxB are internalized into these membrane structures, whose production is dependent on activity of the small G-proteins Cdc42 and Arf1 [8,10,14]. However, the lack of specific proteins known to be involved in the generation of CLICs had hampered the further characterization of this pathway. Since the morphological features and cargo specificity described for CLICs also apply to GRAF1-dependent endocytosis, we propose that GRAF1 is the first non-cargo marker and membrane remodelling protein described for the CLIC pathway. As mentioned above, GRAF1 has the ability to localize to membrane tubular structures via its BAR and PH domains, and to regulate the activity of both Cdc42 and RhoA. Using electron microscopy, GRAF1-positive carriers in cells were confirmed to contain endocytosed cargo and shown to be approx. 40 nm in diameter [23]. Interestingly, the BAR and PH domains of GRAF1 work together to sculpt spherical liposomes to 40 nm diameter tubules in vitro, suggesting that its membrane curvature modulating ability exists similarly in vivo. Furthermore, GRAF1 was found to co-localize with dominant-active Cdc42 at discrete structures at the cell surface, suggesting that it functions together with this small G-protein in endocytic membrane manufacture.

The close interactions between cell membranes and cytoskeletal elements have been extensively reviewed elsewhere [26]. In addition to having likely roles in promoting CLIC membrane manufacture, the actin- and microtubule-based cytoskeletons are expected to be central highways upon which CLIC/GEEC membranes traffic. It has been shown that both N-WASP (neuronal Wiskott–Aldrich syndrome protein; which is activated by Cdc42 and promotes actin polymerization) and actin are involved in the uptake of GPI-linked proteins [27]. Further, the membrane remodelling protein SNX9 (sorting nexin 9), which interacts with N-WASP, has been suggested to participate in clathrin-independent endocytosis [28] (alongside its canonical role in CME). While the GRAF family member oligophrenin interacts with actin directly (via its C-terminus) [29], GRAF1 does not bind directly to actin but participates indirectly in this process via regulation of RhoA/Cdc42 activity. Other proteins that play roles in the CLIC/GEEC pathway include Arf1, and its effector ARHGAP10, which is a GAP for Cdc42 [14]. These observations highlight the importance of GEFs and GAPs in the regulation of membrane transport and it is likely that many similar types of proteins are intimately involved in carrier formation and processing, and that they might be used differentially in distinct cell types. Further work, using live cell microscopy to visualize the recruitment and activation of small G-proteins (including Cdc42 and Arf1) and their modulators (including GRAF1 and ARHGAP10), will be necessary to elucidate how these proteins function spatiotemporally in the CLIC/GEEC endocytic pathway.

Interestingly, CLIC formation and Cdc42 activation have been shown to depend on the presence of cholesterol in the plasma membrane [27], suggesting further that membrane nanodomains are somehow integral to the coupling of small G-protein activity and endocytosis. Flotillins are membrane proteins that are proposed to form a hairpin in the plasma membrane similarly to caveolins, are found highly enriched in cholesterol-rich membranes and are proposed to function in CTxB endocytosis [30]. Although tubules decorated with wild-type GRAF1 did not accumulate flotillin1, expression of dominant-negative GRAF1 stabilized early endocytic tubules that were able to mix with flotillin1–positive membrane regions [23]. This suggests that flotillin1 may act upstream of GRAF1 in specifying domains to be internalized into CLICs, or simply that similar types of membrane regions are involved in these trafficking processes but that they are not interdependent. These questions are currently being answered using siRNA (small interfering RNA) approaches.

Little is known about the driving force for membrane deformation and fission during CLIC generation. GRAF1 has the ability to generate membrane curvature via its BAR domain, but compared with other BAR-containing proteins it is relatively inefficient in this respect. It might be that GRAF1 functions more as a sensor of curvature and thereby localizes to membrane tubules (produced by other proteins) and functions to stabilize their high curvature. It will be necessary to identify the lipids and proteins that collaborate in this process in order to understand more about the mechanism of how carriers form, and perhaps Arf1 acts upstream in the CLIC/GEEC pathway, generating high membrane curvature, which is then stabilized by GRAF1 (Figure 1C). CLIC/GEEC pathway carriers form even in the presence of a mutated dynamin protein that blocks CME. However, it is highly likely that dynamin participates in CLIC/GEEC endocytosis but may not be absolutely required for membrane fission as it is with CME. GRAF1 forms a complex together with dynamin, which suggests that these proteins function together at some stage of membrane carrier processing. The identification of the specific dynamin isoform involved in the CLIC/GEEC pathway will allow further characterization of its role. CLICs mature into GEECs that can eventually fuse with early endosomes in a Rab5-dependent manner, although it is likely that (perhaps most of) the CLIC/GEEC membranes can also traffic directly to perinuclear compartments such as the Golgi apparatus. The sorting and processing of lipids and proteins in these steps will also require an additional arsenal of regulatory proteins that remain to be identified.

**Concluding remarks**

While the molecular and mechanistic characterization of clathrin-independent endocytic pathways is only beginning, recent progress has been made on defining one such pathway, namely the CLIC/GEEC pathway. This pathway is a major mediator of fluid-phase and GPI-linked protein uptake and
can be hijacked by cholera toxin. This pathway requires the function of the membrane sculpting, and small G-protein modulator, GRAF1, which lines its highly curved membrane intermediates, as well as the small G-proteins Cdc42, Arf1 and the ArfGAP ARHGAP10. Further work will identify how these proteins function together in endocytic membrane production, identify other endogenous and exogenous cargoes of the CLIC/GEEC pathway, and determine the cell biological functions of this exciting endocytic portal.

Acknowledgements

We thank Harvey McMahon (MRC Laboratory of Molecular Biology, Cambridge, U.K.) in whose laboratory we had the privilege of performing experiments that led to this review.

Funding

We thank the Swedish Research Council, Swedish Cancer Society and Umeå University for funding.

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Received 16 April 2009
doi:10.1042/BST0371061

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