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

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INTRODUCTION

Heterotrimeric (\(\alpha \cdot \beta\gamma\)) 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-ligated \(\alpha\) subunit and the free \(\beta\gamma\) dimer. Both \(\alpha\)-GTP and \(\beta\gamma\) are capable of mediating effector regulation [1].

At least 20 \(\alpha\), 6 \(\beta\) and 12 \(\gamma\) 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 \(\alpha\) subunits are N-myristoylated, S-palmitoylated or both at their N-termini [2,5]. These modifications are important for membrane targeting of \(\alpha\) subunits and their interaction with G-protein \(\beta\gamma\) dimers [3,5]. The \(\alpha\) subunit of retinal rod transducin, \(\alpha\), is heterogeneously N-acylated at its N-terminal glycine residue [6,7]. G-protein \(\gamma\) 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 (\(\gamma_1\), \(\gamma_2\) and \(\gamma_11\)) or geranylgeranylated (all other \(\gamma\) 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 \(\gamma\) 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 \(\alpha\) 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, \(\alpha\) 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,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

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[32P]ADP-ribosylation of G-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 α · βγ 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 βγ 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 αi and βγ, in the absence of detergents and lipids, which was not observed for αi and βγ, 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 βγ dimers, inhibited the pertussis-toxin-mediated, βγ, stimulated [32P]ADP-ribosylation of αi. In a related study, Rahmatullah and Robishaw [23] found that a recombinant prenylated γ2 subunit that had been expressed in the absence of β specifically interacted with an αi- affinity matrix, whereas the non-prenylated γ2 did not. Parish et al. [24] showed that demethylation of βγ dimer did not affect the pertussis-toxin-mediated [32P]ADP-ribosylation of the αi subunit and had only minor effects on membrane and effector interaction of βγ. In a more recent paper [25], the authors described an ≈ 40–50% inhibition of βγ, stimulated pertussis-toxin-mediated [32P]ADP-ribosylation of αi by FTS (S-trans,farnesyl thiosalicylic acid), FTP [S-(trans,farnesyl-3-thio)propionic acid], FCOOH (S-trans,farnesylthiocarboxylic acid) and FTA [S-(trans,farnesyl-3-thio)acetonic acid], and concluded that the inhibitors interfered with a lipid–lipid-based association of αi and βγ, 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], αi and βγ, 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 αi and βγ, 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 βγ, dimers to αi 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 βγ, dimers for binding to a farnesyl-recognition domain in the αi 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-32P]NAD+ was obtained from PerkinElmer Life Sciences (Brussels, Belgium). [carboxyl-14C]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 Gt (heterotrimeric transducin) and its subunits, αs and βγ**

Purification of Gt from bovine rod outer segments and separation into its subunits, αs and βγ, was performed as described in [29]. Briefly, Gt was eluted from rod outer segment membranes with hypotonic buffer containing 100 mM GTP and separated into αs and βγ, 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)–α322–350**

An expression vector encoding a GST–α322–350 fusion protein was constructed by ligating the FspI–SmaI fragment of the baculovirus transfer vector pVL1392 carrying a synthetic gene encoding bovine αi [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 SDLIEGRGIp linker peptide and the C-terminal-most 29 residues of αs. GST–α322–350 was expressed in Escherichia coli DH5α and was purified by chromatography on glutathione–Sepharose 4B (Amersham Biosciences) as described by the manufacturer. GST–α322–350 was eluted from the affinity resin by adding 300 µl of buffer containing 50 mM Tris/HCl, pH 8.0, and 10 mM reduced glutathione to 250 µl of a 100% slurry in 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.3. The protein was snap-frozen in liquid N2 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 αi mutant, αiA**

