The photoactivatable NAD⁺ analogue [³²P]2-azido-NAD⁺ defines intra- and inter-molecular interactions of the C-terminal domain of the G-protein G α_i

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Recently, we reported the synthesis and use of [³²P]2-azido-NAD⁺ as a probe to study the structural organization of G-proteins. Pertussis toxin was used to 'tether' [³²P]2-azido-ADP-ribose of [³²P]2-azido-NAD⁺ to Cys³⁴⁷ of the α subunit of the G-protein G α_i . Light activation of the azide moiety covalently cross-linked the domain containing Cys³⁴⁷ at the C-terminus of α_i with neighbouring intra- and inter-molecular domains of holo-transducin. The radiolabel from [³²P]2-azido-ADP-ribose was then transferred to the 'acceptor' domain by cleaving the thioglycosidic bond between Cys³⁴⁷ and [³²P]2-azido-ADP-ribose with mercuric acetate. ADP-ribosylation followed by photocross-linking of holo-transducin indicated intra-molecular interactions of the C-terminal domain with other α_i domains and intermolecular interactions with holotransducin α

and γ subunits. The radiolabelled peptides, which were radiolabelled because of the transfer of the photoactive moiety, were identified by utilizing 2-(2'-nitrophenylsulphenyl)-3-methyl-3'-bromoindolenine ('BNPS-skatole') and CNBr. The results indicate that the C-terminus of α_i interacts with both N-terminal and C-terminal domains within the α_i molecule. Mapping the interacting sites between cross-linked α_i dimers and α trimers indicates that the C-terminal domain of α_i is involved in the formation of α_i homopolymers in solution. In addition, our studies place the $\beta\gamma$ subunit in close proximity to Cys³⁴⁷ of α_i , as indicated by the transfer of [³²P]2-azido-ADP-ribose from Cys³⁴⁷ to the γ subunit, which was further localized to the C-terminal half of γ_i . The studies presented here identify the C-terminal intra- and inter-molecular interactions of the α subunit of holo-transducin.

INTRODUCTION

Heterotrimeric G-proteins ($\alpha\beta\gamma$) mediate the transfer of an extracellular signal from a cell-surface receptor to an intracellular enzyme. The transfer of G-protein-mediated information is dependent on the binding and hydrolysis of guanine nucleotides. A number of methods have been used to probe G-protein structure and function, and each method has provided some information about the functional domains involved in GTP/GDP binding, GTPase activity, effector activation, receptor association, and $\beta\gamma$ subunit interaction [1–4]. Structural models of heterotrimeric G-proteins had been proposed on the basis of the crystal structure of the Ras protein and elongation factor EF-Tu [5–8]. More recently, the crystal structures were obtained for G α_i [9–11] and G α_{11} [12]. While the crystal structures have revealed several interesting features of the tertiary structure of G-protein α subunits, recent studies from our laboratory and others have shown that photoaffinity and fluorescent probes can be used to define the intermolecular interactions of G-protein α , β and γ subunits [1,3,13–17]. For example, we have shown using [³²P]2-azido-NAD⁺ that the α subunit exists as homopolymers in solution as a result of intermolecular associations involving their C-terminal domains [15]. In addition, these studies indicated the interaction between the C-terminus of the α subunit and the γ subunit. Using the heterobifunctional photoaffinity probe *N*-(3-[¹²⁵I]iodo-4-azidophenylpropionamido-S-2-thiopyridyl)cysteine ([¹²⁵I]ACTP), we have demonstrated the association between the α subunit domain including Cys-210 and the β subunit [18]. In the present study we analysed and identified the intermolecular interactions of the C-terminal domain of heterotrimeric G-protein subunits relative to the α subunit.

