FEBS Letters 369 (1995) 84–88 FEBS 15692

Minireview

Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles

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Received 18 May 1995

Abstract Heterotrimeric G proteins are involved in hormonal signal transduction across the plasma membrane. Recent evidence suggests that they have a role in vesicular protein transport as well. Biochemical probes that interfere with the classical G protein cycle have been applied to the field of intracellular membrane transport to study their mechanism of action. Evidence has been obtained that intracellular G proteins act both through classical and alternative G protein cycles.

Key words: Heterotrimeric GTP binding protein; Vesicular trafficking; Signal transduction; Golgi apparatus; Secretory pathway; Endocytic pathway

1. Classical heterotrimeric G protein cycle at the plasma membrane

Many hormones and neurotransmitters elicit cellular responses by binding to cell-surface receptors. Most of these receptors at the plasma membrane have a common feature in that they contain seven membrane spanning domains (reviewed in [1,2]). Stimulation of these receptors results in the activation of guanine nucleotide-binding regulatory proteins (G proteins) [3–5]. The proteins of this family have a heterotrimeric structure; The α subunit that binds and hydrolyses GTP, and a β-and γ-subunit which form a stable $\beta\gamma$-dimer. The binding of an agonist to an appropriate receptor catalyzes the exchange of GDP for GTP on the G protein α subunit (Fig. 1). This is followed by dissociation of GTP-bound G_α from the $\beta\gamma$ dimer. Both the activated α subunit and the $\beta\gamma$ subunit can interact with effectors and modulate their activity [6]. Hydrolysis of GTP by the α subunit returns the protein into its basal conformation and the subunits re-associate. Regulatory mechanisms are superimposed on this very general scheme which allows either a very specific or a pleiotypic response to agonist stimulation [5].

Several biochemical probes are available which interfere with the G protein cycle and can be used to study the role of G protein-coupled signal transduction cascades in cellular pathways. Fig. 1 summarizes some of these modulators and their proposed mechanism of action. For discussions on the precise mechanism of action of these modulators the reader is referred to the references. GTPγS, a non-hydrolyzable analogue of GTP can bind and thus activate all GTP binding proteins. In contrast, AlF_4^- only activates heterotrimeric G proteins [7]. Other probes such as G protein derived peptides, Pertussis toxin and mastoparan (and analogues) interfere with coupling of a receptor to the C-terminus of a heterotrimeric G protein. Pertussis toxin (PTX) catalyzed ADP-ribosylation of a cysteine within the C-terminus of G_i or G_o [8] and synthetic C-terminal G_α peptides [9,10] interfere with the binding of a G protein to its receptor (uncoupling) and thus prevent G protein activation. Peptides corresponding to specific regions of the cytoplasmic domains of receptors appear to be able to activate G proteins [11]. Mastoparan, a peptide toxin from wasp venom presumably mimics this cytosolic loop and interacts with the C-terminus of a G_i/G_o protein [12,13] resulting in its activation. A logical consequence is that the effect of mastoparan can be reversed by PTX treatment of G proteins. Since AlF_4^- directly activates heterotrimeric G proteins without nucleotide exchange, bypassing the requirement for a receptor to stimulate GDP release from G proteins, AlF_4^- can still activate G proteins that are uncoupled from their receptor by e.g. ADP-ribosylation with PTX.

In addition, peptides affecting the interaction of G proteins with specific effectors have been developed [14]. These peptides are difficult to design, however, since effectors can interact with various regions of the G protein [15,16]. Recently, purified G_α and $\beta\gamma$ subunits as well as overexpression of constitutively active mutants of G_α have been used to demonstrate their involvement in signaltransduction cascades [17,18].

2. Heterotrimeric G proteins at intracellular organelles

Although the classical signaltransduction cascade as described in Fig. 1 operates at the plasma membrane, it has been known for some time that heterotrimeric G proteins are found on intracellular membranes as well. Pertussis-toxin sensitive G proteins have been detected on membranes of secretory granules [19], the endoplasmic reticulum [20,21], endosomes [22] and the Golgi complex [23].