_Tricltoplasia ni_ 5B1-4 cells (High five cells; Invitrogen) were grown in suspension culture and infected with baculovirus encoding αiA, a N-acylation-resistant mutant of αi, carrying an isoleucine residue in place of the GAGA acylation site present in positions 2–5 of wild-type αi [31]. Infected cells were harvested, resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 2 mM MgCl2, 50 µM GDP, 2 µg/ml aprotinin and 1 µg/ml pepstatin), homogenized and fractionated into soluble and particulate constituents as described in [32]. Recombinant αi 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 PolyAion SI was omitted. The peak fractions obtained by gel filtration, containing ≈ 60 µg of mostly homogeneous αiA, were pooled, concentrated ≈ 10-fold using Microsep 30 K concentrators (Pall
\( ^{25} \)P\ADP-ribosylation by pertussis toxin

Pertussis toxin was activated by incubation for 1 h at 20°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 \( ^{25} \)P\ADP-ribosylation, 5 \( \mu \)l of toxin substrate (\( \alpha \), GST-\( \alpha \), \( \alpha \)-GTP, \( \alpha \), G, or \( \alpha \)-GTP) was preincubated for 10 min at 37°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 \( \beta \gamma \) and the incubation was continued for 60 min at 37°C. The final concentrations of the toxin substrates, \( \beta \gamma \), and the S-prenyl analogues are specified in the figure legends.

\textbf{NAD\(^{+}\) glycohydrolase assay}

The effect of FTS on the NAD\(^{+}\) glycohydrolase activity of pertussis toxin was determined in a mixture (100 \( \mu \)l) containing 1 \( \mu \)g 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 [carbonyl-\( ^{14} \)C\]nicotinamide was eluted with 5 \( \mu \)l of 20 mM Tris/HCl, pH 7.5, according to [33], [carbonyl-\( ^{14} \)C\]nicotinamide was eluted with 5 \( \mu \)l of 20 mM Tris/HCl, pH 7.5 [33], supplemented with 15 \( \mu \)l of scintillation fluid (Ultima Gold, Canberra-Packard, Dreieich, Germany), and the radioactivity was determined in a liquid scintillation counter.

\textbf{Gel-filtration chromatography of FTS-treated \( \alpha \)\(_{t} \)}

Purified \( \alpha \)\(_{t} \) (0.3 nmol) was incubated for 10 min at 37°C in a volume of 200 \( \mu \)l containing 20 mM Tris/HCl, pH 8.0, 1 mM EDTA and 110 \( \mu \)M FTS. This mixture (190 \( \mu \)l) 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). \( \alpha \)\(_{t} \) and FTS eluted at 6–10 min and 30–40 min, respectively, after injection of the sample.

\textbf{Miscellaneous}

The presence of \( \alpha \)\(_{t} \), \( \alpha \)\(_{t}\)\(^{\Delta} \) and GST-\( \alpha \)\(_{322-350} \) in column fractions was monitored by immunoblotting using antibodies reactive against the C-terminal decapetide of \( \alpha \) [34]. Incorporation of \( ^{32} \)P\ADP-riboside into proteins was determined by both autoradiography and PhosphorImager\(^{\text{TM}}\) (Molecular Dynamics, Sunnyvale, CA, U.S.A.) analysis of dried gels. Results obtained by PhosphorImager\(^{\text{TM}}\) analysis were used to calculate the extent of S-prenyl-derivative-mediated inhibition of \( ^{32} \)P\ADP-ribosilation 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.
To determine the influence of FTS on the ADP-ribosyltransferase activity of the toxin, the C-terminal most 29 amino acids of αt were expressed as a recombinant GST fusion protein (GST–αt322–350) 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-riboseylation of synthetic peptides encompassing the last 10–20 amino acids of pertussis-toxin-sensitive α subunits [38]. Figure 2 illustrates that GST–αt322–350 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 βγt. More importantly, FTS (100 µM) had no effect on [32P]ADP-ribosylation of GST–αt322–350, regardless of whether βγt 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 αt by interfering with the enzymic activities of the toxin.

