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Trimeric G-proteins of the *trans*-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules

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Non-hydrolysable analogues of GTP, such as GTPγS and GMP-PNP, have previously been shown to inhibit the formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the *trans*-Golgi network (TGN). Using a cell-free system, we show here that the formation of these vesicles is also inhibited by $[AlF_4]^-$, a compound known to act on trimeric G-proteins. Addition of highly purified G-protein βγ subunits stimulated, in a differential manner, the cell-free formation of both CSVs and ISGs. ADP-ribosylation experiments revealed the presence of a pertussis toxin-sensitive G-protein α subunit in the TGN. We conclude that trimeric G-proteins regulate the formation of secretory vesicles from the TGN.

Trimeric G-protein; Cell-free vesicle formation; βγ Subunits; Guanosine nucleotides; Fluoride; ADP-ribosylation

1. INTRODUCTION

GTPases regulate numerous cellular processes [1–3]. Several of the small, ras-like GTPases have been found to be associated with specific intracellular compartments [4,5]. Studies using yeast secretion mutants and cell-free membrane traffic systems have implicated such GTPases in the targeting of membrane vesicles to, and their fusion with, appropriate acceptor compartments [6–8]. The formation of vesicles from donor compartments has also been found to require GTP hydrolysis [9,10]. Recently, the small GTPase Sar1p has been implicated in the formation of vesicles from the endoplasmic reticulum in yeast [11]. Using a cell-free system [12], we report here that the formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the *trans*-Golgi network (TGN) involves pertussis toxin-sensitive, trimeric G-proteins. Our results suggest that not only small ras-like GTPases, but also trimeric G-proteins have a role in regulating membrane traffic.

2. MATERIALS AND METHODS

2.1. *Cell-free formation of CSVs and ISGs*

A post-nuclear supernatant (PNS) was prepared from sulphate-labelled PC12 cells and incubated at 37°C for 60 min as described previously [12], with the addition of GTPγS, $AlCl_3$ or $[AlF_4]^-$ as indicated in the figure legends. βγ subunits purified from bovine brain [13,14] were added from a 1 mg/ml stock in buffer A (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithioerythritol, 100 mM NaCl and 0.2% cholate). At the highest final concentration of βγ subunits used (400 nM), this buffer contributed 0.4 mM Tris-HCl, 2 μM EDTA, 20 μM dithioerythritol, 2 mM NaCl and 0.004% cholate to the cell-free reaction mixture, and a corresponding amount of buffer A lacking βγ subunits was used as control. In some experiments, an aliquot of the βγ subunit stock was digested to completion with an equal volume of 1 mg/ml trypsin for 10 min at 37°C, boiled for 5 min, supplemented on ice with an equal volume of 1 mg/ml soybean trypsin inhibitor, and then added to the PNS prior to starting the cell-free vesicle formation.

2.2. *Separation of CSVs and ISGs*

ISGs and CSVs were separated from the TGN by velocity sucrose gradient centrifugation [12], and the gradient fractions analysed by SDS-PAGE and fluorography. When indicated, fractions 2–5 of the velocity gradient, containing the bulk of post-TGN vesicles, were subjected to equilibrium centrifugation as described [12] to separate CSVs and ISGs, using a modified linear sucrose gradient of 0.5 M to 1.6 M.

2.3. *Quantitation*

The distribution of sulphate-labelled secretogranin II (SgII) and heparan sulphate proteoglycan (hsPg) across the velocity and equilibrium sucrose gradients was determined as described [9,12]. The budding efficiencies of the ISGs and CSVs were calculated from the values obtained for sulphate-labelled SgII and hsPg, respectively, after quantitation of the velocity sucrose gradients [9,12].