The basic experimental approach for the structural analysis of

heterotrimeric G-protein subunits is outlined as follows. (i) The G-protein α subunit was ADP-ribosylated with pertussis toxin using a radioactive and photoactivatable NAD⁺ derivative. (ii) Excess radiolabel that was not covalently linked to the cysteine residue located at the C-terminus of the α subunit was removed by gel-filtration chromatography. (iii) The radioactive and photoactivatable [³²P]2-azido-ADP-ribose was, therefore, tethered to the α subunit of a holo-G-protein from a fixed position (i.e., Cys³⁴⁷ of α_i). (iv) The ADP-ribosylated α subunit would then be activated with light to photocross-link internally within the α subunit (intramolecular) or into neighbouring polypeptides such as a $\beta\gamma$ subunit or a receptor (intermolecular). (v) The photocross-linked proteins were allowed to react with mercuric acetate in order to cleave the thioglycosidic bond between the sulphur atom of Cys³⁴⁷ and the [³²P]2-azido-ADP-ribose moiety, thus transferring the radiolabel to the 'acceptor' polypeptide. (vi) The polypeptides which had 'radiolabel transferred' to them were purified, then chemically cleaved to generate peptides for Edman degradation. Microsequencing of the N-terminal residues was matched with the cDNA sequence to identify the fragment to which radiolabel was transferred. This domain could only be radiolabelled if it was associated with, or in close proximity to, the C-terminus of the G-protein α subunit.

MATERIALS AND METHODS

Materials

Sources of materials were as follows. Sephadex G-25 and G-50 (fine) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); DMSO and BNPS-skatole were from Pierce Chemical Co. (Rockford, IL, U.S.A.); CNBr, 3-mercaptopropionic acid,

Abbreviations: CAPS, 3-(cyclohexylamino)propane-1-sulphonic acid; BNPS-skatole, 2-(2'-nitrophenylsulphenyl)-3-methyl-3'-bromoindolenine; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PVDF, poly(vinylidene difluoride); ACTP, *N*-(3-iodo-4-azidophenylpropionamido-S-2-thiopyridyl)cysteine.

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Tricine, mercuric acetate and 3-(cyclohexylamino)propane-1-sulphonic acid (CAPS) were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); X-Omat AR X-ray film was from Eastman-Kodak Co. (Rochester, NY, U.S.A.); pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.); aldehyde-free acetic acid was from Fisher Scientific Co. (Chicago, IL, U.S.A.); and poly(vinylidene difluoride) (PVDF) membrane and Bio-Gel P6 were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Synthesis of [32 P]2-azido-NAD $^{+}$

The synthesis and characterization of high-specific-radioactivity [32 P]2-azido-NAD $^{+}$ has been described previously [19].

ADP-ribosylation of transducin

Transducin was ADP-ribosylated with [32 P]2-azido-NAD $^{+}$ exactly as previously described [15]. A time course of pertussis-toxin-dependent ADP-ribosylation of transducin with 25 μ M NAD $^{+}$ or 2-azido-NAD $^{+}$ indicated that the amount of ADP-ribose or 2-azido-ADP-ribose incorporation into α_i respectively was the same for both compounds and reached saturation after 1 h with similar Michaelis-Menten kinetics [19].

Photolysis of [32 P]2-azido-ADP-ribosylated G-proteins

In order to photocross-link the ADP-ribosylated G-proteins, free [32 P]2-azido-NAD $^{+}$ was removed by gel-filtration chromatography using a 3 ml P $_6$ column at 4 $^{\circ}$ C (0.7 cm $\times$ 10 cm; three-drop fractions) under dim light. Fractions containing ADP-ribosylated G-protein were assayed for protein [20], or radiolabelled transducin was detected with a Gieger counter, typically in fractions 8–10. Pooled fractions were photolysed at 4 $^{\circ}$ C through 2-mm-thick Pyrex tubes for 5 s at a distance of 10 cm from a 1 kW mercury lamp AH-6 [21]. After photocross-linking, G-proteins were reduced with 5 mM dithiothreitol (DTT) and alkylated with 25 mM NEM, incubating each for 15 min at ambient temperature. Radiolabelled subunits were analysed by SDS/PAGE [22].