The localization of G proteins to subcellular organelles raises the interesting question whether intracellular G proteins function within signaltransduction cascades similar to those at the plasma membrane or whether alternative, as yet undefined roles for heterotrimeric G proteins exist. For example, most of the receptors containing seven membrane spanning domains that couple to G proteins are found at the plasma membrane [1,2]. If intracellular G proteins do not participate in the classical signalling cascade, one could expect that probes, affecting the interaction between G proteins and these receptors (Fig. 1)

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SSDI 0014-5793(95)00620-6

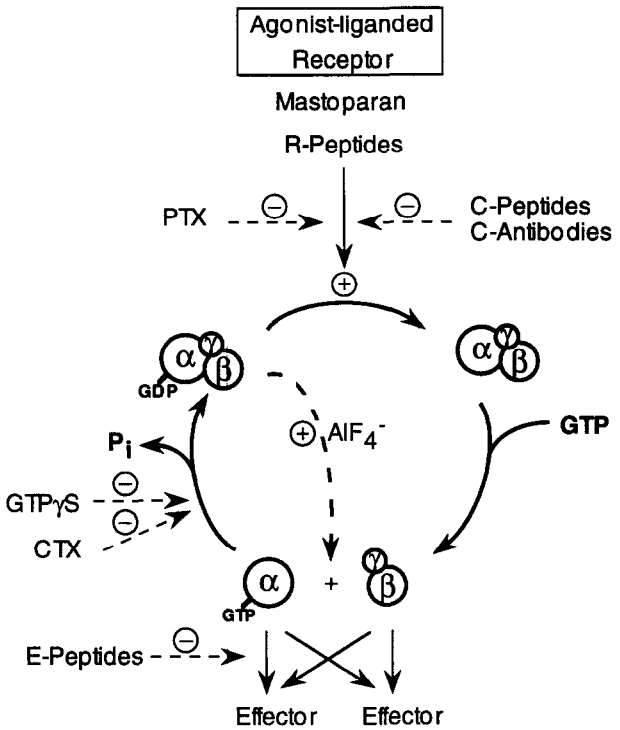

Fig. 1. Probes affecting the G protein cycle. The G protein cycle is based on [5]. Rather than showing the actual target of the probes (e.g. $G_{s\alpha}$ for CTX), the presumed site of action (e.g. inhibition of GTP hydrolysis for CTX) of the probes is indicated. For details, see text. Not shown is the G_α subunit specificity of the probes: the effect of mastoparan is relatively specific for $G_{i\alpha}/G_{o\alpha}$ [12]. Pertussis toxin ADP-ribosylates $G_{i\alpha}/G_{o\alpha}/G_{t\alpha}$ subunits whereas cholera toxin is specific for $G_{s\alpha}$ [8]. AlF_4^- binds directly to G_α (bypassing nucleotide exchange). The presence of GDP and AlF_4^- in the nucleotide binding pocket of G_α mimics the active, GTP-bound G_α conformation [16,78]. Abbreviations: C-peptides, peptides corresponding to the carboxyl-terminus of G_α; E-peptides, effector-peptides, corresponding to the region of G_α that interacts with down-stream targets; R-peptides, receptor-peptides corresponding to specific regions of the cytoplasmic domains of receptors capable of activating G proteins; CTX, cholera toxin; PTX, pertussis toxin.

are ineffective in uncoupling intracellular G protein cycles. Several lines of evidence now suggest that (some of the) intracellular G proteins are involved in membrane trafficking along the endocytic and secretory pathways. Indications for both classical as well as non-classical G protein cycles will be discussed.

3. Heterotrimeric G proteins in vesicular protein transport

Over the past decade, a protein machinery has been discovered which is necessary for vesicular transport of proteins from one compartment to the other (reviewed in [24]). Elucidation of this protein machinery started with the finding that protein transport between organelles can occur in cell-free systems [25] and has since been expanded to numerous in vitro assays that reconstitute the major intracellular protein transport pathways [26]. The first indications that heterotrimeric G proteins might be part of this protein machinery date back to the late 1980s in which it was shown that AlF_4^- inhibits several reconstituted transport pathways along the secretory and endocytic pathways [27–29]. Since AlF_4^- activates heterotrimeric G proteins but not monomeric, small molecular weight GTP binding proteins [7], these results could be taken as a first indication for the involvement of heterotrimeric G proteins, although AlF_4^- can interact with other proteins as well [30]. The current view on G protein sensitive transport pathways is summarized in Fig. 2. Evidence for the involvement of specific sub-types of G proteins in membrane transport pathways will be discussed briefly (for other reviews, see also [31,32]).

