Studies on G-protein $\alpha \cdot \beta\gamma$ heterotrimer formation reveal a putative S-prenyl-binding site in the α subunit

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The α and $\beta\gamma$ subunits of heterotrimeric G-proteins contain specific lipid modifications, which are required for their biological function. However, the relevance of these modifications to the interactions within the heterotrimeric G-protein is not fully understood. In order to explore the role of the S-prenyl moiety of the isoprenylated $\beta\gamma$ dimer of retinal transducin, $\beta\gamma_t$, in the formation of the heterotrimeric complex with the corresponding N-acylated α subunit, α_t , we employed purified fully processed subunits, which are soluble in aqueous solutions without detergents. Pertussis-toxin-mediated [³²P]ADP-ribosylation of α_t is strongly stimulated (≈ 10 -fold) in the presence of $\beta\gamma_t$ and can thus serve as a measure for heterotrimer formation. Using this assay, preincubation of α_t with S-prenyl analogues containing farnesyl or geranylgeranyl moieties was found to inhibit heterotrimer formation in a dose-dependent manner. The inhibition was com-

petitive and reversible, as indicated by its reversal upon increase of the $\beta\gamma_t$ dimer concentration or by removal of the S-prenyl analogue using gel filtration. The competitive nature of the inhibition is supported by the marked attenuation of the inhibition when the S-prenyl analogue was added to α_t together with or after $\beta\gamma_t$. The inhibition does not involve interaction with the α_t acyl group, since an S-prenyl analogue inhibited the [³²P]ADP-ribosylation of an unlipidated α_t mutant. These data suggest the existence of a hitherto unrecognized S-prenyl-binding site in α_t , which is critical for its interaction with prenylated $\beta\gamma_t$.

Key words: farnesylation, G-protein $\beta\gamma$ dimer, heterotrimeric G-protein, isoprenylation, post-translational modification, signal transduction.

INTRODUCTION

Heterotrimeric (α and $\beta\gamma$) G-proteins are essential elements of many transmembrane-signalling systems involved in coupling numerous receptors for extracellular mediators or sensory stimuli to the generation of intracellular signals by effector moieties such as adenylate cyclase, ion channels or phospholipase C (reviewed in [1]). G-protein activation is initiated by the receptor-catalysed release of GDP from the heterotrimeric G-protein, followed by the binding of GTP and the dissociation of the $\alpha \cdot \beta\gamma$ heterotrimer into the GTP-liganded α subunit and the free $\beta\gamma$ dimer. Both α_{GTP} and $\beta\gamma$ are capable of mediating effector regulation [1].

At least 20 G_α , 6 G_β and 12 G_γ subunits are expressed in mammalian cells when splice variants are included [2]. Additional structural diversity of G-protein subunits is caused by co- and/or post-translational modifications of all three subunits (reviewed in [3,4]). G-protein α subunits are N-myristoylated, S-palmitoylated or both at their N-termini [2,5]. These modifications are important for membrane targeting of α subunits and their interaction with G-protein $\beta\gamma$ dimers [3,5]. The α subunit of retinal rod transducin, α_t , is heterogeneously N-acylated at its N-terminal glycine residue [6,7]. G-protein γ subunits are modified post-translationally at their C-termini by isoprenylation, proteolytic cleavage and methyl-esterification (reviewed in [6,8]). The proteins are first either farnesylated (γ_1 , γ_C and γ_{11}) or geranylgeranylated (all other γ subunits) at a cysteine residue at position –4 from the C-terminus by farnesyltransferase or geranylgeranyltransferase

type I respectively. Following S-isoprenylation, an endoprotease associated with the endoplasmic reticulum, Rce1 (for Ras and a-factor-converting enzyme) [9], cleaves the three terminal amino acids and the resultant terminal carboxyl group is methyl-esterified by a membraneous Icmt (isoprenylcysteine carboxymethyltransferase) [10]. The C-terminal γ subunit modifications are not required for the formation of the $\beta\gamma$ dimer [11], but are essential for their binding to membranes and regulate their interactions with α subunits and effectors (see [12] for references). Mice deficient in either Rce1 or Icmt show severe phenotypes, which may, at least in part, be due to the mislocalization of Ras proteins, which use the same enzymes for post-translational processing of their C-termini ([13,14] and references therein).

Structural studies on G-protein heterotrimers or their individual subunits, α and $\beta\gamma$, showed contacts between all three subunits [15–18]. However, because the crystallized subunits were not lipid-modified, these studies did not provide information on possible lipid–protein or lipid–lipid interactions within the heterotrimer. We have previously shown that the S-prenylated cysteine analogue L-AFC (*N*-acetyl-S-*trans,trans*-farnesyl-L-cysteine) suppresses basal and receptor-stimulated binding of GTP[S] [guanosine 5'-O-(3-thiotriphosphate)] to membranes from human peripheral neutrophils and HL-60 granulocytes stimulated by formyl peptide and complement C5a [19,20]. L-AFC also interfered with GTP[S] binding to retinal transducin when stimulated by light-activated rhodopsin in a reconstituted system [20]. As L-AFC did not affect the pertussis-toxin-mediated

Abbreviations used: G_t , heterotrimeric transducin; FTS, *S-trans,trans*-farnesyl thiosalicylic acid; 5-Cl-FTS, *S-trans,trans*-farnesyl-5-Cl-thiosalicylic acid; 4-Cl-FTS, *S-trans,trans*-farnesyl-4-Cl-thiosalicylic acid; 5-F-FTS, *S-trans,trans*-farnesyl-5-F-thiosalicylic acid; 4-F-FTS, *S-trans,trans*-farnesyl-4-F-thiosalicylic acid; FCOOH, *trans,trans*-farnesoic acid; DTT, dithiothreitol; GST, glutathione S-transferase; GTP[S], guanosine 5'-O-(3-thiotriphosphate); GTS, *S-trans*-geranyl thiosalicylic acid; GGTS, *S-all-trans*-geranylgeranyl thiosalicylic acid; L-AFC, *N*-acetyl-S-*trans,trans*-farnesyl-L-cysteine; RhoGDI, Rho GDP dissociation inhibitor. Rce1, Ras and a-factor-converting enzyme; Icmt, isoprenylcysteine carboxymethyltransferase.