Purification of radiolabel-transferred α and γ subunits

Dimers and trimers of α_i were purified away from monomeric α , β and γ subunits by SDS/PAGE as described previously [15]. Labelled subunits were identified by autoradiography before staining the gel with Coomassie Blue and destaining. Radioactive dimers and trimers were excised from the gel with a razor blade and eluted from the gel slice with 0.75 ml of water by rotary agitation for 6 h at ambient temperature. Oligomeric α subunits were then desalted and treated with mercuric acetate in order to generate monomeric α subunits that were photolabelled but not cross-linked. Labelled α_i subunits, which had radiolabel photo-incorporated into themselves as a result of intramolecular cross-linking, were purified by SDS/PAGE. The α subunit, which was radiolabelled in an intramolecular fashion, was excised and eluted from the gel as described above and then treated with mercuric acetate to remove unchanged [32 P]2-azido-ADP-ribose and to transfer the [32 P]2-azido-ADP-ribose moiety to an adjacent domain within α_i . Photolabelled γ subunits were derived by treating cross-linked α - γ dimers with mercuric acetate prior to separation by SDS/PAGE. The photolabelled γ subunit was resolved away from holo-transducin α and β subunits by SDS/PAGE using 16.5% (w/v) acrylamide and 3% (w/v) bisacrylamide [23]. The radiolabelled γ subunit was identified by autoradiography, excised from the gel with a razor blade and

eluted from the gel with 30% ethanol for 6 h at ambient temperature.

Desalting of α and γ subunits and peptides

α and γ subunits, which were eluted from as many as four gel slices, were pooled and concentrated under reduced pressure to 0.2 ml and applied to a 3 ml (0.75 cm $\times$ 10 cm) G-50 (α subunits) or G-25 (γ subunits) column at ambient temperature, washing with a total of 0.8 ml of water and eluting with 1 ml of water.

Chemical cleavage of the thioglycosidic bond

Desalted α subunit was treated with mercuric acetate as described previously [15,24,25] in order to release unchanged [32 P]2-azido-ADP-ribose and to transfer the radiolabel from the thioglycosidic bond at Cys 347 of the ADP-ribosylation site to the photoinsertion site. Briefly, G-proteins, which were desalted ($\sim$ 1 ml), were incubated at a final concentration of 10 mM mercuric acetate, 0.1% SDS, and aq. 0.33% acetic acid for 15 min at 30 $^{\circ}$ C. After reversal with mercuric acetate, the sample was concentrated under reduced pressure and the remaining residue was dissolved in 2.5% acetic acid, then desalted through a Sephadex G-50 column as described above. The eluant was concentrated to dryness under reduced pressure.

Cleavage with BNPS-skatole

Protein, which was desalted and lyophilized in a 1.5 ml Eppendorf tube, was cleaved in 10 μ l H $_2$ O and 30 μ l of 1.3 mg/ml BNPS-skatole in acetic acid at 47 $^{\circ}$ C [26]. After 30 min, the BNPS-skatole reaction was diluted with 0.36 ml of water and extracted once with 0.4 ml of ethyl acetate, followed by centrifugation in a centrifuge at maximum speed (16000 g) for 5 min. The ethyl acetate was removed and the aqueous phase was concentrated under reduced pressure before SDS/PAGE.

Cleavage with CNBr

α and γ subunits were cleaved overnight in 0.1 M HCl/0.2 M CNBr (5 M in acetonitrile) at ambient temperature [27].

Electrophoresis of low-molecular-mass peptides

Peptides between 6 and 25 kDa were resolved with 16.5:3% gels, whereas peptides less than 6 kDa were resolved with 16.5:6% gels, as previously described [23]. Peptides that were prepared for amino acid sequence analysis were purified with the same SDS/PAGE system. The following precautions were taken to prevent N-terminal blocking: (i) the gels were cast with recrystallized SDS [28]; (ii) the gels were allowed to polymerize overnight at ambient temperature; and (iii) during electrophoresis, 1 mM 3-mercaptopropionic acid was added to the electrophoresis buffer [29].