2.4. *ADP-ribosylation*

A PNS prepared from unlabelled PC12 cells was subjected to cell-free vesicle formation as described [12] with the following modifica-

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tion. Samples contained in addition 10 mM thymidine, 100 μM [^{32}P]NAD (75 mCi/mmol), 0.25 mM dithiothreitol, 0.1 mM sodium phosphate, pH 7.0, 1 mM NaCl and either heat-inactivated (5 min, 95°C) or activated [15] 1 μg/ml pertussis toxin (Calbiochem 516560, reconstituted to 0.2 mg/ml stock). After incubation at 37°C for 60 min, samples were subjected to velocity sucrose gradient centrifugation and the fractions analysed by SDS-PAGE and autoradiography. Aliquots of velocity sucrose gradient fractions (see legends) were subjected to equilibrium sucrose gradient centrifugation and the distribution of [^{32}P]ADP-ribosylated G protein α subunit in the gradient fractions was determined.

3. RESULTS AND DISCUSSION

We first explored the possibility that the inhibition of formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the *trans*-Golgi network (TGN) by non-hydrolyzable GTP analogues, previously observed in a cell-free system derived from the neuroendocrine cell line PC12 [9], may reflect the involvement of trimeric G-proteins. For this purpose, we exploited the recent observation [16] that $[AlF_4]^-$, which in the presence of GDP is known to activate both inhibitory and stimulatory trimeric G-proteins by mimicking the γ-phosphate moiety of GTP [17], does not affect small, ras-like GTPases. As shown in Fig. 1, $[AlF_4]^-$ inhibited, in the cell-free system, the formation of ISGs (Fig. 1a) and CSVs (Fig. 1b) characterized by the sulphate-labelled markers secretogranin II (SgII) and heparan sulphate proteoglycan (hsPg), respectively [12]. The degree of inhibition (50% for ISGs; 59% for CSVs) was similar to that observed in the presence of the non-hydrolyzable GTP analogue GTPγS (Fig. 1; cf. [9]). In contrast, aluminium chloride, which was added as a control, did not significantly affect the formation of either CSVs or ISGs (Fig. 1). In addition, no inhibition of formation of ISGs and CSVs was observed when only fluoride, but not aluminium, ions were added to the cell-free reaction (data not shown). One possible interpretation of these data is that an inhibitory trimeric G-protein was activated by the $[AlF_4]^-$, thus resulting in a decrease in the amount of post-TGN vesicles formed in the cell-free system.

To test this possibility, we studied the effect of βγ subunits on the cell-free formation of ISGs and CSVs. An excess of βγ subunits is known to exert the opposite effect of non-hydrolyzable GTP analogues on the activation state of α subunits, presumably by shifting the association equilibrium of these subunits towards the trimeric state in which the α subunits are inactive [2,18,19]. If the inhibition of post-TGN vesicle formation by GTPγS reflected an involvement of an inhibitory α subunit, addition of excess βγ subunits should stimulate the formation of secretory vesicles from the TGN in the cell-free system. Indeed, as shown in Fig. 2, addition of βγ subunits, purified to homogeneity from bovine brain, stimulated the transfer of sulphate-labelled SgII (Fig. 2a) and hsPg (Fig. 2b) from the TGN