Overexpressed $G_{\alpha i\text{-}3}$ in LLC-PK_1 cells co-localizes to the Golgi complex and inhibits transport of a secretory protein (heparan sulfate proteoglycan) through the Golgi complex resulting in an accumulation of its precursors in the medial/*trans*-Golgi. This effect is reversed by pertussis toxin treatment of the cells [33]. Similarly, in polarized cells, transport to the basolateral membrane is also inhibited by a Gαi-subtype [34]. In addition to the effect of $G_{\alpha i\text{-}3}$ at the Golgi complex, this G protein also is involved in stimulation of exocytosis (see below).

The G_s-subclass of G proteins has been shown to affect various intracellular pathways. Peptides, derived from the domain of receptors that couple to G_s, mastoparan analogues with specificity for G_s, as well as cholera toxin activate G_s, resulting in inhibition of endosome fusion [35]. This is in an apparent paradox with the inhibition of endosome fusion by $\beta\gamma$ which supposedly inactivates G proteins but could be explained if a second heterotrimeric G protein is involved to which $\beta\gamma$ specifically binds [36]. In this model activation of Gsα results in free $\beta\gamma$ which associates with the second G protein. With the recent identification of $\beta\gamma$ as a signalling molecule [6], it could however also transduce signals in and of itself. Alternatively, the inhibition is an indirect effect due to the interaction with ARF (see below). In polarized cells, $G_{s\alpha}$ affects both transcytosis as well as transport from the TGN to the apical membrane. In contrast to endosome fusion, activation of $G_{s\alpha}$ stimulates transcytotic vesicle production from early endosomes destined for the apical membrane [17] as well as transport of proteins from the TGN to the apical membrane [34]. In addition, both pathways are inhibited by antibodies directed against the amino-terminal but not carboxyl-terminal region of $G_{s\alpha}$. Given these remarkable similarities, it cannot be excluded that apical transport also occurs via an intermediate compartment such as an endosomal compartment [17]. In this case, the effect of $G_{s\alpha}$ in transcytosis and in apical transport would be on the same transport pathway, namely from the endosomal compartment to the apical membrane.

The *trans*-Golgi network (TGN) is an interesting subcompartment in that multiple heterotrimeric G proteins have been localized to this organelle which affect vesicle budding [37,38]. Both cholera toxin sensitive G proteins ($G_{s\alpha}$, activation of which stimulates vesicle production) as well as pertussis toxin sensitive G proteins ($G_{o\alpha}/G_{i\alpha}$, activation of which inhibits vesicle production) affect the formation of TGN-derived vesicles [38]. The fact that AlF_4^-, an effector that activates both classes at the same time, gives an overall inhibition of vesicle production could be explained by the higher abundance of $G_{o\alpha}/G_{i\alpha}$ over $G_{s\alpha}$ at the TGN [38]. Interestingly, a cytosolic phosphoprotein has been identified that antagonizes the inhibition of vesicle production by AlF_4^- [39]. It is therefore proposed that the phosphoprotein either inhibits the $G_{o\alpha}/G_{i\alpha}$ class of G proteins or stimulate $G_{s\alpha}$. Both membrane transport pathways departing from the TGN, constitutive secretory vesicles and immature granules (destined for regulated secretion) are