To investigate the mechanisms of FTS inhibition of αt, [32P]ADP-ribosylation in more detail, FTS and βγt 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 βγt. Thus FTS inhibition was maximal (> 95% inhibition at 100 µM FTS) when FTS was added before βγt (Figure 3A), and was clearly reduced when this order was reversed. Specifically, the addition of βγt, before FTS (Figure 3B) or together with the inhibitor (Figure 3C) partially protected αt 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).

The observation that FTS inhibited αt [32P]ADP-ribosylation maximally when αt was preincubated with FTS in the absence of βγt, raised the possibility that interaction of FTS with free αt, caused an irreversible structural alteration of αt, thus rendering the protein resistant to [32P]ADP-ribosylation. To challenge this hypothesis, αt 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 αt [32P]ADP-ribosylation induced by FTS preincubation was fully reversed upon the removal of FTS from αt by gel filtration. Irreversible structural alteration of free αt by FTS is, therefore, unlikely to be the cause of FTS-mediated inhibition of αt [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 αt. Figure 5, top panel, shows that inhibition of αt [32P]ADP-ribosylation was
which are FTS derivatives (Figure 5, bottom panel), as well as of up to 100 µM, did not affect 
[32P]ADP-ribosylation (IC50, ≈ 200 µM). In additional experiments (results not shown), we found that the S-prenylated cysteine analogue L-AFC also caused a marked (> 95%) inhibition of βγ-stimulated [32P]ADP-ribosylation of insect-cell-expressed recombinant α2 when preincubated with this α subunit before addition of βγ. Removal of L-AFC from L-AFC-pretreated α2 by gel filtration completely restored βγ-stimulated [32P]ADP-ribosylation of α2. 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 βγ was capable of protecting, at least to some degree, α from FTS-mediated inhibition of [32P]ADP-ribosylation (cf. Figures 3B and 3C), prompted us to determine whether βγ was capable of reversing the inhibition of [32P]ADP-ribosylation of FTS-pretreated α1. To this end, α1 was preincubated at a fixed concentration of FTS followed by incubation with increasing concentrations of βγ during the [32P]ADP-ribosylation reaction (Figure 6). At 0.5 µM βγ, FTS (75 µM) inhibited [32P]ADP-ribosylation of α1 (0.5 µM) by ≈ 90%. Importantly, there was a complete loss of FTS inhibition upon increasing the concentration of βγ, from 0.5 to 5 µM.

The competitive nature of the effects of FTS and βγ on α1 [32P]ADP-ribosylation raised the possibility that FTS inhibited βγ-stimulated α1 [32P]ADP-ribosylation by specifically interfering with α1:βγ heterotrimer formation. The previous suggestion that the isoprenylated C-terminus of γi interacts directly with the acylated N-terminus of αi [24] led us to determine the role of the N-acetyl group of αi in mediating FTS inhibition of [32P]ADP-ribosylation. For that reason, αiΔN, an N-acetylation-resistant mutant of αi, 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 [32P]ADP-ribosylation (Figure 7). In agreement with a previous report on αs [39], the extent of αiΔN [32P]ADP-ribosylation was reduced compared with native αi (results not shown) and βγi (0.5 µM) increased the [32P]ADP-ribosylation of αiΔN (0.5 µM) only 2.3-fold. However, FTS (100 µM) did inhibit αiΔN [32P]ADP-ribosylation in the absence and presence of βγ. Specifically,
Figure 7  Effect of FTS on pertussis-toxin-mediated $^{32}$PAD-ribosylation of the N-acylation-resistant $\alpha_t$ mutant $\alpha_t^A$.

Control buffer (lane 1) or purified $\alpha_t^A$ (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 $\beta\gamma_t$ (lanes 1, 4 and 5) or control buffer (lanes 2 and 3) and the incubation was continued for 60 min. The final concentration of $\alpha_t^A$ and $\beta\gamma_t$ 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 $^{32}$PAD-ribosylated $\alpha_t^A$ is indicated.