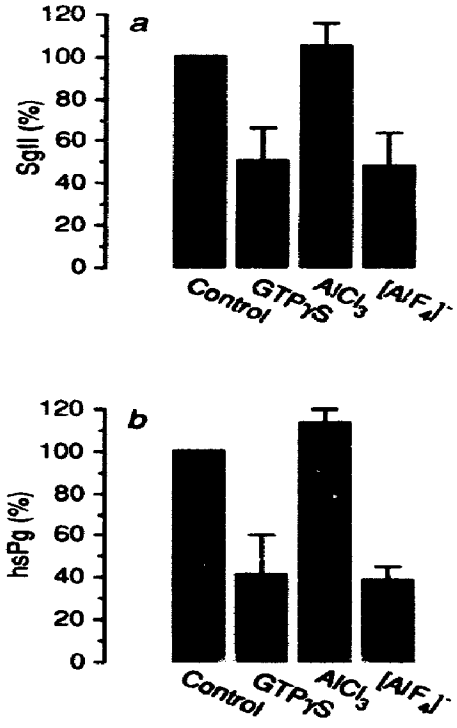

Fig. 1. $[AlF_4]^-$ inhibits the cell-free formation of ISGs (a) and CSVs (b). A PNS prepared from sulphate-labelled PC12 cells was incubated for 60 min in the absence (control) and presence of 10 μM GTPγS, 40 μM Al^{3+} and 6 mM Cl^- ($AlCl_3$), or 40 μM Al^{3+} and 6 mM F^- ($[AlF_4]^-$). ISGs and CSVs were separated from the TGN by velocity sucrose gradient centrifugation, and the gradient fractions analysed by SDS-PAGE and fluorography. The budding efficiencies of the ISGs and CSVs were determined after quantitation of sulphate-labelled SgII and hsPg, respectively, and are expressed as percent of control. The bars indicate the standard deviation (GTPγS, $[AlF_4]^-$, $n = 3$) or the variation of duplicates from the mean ($AlCl_3$).

to post-TGN vesicles. Very little stimulation was observed when only the cholate-containing buffer of the βγ subunit stock was used (squares in Fig. 2). In addition, no stimulation was observed when the βγ subunits were digested with trypsin prior to use in the cell-free system (triangles in Fig. 2). The stimulation of post-TGN vesicle formation showed a linear relationship with the concentration of added βγ subunits. This suggests that the amounts of sulphate-labelled SgII and hsPg were not rate-limiting for the formation of post-TGN vesicles in the cell-free system under any of the present conditions. In agreement with this conclusion, ~25% of both the sulphate-labelled SgII and hsPg were still present in the TGN at the end of the cell-free reaction with the highest concentration of βγ subunits investigated (400 nM). The observed stimulation of post-TGN vesicle formation upon addition of βγ subunits (Fig. 2), in addition to the results obtained with $[AlF_4]^-$

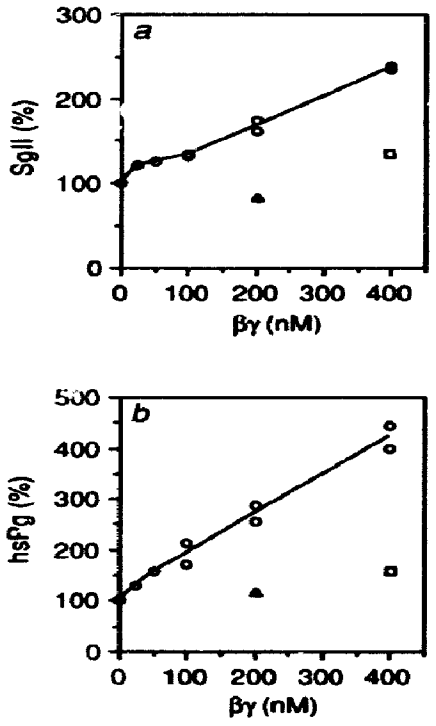

Fig. 2. Purified $\beta\gamma$ subunits stimulate the exit of SgII (a) and the hsPg (b) from the TGN in post-TGN vesicles in a cell-free system. Cell-free formation of post-TGN vesicles was performed in the presence of the indicated final concentrations of: highly purified $\beta\gamma$ subunits (circles), 200 nM trypsin-treated $\beta\gamma$ subunits (triangles), or an amount of buffer A (squares) corresponding to that added when 400 nM $\beta\gamma$ subunits were used. The appearance of SgII and hsPg in post-TGN vesicles was determined after velocity sucrose gradient centrifugation as in Fig. 1 and is expressed as percent of that observed in the absence of added $\beta\gamma$ subunits. The values obtained in the presence of 100, 200 and 400 nM $\beta\gamma$ subunits are from two independent experiments.