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[32 P]ADP-ribosylation of G_i proteins in HL-60 granulocyte membranes, it was speculated that S-prenylated cysteine analogues such as L-AFC inhibit receptor-mediated G-protein activation by interfering with the interaction of activated receptors with G-proteins, rather than by inhibiting $\alpha \cdot \beta\gamma$ heterotrimer formation. However, because these studies employed membrane preparations, no clear answer regarding possible protein–lipid interactions within the heterotrimer itself could be obtained.

The functional significance of the lipid modifications of α and/or $\beta\gamma$ subunits in membrane anchorage and in mediating interactions within heterotrimeric G-proteins has been a subject of debate. Much of the controversy is due to the use of detergents, phospholipids or native membranes in biochemical assays designed to address this issue. In the case of transducin, gradient centrifugation and gel-filtration studies demonstrated a weak interaction between α_i and $\beta\gamma_i$ in the absence of detergents and lipids, which was not observed for α_i and $\beta\gamma_i$ variants lacking either the N-terminal acyl chain or the isoprenylated γ subunit C-terminus [21]. Furthermore, Matsuda et al. [22] showed that synthetic peptides corresponding to the isoprenylated, but not those corresponding to non-isoprenylated γ subunits of $\beta\gamma$ dimers, inhibited the pertussis-toxin-mediated, $\beta\gamma_i$ stimulated [32 P]ADP-ribosylation of α_i . In a related study, Rahmatullah and Robishaw [23] found that a recombinant prenylated γ_2 subunit that had been expressed in the absence of β specifically interacted with an α_o -affinity matrix, whereas the non-prenylated γ_2 did not. Parish et al. [24] showed that demethylation of $\beta_1\gamma_2$ dimer did not affect the pertussis-toxin-mediated [32 P]ADP-ribosylation of the α_{i1} subunit and had only minor effects on membrane and effector interaction of $\beta_1\gamma_2$. In a more recent paper [25], the authors described an ≈ 40 – 50 % inhibition of $\beta\gamma_i$ stimulated pertussis-toxin-mediated [32 P]ADP-ribosylation of α_i by FTS (*S-trans,trans*-farnesyl thiosalicylic acid), FTP [*S-(trans,trans*-farnesyl-3-thio)propionic acid], FCOOH (*trans,trans*-farnesoic acid) and FTA [*S-(trans,trans*-farnesyl-3-thio)acetic acid], and concluded that the inhibitors interfered with a lipid–lipid-based association of α_i and $\beta\gamma_i$, through the lipid modifications present on each subunit. The latter studies, however, were performed in the presence of detergent, making the derivation of conclusions on the presence or absence of lipid–protein interactions rather ambiguous.

To study the role of lipid–protein interactions in G-protein heterotrimer formation, we employed fully processed transducin subunits [6,7], α_i and $\beta\gamma_i$, purified from a natural source. This system is advantageous since the fully processed transducin subunits are soluble without detergent, enabling studies on the interactions of the purified α_i and $\beta\gamma_i$ in aqueous solutions as well as the effects of various S-prenyl analogues on these interactions. We have used here analogues that were shown previously to interfere with the functions of G-protein coupled receptors, such as L-AFC [19,20], as well as other analogues, such as FTS, shown to interfere with Ras functions (reviewed in [26]). We show that binding of farnesylated G-protein $\beta\gamma_i$ dimers to α_i is inhibited by S-prenyl analogues in a manner dependent on the isoprenoid structure and on the concentration. The effect appears to be mediated by competition with $\beta\gamma_i$ dimers for binding to a farnesyl-recognition domain in the α_i subunit.

EXPERIMENTAL

Materials

FTS was synthesized as described in [27]. The synthesis of GTS (*S-trans*-geranyl thiosalicylic acid), GGTS (*S-all-trans*-geranylgeranyl thiosalicylic acid), 5-F-FTS (*S-trans,trans*-farnesyl-

5-F-thiosalicylic acid), 4-F-FTS (*S-trans,trans*-farnesyl-4-F-thiosalicylic acid), 5-Cl-FTS (*S-trans,trans*-farnesyl-5-Cl-thiosalicylic acid) and 4-Cl-FTS (*S-trans,trans*-farnesyl-4-Cl-thiosalicylic acid) is described in [28]. FCOOH was prepared as described in [23]. All isoprenylated compounds were dissolved in DMSO. The final assay concentration of DMSO was 0.5 % (v/v). [*adenylate*- 32 P]NAD $^{+}$ was obtained from PerkinElmer Life Sciences (Brussels, Belgium). [*carbonyl*- 14 C]NAD $^{+}$ was from Amersham Biosciences (Freiburg, Germany). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, U.S.A.). All other materials were from standard vendors or from sources described previously [20].

Purification of native G_i (heterotrimeric transducin) and its subunits, α_i and $\beta\gamma_i$

Purification of G_i from bovine rod outer segments and separation into its subunits, α_i and $\beta\gamma_i$, was performed as described in [29]. Briefly, G_i was eluted from rod outer segment membranes with hypotonic buffer containing 100 μ M GTP and separated into α_i and $\beta\gamma_i$ by chromatography on Blue Sepharose CL-6B. The purity of the proteins was at least 95 %, as judged by analysis of Coomassie Blue-stained SDS/polyacrylamide gels.