Electrotransfer and protein staining on PVDF membranes

Electrotransfer and protein staining were performed as described in [30]. Following electrophoresis, the 0.75 mm gel was equilibrated in transfer buffer [10 mM CAPS (pH 11)/20% methanol] for 5 min. The PVDF membrane, soaked for 5 min in methanol and then for 5 min in transfer buffer, was placed on the gel and sandwiched between three pieces of Whatman 3MM paper into a Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Electrotransfer was performed at 70 V for 30 min at ambient temperature. Following electrotransfer, the PVDF membrane was briefly rinsed with water, then stained with 0.025% (w/v) Coomassie Blue R-250 in 40%

Figure 1 Removal of [32 P]2-azido-ADP-ribose from α_i

After [32 P]2-azido-ADP-ribosylation, transducin was treated with 10 mM mercuric acetate at 30 °C as described in the Materials and methods section. At the indicated time, an aliquot containing 5 μ g of transducin was removed and the reversal reaction was quenched with 2 M β -mercaptoethanol. The samples were resolved by SDS/PAGE, and the α subunits were excised with a razor blade and 32 P incorporation was determined by liquid-scintillation counting. The results show the percentage of the total amount of [32 P]2-azido-ADP-ribose removed from α_i .

methanol for 5 min. Excess stain was removed with 45% methanol/10% acetic acid (aldehyde-free acetic acid for amino-acid-sequencing membranes) for 10 min. The membrane was allowed to dry for 30 min before further manipulations.

Automated Edman degradation

N-terminal amino-acid-sequence analysis was obtained by automated Edman degradation in an Applied Biosystem gas-phase sequencer 477A. Phenylthiohydantoin-amino acid derivatives were identified by HPLC. This service was provided by Joe Leykam (Department of Biochemistry, Michigan State Uni-

versity, East Lansing, MI, U.S.A.) at the Macromolecular Structure Facility.

RESULTS

G_i was [32 P]2-azido-ADP-ribosylated, photolysed and then the photocross-linked products were analysed by SDS/PAGE. Three major photocross-linked polypeptides, which contained [32 P]ADP-ribosylated α_i , were detected as previously described [15] and had apparent molecular masses of 47 (α - γ) of 83 (α - α), and 105 kDa (α - α - α). The identity of the subunits was confirmed by immunoblotting using anti-(α subunit) and anti-(β - γ subunit) antibodies as previously described [15]. The radiolabelled α subunit dimers and trimers were eluted from gel slices and α subunit complexes were treated with mercuric acetate to cleave the thioglycosidic bond. This cleavage resulted in the transfer of the [32 P]2-azido-ADP-ribose moiety to either α or γ subunits (results not shown; see [15]). Removal of non-photoinserted [32 P]2-azido-ADP-ribose from the ADP-ribosylation site was greater than 99% (Figure 1). Thus radioactivity incorporated into the α subunit occurred as a result of light activation of the azide moiety of [32 P]2-azido-ADP-ribose and subsequent transfer of radiolabel from Cys 347 to an adjacent domain of α_i .

To generate unique peptide fragments that could be readily separated and identified by SDS/PAGE, a chemical cleavage method which is specific for tryptophan was utilized. BNPS-skatole cleaves peptide bonds at the C-terminus of tryptophan residues. There are two tryptophan residues at positions 127 and 207 of the α_i amino acid sequence and these residues are highly conserved among α subunits [31]. The prediction is that complete cleavage of α_i with BNPS-skatole would yield three peptides of 127, 80, and 143 amino acids, whereas incomplete cleavage would yield two additional fragments consisting of amino acids 1–207 and 127–350 respectively (Figures 2a–2c). Cleavage of α_i