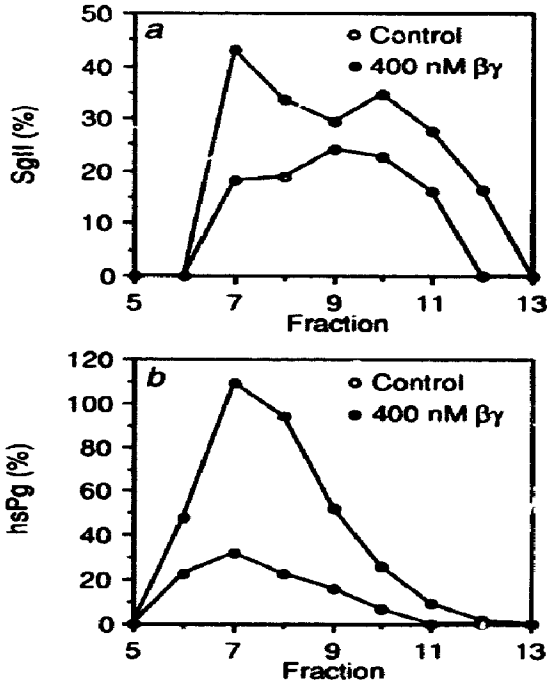

Fig. 3. The effect of purified $\beta\gamma$ subunits on the packaging of SgII (a) and the hsPg (b) into ISGs and CSVs. Cell-free packaging of SgII and hsPg was performed in the absence (control, open circles) and presence (filled circles) of highly purified 400 nM $\beta\gamma$ subunits as described in Fig. 2. After velocity sucrose gradient centrifugation, fractions 2–5, containing the bulk of post-TGN vesicles, were subjected to equilibrium centrifugation to separate CSVs and ISGs, and the distribution of sulphate-labelled SgII and hsPg in the gradient fractions (fraction 13 = bottom of gradient) was determined. In the control condition, the individual values of each sulphate-labelled marker are expressed as percent of the total recovered. The individual values obtained in the presence of 400 nM $\beta\gamma$ subunits are expressed relative to the respective control values.

(Fig. 1), provides further evidence for a role of trimeric G-protein(s) in the formation of ISGs and CSVs.

We examined the effect of added $\beta\gamma$ subunits on the packaging of sulphate-labelled SgII and hsPg into ISGs and CSVs by separating these two post-TGN vesicle populations using equilibrium sucrose gradient centrifugation [12]. As shown in Fig. 3b, the sulphate-labelled hsPg was found to peak in the position characteristic of CSVs (fraction 7) in both the absence and presence of 400 nM added $\beta\gamma$ subunits. In contrast, the addition of 400 nM $\beta\gamma$ subunits exerted a dual effect on the packaging of SgII into post-TGN vesicles (Fig. 3a). First, it increased the amount of sulphate-labelled SgII in the position characteristic of ISGs (peak in fractions 9–10), indicating that the formation of ISGs was stimulated in this condition. Second, it led to the packaging of a significant amount of the sulphate-labelled SgII into vesicles (peak in fraction 7) which were indistinguishable from CSVs by their buoyant density. Apparently, the $\beta\gamma$ subunit-induced stimulation of SgII exit from the TGN exceeded the capacity for packaging of this protein into ISGs.

To investigate which class of α-subunit might be mediating the inhibition of CSV and ISG formation by non-hydrolyzable GTP analogues and $[AlF_4]^-$, we used bacterial toxins which specifically ADP-ribosylate certain α subunits of trimeric G-proteins [3]. Thus, cholera toxin catalyzes the ADP-ribosylation of stimulatory α subunits ($G\alpha_s$) [20] whereas pertussis toxin catalyzes the ADP-ribosylation of different α subunits including inhibitory ($G\alpha_i$) and other ($G\alpha_o$) subunits [21,22]. We therefore searched for the presence of proteins in the TGN of PC12 cells which were substrates for bacterial toxin-catalyzed ADP-ribosylation. A post-nuclear supernatant (PNS) derived from PC12 cells was incubated with [^{32}P]NAD, the co-substrate for ADP-ribosyl transfer-