FTS inhibition was $\approx$75 and 95 %, respectively, under these conditions. These data clearly indicate that an interaction of the the S-prenyl analogues with the acylated N-terminus of $\alpha_t$ is unlikely to be the basis of their inhibitory effect on $\alpha_t$ $^{32}$PAD-ribosylation.

**DISCUSSION**

In the present study, we investigated the interactions of the acylated $\alpha_t$ subunit of retinal transducin, $\alpha_t$, and the prenylated $\beta\gamma_t$ dimer, $\beta\gamma_t$, 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 $\alpha_t$ and $\beta\gamma_t$, which are soluble in aqueous solutions without detergents [41], and examined the $\beta\gamma_t$ dimer-stimulated, pertussis-toxin-mediated $^{32}$PAD-ribosylation of a G-protein $\alpha_t$ subunit, a well-established method to detect G-protein heterotrimer formation [35]. We investigated the interactions of processed $\beta\gamma_t$ and $\alpha_t$ 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 $\alpha_t$ with increasing concentrations of S-prenyl analogues such as FTS, GGTS or L-AFC markedly inhibits the $\beta\gamma_t$ dimer-stimulated, pertussis-toxin-mediated $\alpha_t$ subunit $^{32}$PAD-ribosylation. The results presented herein suggest that the prenyl group of the $\beta\gamma_t$ dimer interacts with the $\alpha_t$ 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 $\alpha_t$. FTS failed to inhibit both activities, indicating the lack of a direct effect on pertussis toxin itself. The fact that $\beta\gamma_t$ did not affect $^{32}$P ADP-ribosylation of GST–$\alpha_t$ also argues against the notion that $\beta\gamma_t$ dimers enhance $^{32}$P ADP-ribosylation of $\alpha_t$ by interacting with the S1 subunit of pertussis toxin rather than with $\alpha_t$. A similar conclusion has previously been reached on the basis of studies with a C-terminal peptide of $\alpha_t$ [38, 42]. Secondly, the inhibition by FTS or L-AFC was reversible, since it was eliminated by filtering away the inhibitor before preincubation with the $\alpha_t$ 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 $\alpha_t$ 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 $\alpha_t$ and the prenyl group of $\beta\gamma_t$.

Fourthly, we demonstrated that inhibition of pertussis-toxin-mediated $\alpha_t$ subunit $^{32}$PAD-ribosylation by FTS was abolished by increasing the concentration of $\beta\gamma_t$ dimers. This shows the apparently competitive nature of FTS and $\beta\gamma_t$ interaction with the $\alpha_t$ subunit. Since the most obvious common chemical entity in $\beta\gamma_t$ and the inhibitors is the S-prenyl moiety, and since FTS effectively inhibited the $^{32}$P ADP-ribosylation of a non-acylated $\alpha_t$ subunit in the absence of $\beta\gamma_t$, our results suggest that the $\alpha_t$ subunit contains an S-prenyl-binding site; moreover, we demonstrate that FTS binding to this site competes with binding of $\beta\gamma_t$ to $\alpha_t$.

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 $\alpha_t$ adapts better to farnesylated FTS than to geranylgeranylated GGTS, it is important to note that recombinant $\beta\gamma_t$ carrying a mutation in the $\gamma_t$ 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 $\alpha_t$ than wild-type recombinant $\beta\gamma_t$ [43]. Neither the C10 GTS analogue, with its apparently too short structure of the inhibitory S-prenyl analogues (FTS, its halogenic analogues and L-AFC) did not affect $^{32}$PAD-ribosylation of $\alpha_t$ subunit. This rules out the possibility that the observed inhibition by FTS or L-AFC was abolished by increasing the concentration of $\beta\gamma_t$ dimers. This shows the apparently competitive nature of FTS and $\beta\gamma_t$ interaction with the $\alpha_t$ subunit. Since the most obvious common chemical entity in $\beta\gamma_t$ and the inhibitors is the S-prenyl moiety, and since FTS effectively inhibited the $^{32}$P ADP-ribosylation of a non-acylated $\alpha_t$ subunit in the absence of $\beta\gamma_t$, our results suggest that the $\alpha_t$ subunit contains an S-prenyl-binding site; moreover, we demonstrate that FTS binding to this site competes with binding of $\beta\gamma_t$ to $\alpha_t$.