Production of GST (glutathione S-transferase)- $\alpha_i^{322-350}$

An expression vector encoding a GST- $\alpha_i^{322-350}$ fusion protein was constructed by ligating the *FspI*-*SnaBI* fragment of the baculovirus transfer vector pVL1392 carrying a synthetic gene encoding bovine α_i [30] into the *SmaI* site of pGEX-3X (Amersham Biosciences). The resulting expression vector codes for a fusion protein composed of *Schistosoma japonicum* GST, followed by a SDLI₂GRGIP linker peptide and the C-terminal-most 29 residues of α_i . GST- $\alpha_i^{322-350}$ was expressed in *Escherichia coli* DH5 α and was purified by chromatography on glutathione-Sepharose 4B (Amersham Biosciences) as described by the manufacturer. GST- $\alpha_i^{322-350}$ was eluted from the affinity resin by adding 300 μ l of buffer containing 50 mM Tris/HCl, pH 8.0, and 10 mM reduced glutathione to 250 μ l of a 100 % slurry in 140 mM NaCl, 2.7 mM KCl, 10 mM Na $_2$ HPO $_4$ and 1.8 mM KH $_2$ PO $_4$, pH 7.3. The protein was snap-frozen in liquid N $_2$ and was stored at -80 °C. The purity of the protein was at least 90 %, as judged by analysis of Coomassie Blue-stained SDS/polyacrylamide gels.

Production of the N-acylation-resistant α_i mutant, α_i^{Δ}

Trichoplusia ni 5B1-4 cells (High five cells; Invitrogen) were grown in suspension culture and infected with baculovirus encoding α_i^{Δ} , a N-acylation-resistant mutant of α_i carrying an isoleucine residue in place of the GAGA acylation site present in positions 2–5 of wild-type α_i [31]. Infected cells were washed, resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 2 mM MgCl $_2$, 50 μ M GDP, 2 μ g/ml aprotinin and 1 μ g/ml pepstatin), homogenized and fractionated into soluble and particulate constituents as described in [32]. Recombinant α_i^{Δ} was purified from the soluble fraction (70 mg of protein) using a protocol similar to that described in [31], except that gel-filtration chromatography was performed using a Superdex 75 HR 10/30 column (Amersham Biosciences) and chromatography on Polyanion SI was omitted. The peak fractions obtained by gel filtration, containing ≈ 60 μ g of mostly homogeneous α_i^{Δ} , were pooled, concentrated ≈ 10 -fold using Microsep 30 K concentrators (Pall

Filtron, Dreieich, Germany), snap-frozen in liquid N_2 and stored at $-80^\circ C$.

$[^{32}P]$ ADP-ribosylation by pertussis toxin

Pertussis toxin was activated by incubation for 1 h at $20^\circ C$ at a concentration of $40 \mu g/ml$ in buffer containing 100 mM Tris/HCl, pH 8.0, and 50 mM DTT (dithiothreitol). To examine the effect of S-prenyl analogues on pertussis-toxin-mediated $[^{32}P]$ ADP-ribosylation, $5 \mu l$ of toxin substrate (α_i , GST- $\alpha_i^{322-350}$, G_i or α_i^A) was preincubated for 10 min at $37^\circ C$ with $2.5 \mu l$ of compound, $25 \mu l$ of activated pertussis toxin and $15 \mu l$ of a solution containing 6.7 mM ATP and 105 nM $[^{32}P]NAD^+$ (800 Ci/mmol). The samples were then supplemented with $5 \mu l$ of $\beta\gamma_i$ and the incubation was continued for 60 min at $37^\circ C$. The final concentrations of the toxin substrates, $\beta\gamma_i$ and the S-prenyl analogues are specified in the figure legends.

NAD^+ glycohydrolase assay

The effect of FTS on the NAD^+ glycohydrolase activity of pertussis toxin was determined in a mixture (100 μl) containing 1 μg of pertussis toxin, 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 10 mM DTT, 1 mM ATP, 16.3 mM CHAPS and 19 nM [*carboxyl*- ^{14}C]NAD $^+$ (53 mCi/mmol) ([33] and references therein). The reactions were allowed to proceed for 3 h at $37^\circ C$ in the absence or presence of 100 μM FTS. The samples were then supplemented with 100 μl of ice-cold water and applied to columns containing 0.8 ml of Dowex AG 1-X2 resin (hydroxide form; 200–400 mesh; Bio-Rad, Munich, Germany) that had been converted to the chloride form and equilibrated with 20 mM Tris/HCl, pH 7.5, according to [33]. [*carboxyl*- ^{14}C]Nicotinamide was eluted with 5 ml of 20 mM Tris/HCl, pH 7.5 [33], supplemented with 15 ml of scintillation fluid (Ultima Gold, Canberra-Packard, Dreieich, Germany), and the radioactivity was determined in a liquid scintillation counter.

Gel-filtration chromatography of FTS-treated α_i

Purified α_i (0.3 nmol) was incubated for 10 min at $37^\circ C$ in a volume of 200 μl containing 20 mM Tris/HCl, pH 8.0, 1 mM DTT, 1 mM EDTA and 110 μM FTS. This mixture (190 μl) was applied to a Fast Desalting PC 3.2/10 column (Amersham Biosciences) equilibrated and run at a flow rate of 50 $\mu l/min$ in buffer containing 20 mM Tris/HCl, pH 8.0, 1 mM DTT and 1 mM EDTA using a SMART System (Amersham Biosciences). α_i and FTS eluted at 6–10 min and 30–40 min, respectively, after injection of the sample.

Miscellaneous

The presence of α_i , α_i^A and GST- $\alpha_i^{322-350}$ in column fractions was monitored by immunoblotting using antibodies reactive against the C-terminal decapeptide of α_i [34]. Incorporation of $[^{32}P]$ ADP-ribose into proteins was determined by both autoradiography and PhosphorImagerTM (Molecular Dynamics, Sunnyvale, CA, U.S.A.) analysis of dried gels. Results obtained by PhosphorImagerTM analysis were used to calculate the extent of S-prenyl-derivative-mediated inhibition of $[^{32}P]$ ADP-ribosylation given in the text. All experiments were repeated at least three times. Similar results and identical trends were obtained each time. Data from representative experiments are shown.