ases, in the absence or presence of either cholera toxin or pertussis toxin. The TGN was then separated from other subcellular organelles using the velocity sucrose gradient centrifugation employed above. In the presence of heat-inactivated pertussis toxin, no significant ADP-ribosylation of proteins in the molecular weight range characteristic of α subunits was detected in any of the gradient fractions (Fig. 4a, Con). Incubation of the PNS in the presence of activated pertussis toxin (Fig. 4a, Ptx) led to the ADP-ribosylation of a ~40 kDa protein which upon velocity sucrose gradient centrifugation was detected in two peaks, one in the top three fractions of the gradient which are known to contain various subcellular organelles of PC12 cells including post-TGN vesicles [12] and plasma membrane [23], and the other in the bottom half of the gradient which is known to contain TGN membranes (see bar in Fig. 4a and [12]). When the latter material was subjected to a second equilibrium sucrose gradient centrifugation, the protein ADP-ribosylated by pertussis toxin co-migrated with the TGN (Fig. 4b), as identified by sulphate-labelled hsPg (Fig. 4c) and SgII (Fig. 4d). No ADP-ribosylated α subunit co-migrating with the TGN was observed when cholera toxin was used (data not shown). These results strongly suggest that a pertussis toxin-sensitive α subunit ($G\alpha_i$ and/or $G\alpha_o$) is present on the TGN membrane of PC12 cells.

ADP-ribosylation by pertussis toxin blocks the interaction of α subunits with receptors, and thereby prevents the receptor-mediated GDP-GTP exchange and hence activation of the G-protein [18,24]. If, under our cell-free conditions, the inhibitory effect of GTPγS on the formation of post-TGN vesicles involves a receptor-mediated guanine nucleotide exchange, and if this guanine nucleotide exchange occurs at a pertussis toxin-sensitive α subunit, pertussis toxin-catalyzed ADP-ribosylation of this α subunit prior to cell-free vesicle formation should reduce the inhibitory effect of GTPγS on this process. Indeed, when the PNS was incubated for 5 min in the presence of activated pertussis toxin and NAD prior to the addition of 20 μM GTPγS, the cell-free formation of ISGs and CSVs was 52% and 37% of