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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 $\beta$-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 geranylgeranylated isoprenoid moiety into a hydrophobic pocket residing between the two layers of the $\beta$ sheets [46]. Thus isoprenoid moieties of other proteins may also associate with specific hydrophobic pockets of their binding partners. Our biochemical experiments with $\alpha_i$ 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 $\alpha_i$, but also the [$^{32}$P]ADP-ribosylation of $\alpha_i$ 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 $\alpha_i$. This site is on the $\alpha_i$ polypeptide chain and does not involve interactions with the $\alpha_i$ acyl moiety, since FTS is also capable of inhibiting the [$^{32}$P]ADP-ribosylation by pertussis toxin of the $\alpha_i$ non-acylated mutant (Figure 7). Although we cannot formally exclude the possibility that purified $\alpha_i$ contained $\beta\gamma_i$ dimers as a contaminant, we think that this is an unlikely possibility, since non-myristoylated $\alpha_i$ subunits have an exceedingly low affinity for isoprenylated $\beta\gamma_i$ dimers [21], and the presence of $\beta\gamma_i$ 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 $\mu$M) caused a substantial ($\approx 50\%$) inhibition of pertussis-toxin-mediated [$^{32}$P]ADP-ribosylation of bacterially expressed, non-ribosylated rat $\alpha_i$; that had been purified via an internal hexahistidine tag. Since $\beta\gamma_i$ 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 $\alpha$ subunits in the definite absence of $\beta\gamma_i$ dimers.

Thus the direct effect of FTS on $\alpha_i$, [$^{32}$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 $\alpha_i$. In favour of the first possibility is the finding that [$^{32}$P]ADP-ribosylation by pertussis toxin of GST–$\alpha_i$135–350, 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-ACF [48]. Thus FTS has a dual effect on $\alpha_i$: it competes with $\beta\gamma_i$ for binding to $\alpha_i$ and, in addition, appears to mediate a conformational change in $\alpha_i$ that inhibits pertussis-toxin-mediated ADP-ribosylation. The isoprenyl group of the $\gamma$ 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 $\gamma$ 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 moieties binding in the $\alpha$ or $\beta\gamma_i$ subunits. Interestingly, the contacts between the $\alpha$ subunit and the $\beta\gamma_i$ dimer occur primarily between the $\alpha$ and $\beta_i$ subunits, while the $\gamma$ subunit lies across the face of the $\beta$ subunit without direct contact with the $\alpha$ subunit. However, the C-terminus of the $\gamma$ subunit lies within 18 Å (1Å ≈ 0.1 nm) of the N-terminus of the $\alpha$ subunit and is also relatively close to the $\alpha$ subunit’s C-terminus [15]. Although it was proposed that the proximity of the N-terminus of the $\alpha$ to the C-terminus of the $\gamma$ 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 $\alpha_i$ subunits indicated that their N-termini are disordered and become $\alpha$-helical only upon interaction with $\beta\gamma_i$ [40]. In this context, it is important to note that a monoclonal antibody directed against the N-terminal-most portion of $\alpha_i$ [52] has previously been shown to block $\alpha_i · \beta\gamma_i$, heterotrimer formation [53]. Furthermore, mutations in $\alpha_i$ corresponding to Lys-17 and Lys-31 of $\alpha_i$ 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 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 $\beta$ propeller of $\beta\gamma_i$, such that the farnesyl moiety becomes effectively buried in a cavity formed between blades 6 and 7 of the $\beta$ 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-ribosylated $\beta\gamma_i$, but not in the crystal structures of free $\beta\gamma_i$ or heterotrimeric G, [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 $\alpha_i$ subunits, are studied remains to be investigated.

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

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