Figure 1 Effect of FTS on pertussis toxin-mediated $[^{32}P]$ ADP-ribosylation of α_i

Purified α_i was preincubated for 10 min with $[^{32}P]NAD^+$, DTT-activated pertussis toxin, and increasing concentrations of FTS. The samples were then supplemented with either purified $\beta\gamma_i$ (●) or buffer (○), and the incubation was continued for 60 min. The final concentration of both α_i and $\beta\gamma_i$ was 0.5 μM , the final concentrations of FTS are indicated on the abscissa. The samples were subjected to SDS/PAGE and the incorporation of $[^{32}P]$ ADP-ribose into α_i was determined by PhosphorImagerTM analysis or autoradiography (inset) of the dried gels. Only the ≈ 39 kDa regions of the autoradiographs are shown. No other radiolabelled bands were seen. See the Experimental section for details.

RESULTS

To monitor the interaction of $\beta\gamma_i$ with α_i , we used a well-established method based on the ability of G-protein $\beta\gamma$ dimers to support the pertussis-toxin-mediated $[^{32}P]$ ADP-ribosylation of certain G-protein α subunits ([35], reviewed in [36]). This method gives an apparent measure of the interaction due to the far stronger ADP-ribosylation of the α subunit in the heterotrimeric complex compared with the free α subunit. Figure 1 shows that $\beta\gamma_i$ (0.5 μM) indeed caused an ≈ 10 -fold stimulation of the $[^{32}P]$ ADP-ribosylation of α_i (0.5 μM) by pertussis toxin. Preincubation of α_i with increasing concentrations of the S-prenyl derivative FTS led to a marked ($>95\%$) inhibition of its $[^{32}P]$ ADP-ribosylation, regardless of whether $\beta\gamma_i$ was absent or present during the $[^{32}P]$ ADP-ribosylation reaction (Figure 1). The sensitivity of α_i $[^{32}P]$ ADP-ribosylation to inhibition by FTS was similar in the absence and presence of added $\beta\gamma_i$. Thus half-maximal and maximal inhibition were observed at ≈ 40 and 80 μM FTS, respectively, for both experimental conditions. Note that these concentrations are lower than the critical micelle concentration of FTS, which has been determined to be $\approx 125 \mu M$ [37].

The enzymic activities of the S1 subunit of pertussis toxin, NAD^+ glycohydrolase activity and ADP-ribosyltransferase activity are sensitive to a variety of substances, including thiol reagents, adenine nucleotides, certain phospholipids and detergents (reviewed in [36]). We therefore set out to determine whether the inhibition of α_i $[^{32}P]$ ADP-ribosylation by FTS was due to a direct effect of FTS on the toxin. Table 1 shows that the NAD^+ glycohydrolase activity of pertussis toxin caused the formation of [*carboxyl*- ^{14}C]nicotinamide from [*carboxyl*- ^{14}C]NAD $^+$ and that FTS (100 μM) did not affect this activity of the toxin.

Table 1 Effect of FTS on the NAD⁺ glycohydrolase activity of pertussis toxin

NAD⁺ glycohydrolase activity: [*carbonyl*-¹⁴C]NAD⁺ (1.9 nmol/sample) was incubated in the absence or presence of pertussis toxin (1 µg/sample). The incubation was performed in the absence or presence of 100 µM FTS. Samples were analysed for [*carbonyl*-¹⁴C]nicotinamide as described in the Experimental section. Each value represents the mean ± S.E.M. of triplicate determinations.

	[<i>carbonyl</i> - ¹⁴ C]Nicotinamide (pmol)	
	Control	Pertussis toxin
Control	27.2 ± 2.2	126.9 ± 7.9
FTS	23.9 ± 2.3	130.0 ± 1.0

Figure 2 Effect of FTS on the ADP-ribosyltransferase activity of pertussis toxin

Purified GST- $\alpha_1^{322-350}$ was preincubated for 10 min in the absence or presence of FTS with [³²P]NAD⁺ and DTT-activated pertussis toxin. The samples were then supplemented with either purified $\beta\gamma_1$ or control buffer, and the incubation was continued for 60 min. The final concentration of both GST- $\alpha_1^{322-350}$ and $\beta\gamma_1$ was 0.5 µM. FTS was present at a final concentration of 100 µM. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The position of the [³²P]ADP-ribosylated GST- $\alpha_1^{322-350}$ fusion protein is indicated.

To determine the influence of FTS on the ADP-ribosyltransferase activity of the toxin, the C-terminal-most 29 amino acids of α_1 were expressed as a recombinant GST fusion protein (GST- $\alpha_1^{322-350}$) and used as a substrate for [³²P]ADP-ribosylation by pertussis toxin. This assay is based on the previous observation that pertussis toxin catalyses the [³²P]ADP-ribosylation of synthetic peptides encompassing the last 10–20 amino acids of pertussis-toxin-sensitive α subunits [38]. Figure 2 illustrates that GST- $\alpha_1^{322-350}$ did in fact serve as a substrate for [³²P]ADP-ribosylation by pertussis toxin and that the degree of [³²P]ADP-ribosylation was not affected by $\beta\gamma_1$. More importantly, FTS (100 µM) had no effect on [³²P]ADP-ribosylation of GST- $\alpha_1^{322-350}$, regardless of whether $\beta\gamma_1$ was absent or present in the incubation medium. Taken together, these findings argue against the notion that FTS inhibits pertussis-toxin-mediated [³²P]ADP-ribosylation of α_1 by interfering with the enzymic activities of the toxin.

To investigate the mechanisms of FTS inhibition of α_1 [³²P]ADP-ribosylation in more detail, FTS and $\beta\gamma_1$ were added to the incubation medium at different time points during the course of the [³²P]ADP-ribosylation reaction. Figure 3 shows that the degree of FTS-mediated inhibition was markedly dependent on the order of addition of FTS and $\beta\gamma_1$. Thus FTS inhibition was maximal (> 95 % inhibition at 100 µM FTS) when FTS was added before $\beta\gamma_1$ (Figure 3A), and was clearly reduced when this order was reversed. Specifically, the addition of $\beta\gamma_1$ before FTS (Figure 3B) or together with the inhibitor (Figure 3C) partially protected α_1 from FTS inhibition (to only 65 % at 100 µM FTS). An even stronger reduction in the efficacy of FTS was observed when a preformed heterotrimer was incubated with the inhibitor (Figure 3D).