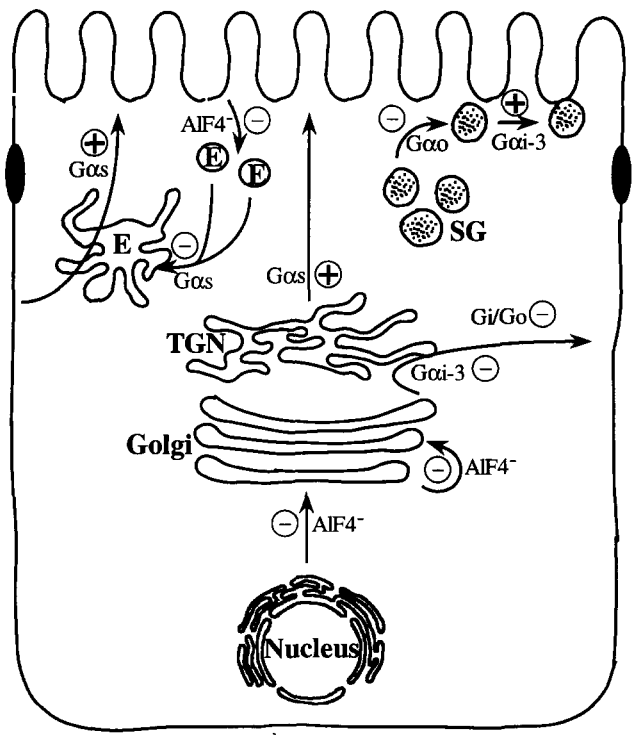

Fig. 2. Involvement of heterotrimeric G proteins in intracellular transport pathways. A polarized cell is depicted in order to be able to show all pathways affected by heterotrimeric G proteins. The data are compiled, however, both from non-polarized and polarized cells. For details, see references in text. The effect of AlF_4^- in receptor-mediated endocytosis is described by Carter et al. [79]. Abbreviations: ER, endoplasmic reticulum; SG, secretory granules; TGN, *trans*-Golgi network; E, endosomal compartment.

affected equally by toxins and probes affecting G protein activity [38].

In summary, one G protein subtype can either stimulate or inhibit vesicular protein transport, depending on the transport pathway. The transport pathways discussed so far fulfill the requirements of a classical G protein cycle such as reversal of the effect of mastoparan [38] or G_α overexpression [33] by PTX as depicted in Fig. 1. Below, some indications for non-classical G protein cycles will be discussed.

4. Indications for non-classical G protein cycles

Vesicular protein transport between the ER and Golgi complex is inhibited by AlF_4^-, mastoparan and $\beta\gamma$ subunits [40]. However, the inhibition by mastoparan is not reversed by PTX [40] although both probes act on the same class of G_i/G_o proteins [8,12]. PTX treatment of cells does not affect transport of the VSV-G protein from the ER to the medial-Golgi [40] whereas PTX stimulates secretion at the medial/*trans* Golgi [33]. Thus, in early stages of protein transport, probes interfering with G protein cycles do not behave as expected in a classical G protein cycle (Fig. 1) and therefore, alternative G protein cycles might be involved. One has to be cautious about the introduction of artifacts due to the use of amphiphilic peptides such as mastoparan [41]. Reversal of the effect of mastoparan by PTX is indicative for a specific interaction of mastoparan with a G_i/G_o protein. However, if the effect of mastoparan is not reversed by PTX, this does not indicate a non-specific effect of mastoparan per se, as will be discussed next.

In exocytosis (regulated secretion) both classical and non-classical G protein cycles seem to operate. Exocytosis from several cells such as adrenal chromaffin cells can be dissected into an ATP-dependent priming step and a calcium-dependent secretion step [42–44]. It is beyond the scope of this review to discuss exocytosis in detail, but the emerging picture is that two heterotrimeric G proteins act in series in the exocytotic pathway. $G_{o\alpha}$, localized to the chromaffin granules [45] seems to be involved in the priming step. $G_{\alpha i\text{-}3}$, localized to the plasma membrane is involved in the calcium-dependent (fusion) step. $G_{\alpha i\text{-}3}$ fulfills the requirements for involvement in a classical G protein cycle. Activation of $G_{\alpha i\text{-}3}$ by secretagogues that mimic receptor activation (i.e. interaction with G proteins via the C-terminal region of G proteins) is reversed both by synthetic peptides that correspond to the C-terminus of $G_{\alpha i\text{-}3}$ and by antibodies directed against this region [46]. In addition, the activation of a G protein in the calcium-dependent stage (presumably $G_{\alpha i\text{-}3}$) with mastoparan is reversed by pertussis toxin treatment [47]. Finally, receptor-mimicking peptides, pre-activated G_i/G_o proteins, as well as transient expression of active mutants of G_i/G_o inhibited the calcium-dependent step of exocytosis [18]. In contrast, the G protein involved in the ATP-dependent priming step is likely subject to a non-classical mechanism of activation. Activation of this G protein by mastoparan results in inhibition of secretion which can not be reversed by PTX [47]. The inhibition is reversed, however, by antibodies directed against the C-terminus of $G_{o\alpha}$ [48]. For $G_{o\alpha}$ an alternative mechanism of activation has been described. Growth-cone-associated protein (GAP-43) can activate $G_{o\alpha}$ in its depalmitoylated (cytosolic) form [49,50]. Interestingly, GAP-43 mimics the action of mastoparan and inhibits the priming reaction. Like mastoparan, the inhibition of GAP-43 is reversed by antibodies directed against the C-terminus of G_o [51].