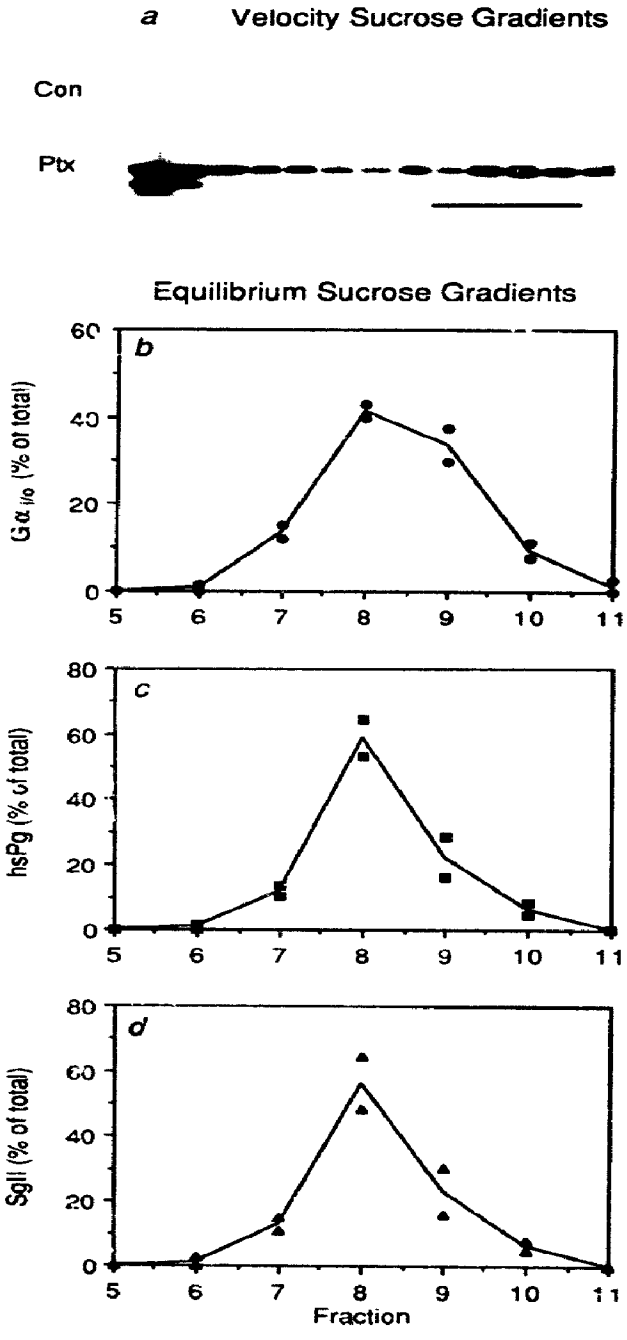

Fig. 4. Presence of a pertussis toxin-sensitive G protein α subunit in the TGN of PC12 cells. (a,b) A PNS prepared from unlabelled PC12 cells was subjected to ADP-ribosylation, under cell-free vesicle formation conditions, in the presence of [^{32}P]NAD and either heat-inactivated (Con) or activated [15] (Ptx) pertussis toxin, followed by velocity sucrose gradient centrifugation. (a) Aliquots of the gradient fractions (top = left) were analysed by SDS-PAGE and autoradiography; only the 35–45 kDa region of the gel is shown. The bar indicates the fractions known to contain TGN [12]. (b) Aliquots of such fractions, from cell-free reactions with activated pertussis toxin, were subjected to equilibrium sucrose gradient centrifugation as described in Fig. 3. The distribution of [^{32}P]ADP-ribosylated G protein α subunit in the gradient fractions was determined and is expressed as percent of the total recovered, for two independent experiments. (c,d) A PNS was prepared from sulphate-labelled PC12 cells, incubated at 4°C for 60 min as described except that the sample contained in addition 10 mM thymidine, and was subjected to velocity sucrose gradient centrifugation. Aliquots of fractions containing TGN were subjected to equilibrium sucrose gradient centrifugation. The distribution of sulphate-labelled hsPg (c) and SgII (d) in the gradient fractions was determined and is expressed as percent of total recovered.

control (no toxin, no GTPγS), respectively, whereas the formation of ISGs and CSVs in the presence of GTPγS was 34% and 22% of control, respectively, when the pertussis toxin had been heat-inactivated.

In conclusion, the present results show that one or more pertussis toxin-sensitive trimeric G-proteins are involved in the formation of CSVs and ISGs from the TGN. Although the identity of the α subunit involved is not yet known, our data are consistent with this subunit belonging to the $G\alpha_i$ or $G\alpha_o$ class and exerting an inhibitory effect on vesicle formation. In this regard, it is interesting to note that $G\alpha_{i3}$, which, like the constitutive secretory pathway, is found in virtually all cell types [3], is concentrated in the Golgi complex [25]. Thus, the α subunit involved in the formation of CSVs from the TGN may well be $G\alpha_{i3}$.

On a more general note, our results have two implications. First, they indicate that not only small, ras-like GTPases [8], but also trimeric G-proteins have a fundamental role in membrane traffic. Second, they imply the existence of signal-transduction mechanisms in the membranes of intracellular organelles which are linked to vesicle formation.

NOTE

After submission of this study, Stow et al. [26] reported that the overexpression of $G\alpha_{i3}$ inhibits the secretion of a heparan sulfate proteoglycan from epithelial cells, an effect reversed by pertussis toxin. These results are consistent with those reported here.

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