Figure 3 Pretreatment of purified α_1 with FTS enhances pertussis-toxin-mediated [³²P]ADP-ribosylation of α_1

Purified α_1 was preincubated for 10 min with FTS (A), $\beta\gamma_1$ (B) or both (C) in the presence of [³²P]NAD⁺ and DTT-activated pertussis toxin. The samples were then supplemented with purified $\beta\gamma_1$ (A), FTS (B) or control buffer (C), and the incubation was continued for 60 min. In (D), purified heterotrimeric G_i was incubated for 70 min at increasing concentrations of FTS with [³²P]NAD⁺ and DTT-activated pertussis toxin. The final concentration of α_1 , $\beta\gamma_1$ and G_i was 0.5 µM; the final concentrations of FTS are indicated. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The positions of [³²P]ADP-ribosylated α_1 are indicated.

Figure 4 Reversibility of the effect of FTS on pertussis-toxin-mediated [³²P]ADP-ribosylation of α_1

Purified α_1 (0.3 nmol) was pretreated in a volume of 200 µl as indicated at the abscissa by incubation for 10 min in the absence (lanes 1 and 2, reading from the left) or presence (lanes 3 and 4) of 110 µM FTS. An aliquot (10 µl) of each sample was kept on ice and the remainder of each sample (190 µl) was subjected to gel-filtration chromatography to separate α_1 from FTS or its solvent. The two aliquots kept on ice (lanes 1 and 3) and aliquots (10 µl) of the peak fractions obtained by gel filtration containing α_1 (lanes 2 and 4) were then supplemented with purified $\beta\gamma_1$ (0.5 µM) and incubated for 60 min with [³²P]NAD⁺ and DTT-activated pertussis toxin. See the Experimental section for details. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The position of [³²P]ADP-ribosylated α_1 is indicated.

The observation that FTS inhibited α_1 [³²P]ADP-ribosylation maximally when α_1 was preincubated with FTS in the absence of $\beta\gamma_1$ raised the possibility that interaction of FTS with free α_1 caused an irreversible structural alteration of α_1 , thus rendering the protein resistant to [³²P]ADP-ribosylation. To challenge this hypothesis, α_1 was preincubated with FTS (110 µM) and was then subjected to gel-permeation chromatography to remove the low-molecular-mass S-prenyl derivative. Figure 4 shows that the inhibition of α_1 [³²P]ADP-ribosylation induced by FTS preincubation was fully reversed upon the removal of FTS from α_1 by gel filtration. Irreversible structural alteration of free α_1 by FTS is, therefore, unlikely to be the cause of FTS-mediated inhibition of α_1 [³²P]ADP-ribosylation.

Next, we set out to characterize the structure–function relationships of S-prenyl analogues with respect to their ability to suppress pertussis-toxin-mediated [³²P]ADP-ribosylation of α_1 . Figure 5, top panel, shows that inhibition of α_1 [³²P]ADP-ribosylation was

Figure 5 Comparison of the effects of FTS and structurally related prenylated compounds on pertussis-toxin-mediated [32 P]ADP-ribosylation of α_i

Purified α_i was preincubated for 10 min as indicated in the presence of increasing concentrations of GTS, FTS, GGTS or L-AFC (top panel), or FTS, FCOOH, 5-F-FTS, 4-F-FTS, 5-Cl-FTS or 4-Cl-FTS (bottom panel) with [32 P]NAD $^{+}$ and DTT-activated pertussis toxin. The samples were then supplemented with purified $\beta\gamma_i$ and the incubation was continued for 60 min. The final concentration of α_i was 0.5 μ M; the final concentrations of FTS and its analogues are indicated. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The positions of [32 P]ADP-ribosylated α_i are indicated.

critically dependent on the length of the isoprenyl moiety of the S-prenylcysteine mimetic. Among the isoprenyl analogues tested, the C $_{20}$ compound GGTS was the most potent inhibitor of [32 P]ADP-ribosylation (IC $_{50}$, $\approx$ 20 μ M), followed by the C $_{15}$ compound FTS (IC $_{50}$, $\approx$ 40 μ M). GTS, which has a C $_{10}$ isoprenyl group, did not affect [32 P]ADP-ribosylation of α_i at concentrations of up to 100 μ M. In contrast with GTS, other S-prenyl analogues, which are FTS derivatives (Figure 5, bottom panel), as well as L-AFC (Figure 5, top panel), inhibited α_i [32 P]ADP-ribosylation. The rank order of potency of these compounds, as determined at 50 and 100 μ M (Figure 5, bottom panel) was: 4-Cl-FTS > 5-Cl-FTS > 4-F-FTS > 5-F-FTS $\approx$ FTS. Farnesoic acid (FCOOH),

Figure 6 Reversal of FTS inhibition of pertussis-toxin-mediated [32 P]ADP-ribosylation of α_i by $\beta\gamma_i$

Purified α_i was preincubated as indicated for 10 min in the absence (lanes 1, 3, 5 and 7, reading from the left) or presence (lanes 2, 4, 6 and 8) of 75 μ M FTS with [32 P]NAD $^{+}$ and DTT-activated pertussis toxin. The samples were then supplemented with increasing concentrations of $\beta\gamma_i$ and the incubation was continued for 60 min. The final concentration of α_i was 0.5 μ M; the final concentrations of $\beta\gamma_i$ are indicated. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The position of [32 P]ADP-ribosylated α_i is indicated.

which lacks a thioether, had no detectable inhibitory activity (Figure 5, bottom panel).