There has been much speculation that G proteins involved in vesicular transport interact with ADP-ribosylation factor (ARF) [31,52]. ARF is a small GTP-binding protein involved in various membrane transport pathways [24,52]. Speculation on the interaction of a G protein with ARF is based on the initial discovery of ARF, in which it was shown that ARF is a necessary component in the CTX-stimulated ADP-ribosylation of $G_{s\alpha}$ [53]. In addition, AlF_4^- stimulates binding of ARF and coatomer to Golgi membranes [54,55], although AlF_4^- cannot activate ARF itself [7]. Furthermore, purified $\beta\gamma$ subunits inhibit ARF binding to Golgi membranes [54]. This however, can now also be explained by the recent finding that GDP-bound ARF can interact directly with $\beta\gamma$ [56] rather than indicating the involvement of a heterotrimeric G protein. Thus, an alternative interpretation of the inhibitory effect of added $\beta\gamma$ would be the interaction with cytosolic (GDP-bound) ARF which prevents binding to Golgi membranes. In addition, this could implicate that the effect of addition of exogenous $\beta\gamma$ on several membrane transport pathways [17,36,37,40] cannot be taken anymore as conclusive evidence for the involvement of a heterotrimeric G protein. It remains to be established whether the GDP-bound ARF–$\beta\gamma$ interaction has physiological significance. First of all, $\beta\gamma$ resides in membranes rather then cytosol and only GTP-bound ARF is bound to membranes [7,54,57]. Second, the interaction is relatively weak and more likely reflects the fact that among all small GTP binding proteins, ARF

has the highest homology to G_α-subunits [58]. It does add support to the speculation that β'-COP might be the subunit of coatomer that binds to ARF [59,60]. β'-COP contains the WD-repeat consensus sequence which is also found in $G\beta$ (reviewed in [61]).

Two types of proteins, β-COP (part of the coatomer complex) and p200 (a phosphoprotein) dissociate from the Golgi complex in the presence of Brefeldin A (BFA) [62,63]. BFA is a fungal metabolite that inhibits an enzyme in a Golgi-enriched fraction that catalyses guanine nucleotide (GDP-GTP) exchange on ARF protein [64,65]. This BFA effect indicates the involvement of ARF in membrane binding of β-COP and of p200. Membrane binding of both proteins is induced by GTPγS and AlF_4^- [54,66]. Interestingly, pertussis toxin pretreatment of Golgi membranes to selectively inactivate $G_{\alpha i\text{-}3}$, reduced the mastoparan-induced binding of p200 to Golgi membranes. Stimulation of membrane-binding of β-COP is less sensitive to mastoparan and the effect could not be reversed by PTX. Recently, p200 has been localized to the TGN [67] whereas β-COP operates in early steps of the secretory pathway (reviewed in [24]). Thus, there is an interesting parallel with the effect of mastoparan and PTX on protein transport in which different effects where observed between early and late stages of the secretory pathway (see above) and adds further support to the hypothesis that non-classical G protein cycles operate at early membrane transport pathways. It is of note that we have also found that β-COP binding to Golgi membranes is insensitive to PTX treatment of Golgi membranes (J.B.H., unpublished) but it is in conflict with data presented by Ktistakis et al. [68].