Interestingly, the S-prenylated cysteine analogue L-AFC also inhibited [32 P]ADP-ribosylation of α_i by pertussis toxin, albeit only at higher concentrations (IC $_{50}$, $\approx$ 200 μ M). In additional experiments (results not shown), we found that the S-prenylated cysteine analogue L-AFC also caused a marked (>95%) inhibition of $\beta\gamma_i$ -stimulated [32 P]ADP-ribosylation of insect-cell-expressed recombinant α_{i2} when preincubated with this α subunit before addition of $\beta\gamma_i$. Removal of L-AFC from L-AFC-pretreated α_{i2} by gel filtration completely restored $\beta\gamma_i$ -stimulated [32 P]ADP-ribosylation of α_{i2} . These findings have important structural implications, because L-AFC shares with FTS the S-farnesyl and the carboxyl moieties; however, unlike in FTS, these moieties can rotate relative to each other in L-AFC. Therefore, although the farnesyl and carboxyl groups are important for the inhibitory effects, their rigid orientation is not.

The observation that $\beta\gamma_i$ was capable of protecting, at least to some degree, α_i from FTS-mediated inhibition of [32 P]ADP-ribosylation (cf. Figures 3B and 3C), prompted us to determine whether $\beta\gamma_i$ was capable of reversing the inhibition of [32 P]ADP-ribosylation of FTS-pretreated α_i . To this end, α_i was preincubated at a fixed concentration of FTS followed by incubation with increasing concentrations of $\beta\gamma_i$ during the [32 P]ADP-ribosylation reaction (Figure 6). At 0.5 μ M $\beta\gamma_i$, FTS (75 μ M) inhibited [32 P]ADP-ribosylation of α_i (0.5 μ M) by $\approx$ 90%. Importantly, there was a complete loss of FTS inhibition upon increasing the concentration of $\beta\gamma_i$ from 0.5 to 5 μ M.

The competitive nature of the effects of FTS and $\beta\gamma_i$ on α_i [32 P]ADP-ribosylation raised the possibility that FTS inhibited $\beta\gamma_i$ -stimulated α_i [32 P]ADP-ribosylation by specifically interfering with $\alpha_i \cdot \beta\gamma_i$ heterotrimer formation. The previous suggestion that the isoprenylated C-terminus of γ_i interacts directly with the acylated N-terminus of α_i [24] led us to determine the role of the N-acyl group of α_i in mediating FTS inhibition of [32 P]ADP-ribosylation. For that reason, α_i^{Δ} , an N-acylation-resistant mutant of α_i was produced as a recombinant cytosolic protein in baculovirus-infected insect cells, purified to near homogeneity, and used as a substrate for pertussis-toxin-mediated [32 P]ADP-ribosylation (Figure 7). In agreement with a previous report on α_o [39], the extent of α_i^{Δ} [32 P]ADP-ribosylation was reduced compared with native α_i (results not shown) and $\beta\gamma_i$ (0.5 μ M) increased the [32 P]ADP-ribosylation of α_i^{Δ} (0.5 μ M) only 2.3-fold. However, FTS (100 μ M) did inhibit α_i^{Δ} [32 P]ADP-ribosylation in the absence and presence of $\beta\gamma_i$. Specifically,

Figure 7 Effect of FTS on pertussis-toxin-mediated [32 P]ADP-ribosylation of the N-acylation-resistant α_i mutant α_i^Δ

Control buffer (lane 1) or purified α_i^Δ (lanes 2–5) were preincubated for 10 min in the absence (lanes 1, 2 and 4) or presence (lanes 3 and 5) of FTS with [32 P]NAD $^+$ and DTT-activated pertussis toxin. The samples were then supplemented with purified $\beta\gamma_i$ (lanes 1, 4 and 5) or control buffer (lanes 2 and 3) and the incubation was continued for 60 min. The final concentration of α_i^Δ and $\beta\gamma_i$ was 0.5 μ M. FTS was present at a final concentration of 100 μ M. The samples were subjected to SDS/PAGE and autoradiography of the dried gel was performed. The position of [32 P]ADP-ribosylated α_i^Δ is indicated.

FTS inhibition was ≈ 75 and 95 %, respectively, under these conditions. These data clearly indicate that an interaction of the S-prenyl analogues with the acylated N-terminus of α_i is unlikely to be the basis of their inhibitory effect on α_i [32 P]ADP-ribosylation.

DISCUSSION

In the present study, we investigated the interactions of the acylated α subunit of retinal transducin, α_i , and the prenylated $\beta\gamma$ dimer, $\beta\gamma_i$, in an attempt to gain knowledge of possible protein–lipid interactions among the G-protein subunits. Such interactions may be of regulatory importance for both the intrinsic functions and the membrane interaction of the G-protein. The possible existence and role of protein–lipid interactions within heterotrimeric G-proteins have not been thoroughly studied previously; biochemical experiments have so far been performed mostly with membrane-bound and/or detergent-solubilized preparations. Crystallographic [15–18] or EPR studies [40] have employed truncated, unmodified versions of G-protein subunits.

Here we used purified, fully processed α_i and $\beta\gamma_i$, which are soluble in aqueous solutions without detergents [41], and examined the $\beta\gamma$ dimer-stimulated, pertussis-toxin-mediated [32 P]ADP-ribosylation of a G-protein α subunit, a well-established method to detect G-protein heterotrimer formation [35]. We investigated the interactions of processed $\beta\gamma$ and α subunits in a cell-free system free of membranes, lipids or detergents. This enabled studies on the formation of the lipid-modified heterotrimer without the interference by lipids or membrane constituents, in a system where the concentration of all protein subunits, the periods of incubation with and without S-prenyl analogues, and the order of the addition of reactants are fully controlled. We show that preincubation of α_i with increasing concentrations of S-prenyl analogues such as FTS, GGTS or L-AFC markedly inhibits the $\beta\gamma$ dimer-stimulated, pertussis-toxin-mediated α subunit [32 P]ADP-ribosylation. The results presented herein suggest that the prenyl group of the $\beta\gamma$ dimer interacts with the α subunit and that the S-prenyl analogues interfere with this interaction.

Several lines of evidence are consistent with the above notion. First, we ruled out the possibility of an inhibitory effect of FTS on the pertussis toxin itself. To this end, we tested the influence of

FTS on the NAD $^+$ glycohydrolase activity of pertussis toxin and the toxin-mediated ADP-ribosylation of a small peptide derived from the C-terminus of α_i . FTS failed to inhibit both activities, indicating the lack of a direct effect on pertussis toxin itself. The fact that $\beta\gamma_i$ did not affect [32 P]ADP-ribosylation of GST- $\alpha_i^{322-350}$ also argues against the notion that $\beta\gamma$ dimers enhance [32 P]ADP-ribosylation of α_i by interacting with the S1 subunit of pertussis toxin rather than with α_i . A similar conclusion has previously been reached on the basis of studies with a C-terminal peptide of α_{i3} [38,42]. Secondly, the inhibition by FTS or L-AFC was reversible, since it was eliminated by filtering away the inhibitor after preincubation with the α subunit. This ruled out irreversible effects of the inhibitors. Thirdly, we showed that the inhibitory effects of FTS are independent of the acyl group of the α subunit. This rules out the possibility that the observed inhibition by S-prenyl analogues is due to interference with an interaction between the acyl group of α and the prenyl group of $\beta\gamma$. Fourthly, we demonstrated that inhibition of pertussis-toxin-mediated α subunit [32 P]ADP-ribosylation by FTS was abolished by increasing the concentration of $\beta\gamma$ dimers. This shows the apparently competitive nature of FTS and $\beta\gamma$ interaction with the α subunit. Since the most obvious common chemical entity in $\beta\gamma$ and the inhibitors is the S-prenyl moiety, and since FTS effectively inhibited the [32 P]ADP-ribosylation of a non-acylated α subunit in the absence of $\beta\gamma$, our results suggest that the α subunit contains an S-prenyl-binding site; moreover, we demonstrate that FTS binding to this site competes with binding of $\beta\gamma$ to α .

Our structure–activity data also support this possibility. We showed that only S-prenyl analogues with an S-farnesyl moiety (FTS, its halogenic analogues and L-AFC) or with an S-geranylgeranyl moiety (GGTS) inhibited heterotrimer formation. Although one would expect that the putative S-prenyl-binding site on α_i adapts better to farnesylated FTS than to geranylgeranylated GGTS, it is important to note that recombinant $\beta_1\gamma_1$ carrying a mutation in the γ_1 subunit directing C-terminal geranylgeranylation rather than farnesylation was previously shown to be a more potent stimulator of pertussis-toxin-mediated [32 P]ADP-ribosylation of α_i than wild-type recombinant $\beta_1\gamma_1$ [43]. Neither the C $_{10}$ GTS analogue, with its apparently too short S-prenyl group, nor FCOOH, lacking the thioether bond, inhibited the pertussis toxin-mediated α subunit [32 P]ADP-ribosylation. The observation that FCOOH, a close mimic of the amphiphilic structure of the inhibitory S-prenyl analogues (FTS, its halogenic analogues and L-AFC) did not affect $\beta\gamma_i$ -stimulated α_i [32 P]ADP-ribosylation argues against the notion that the inhibitors take effect by virtue of a non-specific, detergent-like property. Along the same lines, the order of the K_a values of the polar head groups of the S-prenyl analogues is 5-Cl-benzoic acid > 5-F-benzoic acid > 4-Cl-benzoic acid > 4-F-benzoic acid > benzoic acid [44], which is different from the order of potency of their S-prenyl derivatives, determined here to be 4-Cl-FTS > 5-Cl-FTS > 4-F-FTS > 5-F-FTS $\approx$ FTS. Thus differences in the ionization of the aromatic carboxyl group are unlikely to be the basis of the different potencies of FTS and its congeners to inhibit α_i [32 P]ADP-ribosylation.

A large body of evidence supports the notion that lipid modifications of proteins contribute to their membrane targeting, subcellular localization and functions via both lipid–lipid and lipid–protein interactions (reviewed in [45]). For example, most acylated proteins (S-palmitoylated and/or N-myristoylated) and glycosylphosphatidylinositol-anchored proteins are enriched in cholesterol/glycosphingolipid membrane microdomains, termed lipid rafts [45]. Localization to rafts strongly depends on the long-chain acyl groups that favour ordered membrane structure [45]. It is less clear how the branched and bulky structure of

isoprenoid groups supports membrane interactions of prenylated proteins, such as the $\beta\gamma$ dimers of heterotrimeric G-proteins and members of the Ras, Rho and Rab families of GTPases. Nonetheless, recent structural and biochemical data provided strong evidence for direct lipid–protein interactions of prenylated proteins. The best example is given by structures of the co-crystals of the geranylgeranylated Rho GTPases and their inhibitory proteins RhoGDIs (Rho GDP dissociation inhibitors). The crystal structures of RhoGDI-1 in complex with fully processed Cdc42 [46] and the structure of RhoGDI-2 (or LyGDI, a RhoGDI originally identified in lymphocytes, but subsequently also found in other cells) in complex with fully processed Rac2 have been solved [47]. The results of these studies show that the RhoGDIs have two main domains: the regulatory domain that includes the regulatory arm, which binds to switch 1 and 2 of Cdc42 or Rac2 (blocking GDP dissociation), and an immunoglobulin-like domain [48]. The latter domain folds into a β -sandwich, shown to be responsible for extracting Cdc42 from the cell membrane [49]. The immunoglobulin-like domain binds the C-terminal region of Cdc42 and its structure allows the insertion of the geranylgeranyl isoprenoid moiety into a hydrophobic pocket residing between the two layers of the β sheets [46]. Thus isoprenoid moieties of other proteins may also associate with specific hydrophobic pockets of their binding partners. Our biochemical experiments with α_i and $\beta\gamma_i$ dimers are consistent with this possibility.