In addition to G_s, G_i and G_o proteins found on intracellular membranes, a new type of cholera toxin sensitive G_α has been identified which localizes to the TGN [69]. In addition to the highly homologous $G_{s\alpha}$ sequence, it contains a 51 kDa N-terminal extension resulting in an 'extra large' (XL) G_α. Although its function is unknown, this protein only occurs in cells containing both the constitutive and regulated pathway of protein secretion. It will be of interest to determine whether this extra N-terminal domain contains enzymatic activity: another large G protein (G_h) mediates receptor stimulation of phospholipase C activity but also contains GTPγS-sensitive transglutaminase activity [70]. Apparently, receptor-stimulated GTP-binding switches the function of $G_{h\alpha}$ from transglutamination to receptor signalling [70]. Alternatively, the XL G_α could reflect a covalent coupling of a G protein to a receptor-like activity which allows a more potent and productive agonist-dependent signal transduction pathway [71].

5. Emerging concepts

5.1. Targeting of G_α subunits to intracellular membranes

It has become clear that G proteins are found not only at the plasma membrane but on intracellular membranes as well. Using immuno-fluorescence and/or immuno-EM it appears that one subclass of G_α subunit can be detected on several membranes that varies between cells [72]. For example, depending on the cell-type, $G_{\alpha i\text{-}3}$ is localized exclusively to the Golgi complex [33] or to the plasma membrane as well [46]. Changes in subcellular localization of overexpressed $G_{i\alpha}$-2/$G_{\alpha i\text{-}3}$ chimeras suggest that the targeting information is contained in the carboxyl-terminus of G_α subunits [73]. This agrees well with the discovery of a $G_{\alpha i\text{-}2}$ with an alterative spliced carboxyl-terminal region which changes its cellular distribution from the plasma membrane to the Golgi complex [74]. In contrast to retention mechanisms of membrane-spanning proteins to the Golgi complex [75] very little is known about the specific targeting of peripheral proteins to the Golgi or other subcellular compartments. But given the fact that the subcellular distribution of G proteins varies between different cell-types, it seems more likely that G proteins are retained in an organelle due to its interaction with receptor-type and/or effector-type molecules. This would also explain the targeting information residing in the carboxyl-terminal region. Variations in expressions of receptor/effector type molecules between cell-types thus result in variations of the subcellular localization of G proteins. In agreement with this model is has been found that a modest (three-fold over endogenous) overexpression of $G_{\alpha i\text{-}3}$ results in correct targeting to the Golgi complex whereas a high level of overexpression of $G_{\alpha i\text{-}3}$ subunits results in mistargeting of the $G_{\alpha i\text{-}3}$ and as a result it is found throughout the cell. This indicates a saturable targeting process [33].

5.2. Classical versus non-classical G protein cycles

Membrane transport pathways near the plasma membrane such as endosome fusion, transcytosis and budding of vesicles from the TGN seem to involve the classical G protein cycle. It is known that receptors containing seven membrane-spanning domains can be endocytosed and recycled to the plasma membrane [76,77], it remains to be established whether intracellular organelles that are connected to the plasma membrane by transport pathways, can apply this as a potential mechanism of activation of intracellular G proteins. In the early stages of exocytosis (priming) it seems plausible that a non-classical G protein cycle operates which involves $G_{o\alpha}$ and GAP-43. Could a similar non-classical G protein cycle operate in early stages of the constitutive secretory pathway? Overexpression of $G_{o\alpha\text{-}1}$, but not $G_{o\alpha\text{-}2}$, in C6 glial cells results in a very punctuate subcellular labeling reminiscent of transport vesicles (Bockaert and Homburger, pers. commun.) In addition, the constitutive secretion of only a subset of proteins is affected by the overexpression of $G_{o\alpha\text{-}1}$ (Bockaert and Homburger, pers. commun).

The classical G protein cycle at the plasma membrane is very cell-type specific, since cells usually express only a subset of the components of the G protein cycle. It is likely that similar variations affect the function of intracellular G proteins in intracellular membrane transport pathways. Now that the involvement of G proteins in vesicular protein transport is well established, the stage is set to determine their mode of action which requires identification of receptor- and effector-type molecules, keeping in mind a cell-type specific environment. This will yield new insights not only in the mechanism of vesicular protein transport, but of signal transduction cascades in general.

Acknowledgements: J.B.H. is supported by the Alexander von Humboldt Foundation. I am grateful to Felix Wieland, Walter Nickel, Marie-France Bader and Jochen Lang for critical reading and comments on the manuscript. I thank Drs. Lang and Wollheim, and Drs. Homburger and Bockaert for sharing their data prior to publication.

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