Interestingly, FTS inhibits not only the $\beta\gamma_i$ -stimulated [32 P]ADP-ribosylation of α_i , but also the [32 P]ADP-ribosylation of α_i in the absence of $\beta\gamma_i$ (Figure 1). In view of the lack of effect of FTS on pertussis toxin itself (Table 1), this suggests that the S-prenyl analogue mediates the effect by binding to a site in α_i . This site is on the α_i polypeptide chain and does not involve interactions with the α_i acyl moiety, since FTS is also capable of inhibiting the [32 P]ADP-ribosylation by pertussis toxin of the α_i^{Δ} non-acylated mutant (Figure 7). Although we cannot formally exclude the possibility that purified α_i^{Δ} contained $\beta\gamma$ dimers as a contaminant, we think that this is an unlikely possibility, since non-myristoylated α_i subunits have an exceedingly low affinity for isoprenylated $\beta\gamma$ dimers [21], and the presence of $\beta\gamma$ subunits in soluble fractions of Sf9 insect cells has previously been ruled out by stringent criteria [50]. In additional experiments (results not shown), we found that FTS (100 μ M) caused a substantial ($\approx 50\%$) inhibition of pertussis-toxin-mediated [32 P]ADP-ribosylation of bacterially expressed, non-lipidated rat α_{i1} that had been purified via an internal hexahistidine tag. Since $\beta\gamma$ dimers are absent from and cannot be made in bacteria [11], the latter results attest to the fact that FTS inhibits pertussis-toxin-mediated [32 P]ADP-ribosylation of G-protein α subunits in the definite absence of $\beta\gamma$ dimers.

Thus the direct effect of FTS on α_i [32 P]ADP-ribosylation by pertussis toxin may arise either due to a conformational change mediated by FTS binding, or by direct interference of FTS with pertussis-toxin binding to α_i . In favour of the first possibility is the finding that [32 P]ADP-ribosylation by pertussis toxin of GST- $\alpha_i^{325-350}$, which contains the ADP-ribosylation site, is not inhibited by FTS (Figure 2). Consistent with our suggestion of an isoprenoid-mediated conformational change is the precedence of the conformational change induced in RhoGDI-1 by Cdc42 binding [46] or upon interaction with L-AFC [48]. Thus FTS has a dual effect on α_i : it competes with $\beta\gamma_i$ for binding to α_i and, in addition, appears to mediate a conformational change in α_i that inhibits pertussis-toxin-mediated ADP-ribosylation. The isoprenyl group of the γ subunit most likely binds to the same site and may, in principle, mediate an analogous inhibitory conformational change. If this is the case, the stimulatory effect of the isoprenylated $\beta\gamma$ dimer suggests that there must be regions

in the $\beta\gamma$ polypeptide that overcome the inherent inhibitory effect of the γ subunit isoprenyl group.

It is important to emphasize that the crystal structures of G-protein heterotrimers [15–18] did not provide information on possible sites for lipid moiety binding in the α or $\beta\gamma$ subunits. Interestingly, the contacts between the α subunit and the $\beta\gamma$ dimer occur primarily between the α and β subunits, while the γ subunit lies across the face of the β subunit without direct contact with the α subunit. However, the C-terminus of the γ subunit lies within 18 Å (1 Å $\equiv$ 0.1 nm) of the N-terminus of the α subunit and is also relatively close to the α subunit's C-terminus [15]. Although it was proposed that the proximity of the N-terminus of the α to the C-terminus of the γ would favour an insertion of both lipid modifications in the membrane [15,17], the presence of lipid modifications might also change the conformation of neighbouring protein structures, as described for the hepatitis virus delta antigen [51]. Thus EPR of cysteine-modified α_{i1} subunits indicated that their N-termini are disordered and become α -helical only upon interaction with $\beta\gamma$ [40]. In this context, it is important to note that a monoclonal antibody directed against the N-terminal-most portion of α_i [52] has previously been shown to block $\alpha_i \cdot \beta\gamma_i$ heterotrimer formation [53]. Furthermore, mutations in α_o corresponding to Lys-17 and Lys-31 of α_i are not [32 P]ADP-ribosylated by pertussis toxin in the presence of $\beta\gamma_i$ [54]. Both residues are exposed to the solvent and neither contacts non-isoprenylated $\beta\gamma_i$ [15].

On a final note, we would like to mention that the analysis of the crystal structure of a complex of bovine retinal phosducin and $\beta\gamma_i$ has previously shown that the binding of phosducin induces a distinct structural change in the β propeller of $\beta\gamma_i$, such that the farnesyl moiety becomes effectively buried in a cavity formed between blades 6 and 7 of the β subunit [55]. This cavity is specifically induced by the association of $\beta\gamma_i$ and is also observed in a complex made up of phosducin and non-prenylated $\beta\gamma_i$, but not in the crystal structures of free $\beta\gamma_i$ or heterotrimeric G_i [55]. The contribution, if any, of this potential isoprenyl-binding site to the effects of S-prenyl analogues when heterotrimeric G-proteins, rather than monomeric α subunits, are studied remains to be investigated.

In conclusion, our data point to the existence of a hitherto unknown S-prenyl site in α_i , in addition to the two known $\beta\gamma$ -binding sites. We propose that such a site could reside within the yet unresolved N-terminus of the α subunit, because no hydrophobic pocket was observed in the published structures.

This work was supported in part by grant no. I-599-165.13/98 from the German-Israeli Foundation for Scientific Research and Development (to Y.I.H. and P.G.) and by a grant from the Deutsche Forschungsgemeinschaft (SFB 497; to P.G. and D.I.). We are grateful to Dr Eva Faurobert, Dr Joëlle Bigay and Dr Christiane Kleuss for generously providing the cDNAs used in this study. The expert technical assistance of Sandra Beier is greatly appreciated.

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Received 17 April 2003/14 August 2003; accepted 3 September 2003

Published as BJ Immediate Publication 3 September 2003, DOI 10.1042/BJ20030578