Figure 2 Putative peptides obtained from BNPS-skatole cleavage of α_i

The α subunit has two tryptophan residues (W) at positions 127 and 207 (a). Cleavage with BNPS-skatole at both sites would yield peptides with apparent molecular masses of 15, 14, and 9 kDa (b). The 14 and 15 kDa peptides would be derived from the N- and C-terminus respectively. The 24 and 25 kDa peptides would be obtained if only one tryptophan site were cleaved with BNPS-skatole (c). α_i was ADP-ribosylated with [32 P]2-azido-NAD⁺ and photolysed. Then the α subunit monomer was purified by SDS/PAGE and treated with mercuric acetate to transfer the [32 P]2-azido-ADP-ribose from Cys 347 to other domains of α_i . The purified α subunit was then cleaved with BNPS-skatole. The peptides were resolved by SDS/16.5:3% PAGE [23]. The gel was blotted on to a PVDF membrane and then stained with Coomassie Blue (d). The corresponding autoradiogram is shown. The molecular mass of the BNPS-skatole fragments is indicated on the left.

Figure 3 BNPS-skatole and cyanogen bromide peptides derived from photolysed α_1 .

Photolysed α_1 was cleaved with BNPS-skatole, and the 15 kDa peptide was sequenced by Edman degradation (a). The amino acid sequence which was obtained corresponded to positions 208–222. The tryptophan residues are identified by arrows at positions 127 and 207, and the hatched boxes represent the guanine-nucleotide-binding domains. Photolysed α_1 monomer was treated with CNBr, and a 5 kDa peptide was sequenced by Edman degradation (b). The sequence which was obtained corresponded to positions 240–251, which further localized the radiolabel of photolysed α_1 within the 15 kDa BNPS-skatole peptide.

with BNPS-skatole produced six peptides that were easily detected by staining with Coomassie Blue (Figure 2d). BNPS-skatole cleavage produced peptides with apparent molecular masses of 25, 24, 15, 14, and 9 kDa, as well as uncleaved α subunit, when resolved on an SDS/16.5:3 %-PAGE system [23]. However, the products of incomplete BNPS-skatole hydrolysis, the 24 kDa peptide, derived from the N-terminus (1–207), and the 25 kDa peptide, derived from the carboxyl-terminus (127–350), were not resolved well with this gel system. The identity of the 15 kDa BNPS-skatole fragment that was blotted on to PVDF membrane was confirmed by microsequencing. The amino acid sequence IH(C)PEGVT(C)IIFAA was obtained and localized to positions 208–222 of the α_1 amino acid sequence, which is predicted from the cDNA sequence. This result showed that BNPS-skatole cleaved α_1 at the C-terminus of tryptophan 207 (Figure 2b) and thus confirmed the position of the 15 kDa peptide as the C-terminus of α_1 . Edman degradation of the 14 kDa peptide was unsuccessful, presumably owing to post-translational myristoylation of the N-terminal amino acid of α_1 [32–38]. On the basis of this observation and the molecular size of the 14 kDa BNPS-skatole peptide, this peptide is predicted to be the N-terminal domain of α_1 .

Cysteine-347, the site of pertussis-toxin-dependent ADP-ribosylation on α_1 [39], is located on the 15 kDa BNPS-skatole peptide (Figure 2a). Photocross-linking followed by mercuric acetate cleavage of the thioglycosidic bond resulted in the

Figure 4 BNPS-skatole treatment of oligomeric of α_1 .

Holo-transducin was ADP-ribosylated with [32 P]2-azidoNAD $^{+}$ and then photolysed. Oligomeric dimers (α – α) and trimers (α – α – α) were purified by SDS/PAGE, treated with mercuric acetate, and then treated with BNPS-skatole. The BNPS-skatole fragments were resolved by SDS/16.5:3 %-PAGE [23] and blotted on to PVDF membrane. Monomers of α_1 were kept in the dark (lane a) or photolysed (lane b). There was more sample loaded in lane a than in lane b in this experiment, which accounts for the differences in the dark controls between Figures 2 and 4. Nevertheless, photoincorporation of [32 P]2-azido-ADP-ribose occurs in unique peptides from α_1 dimers and trimers. Owing to the relatively low abundance of a trimers, lane d had to be obtained from a longer autoradiographic exposure of the PVDF membrane shown on the left.

radiolabel being transferred to the 14 and 15 kDa peptides of photolysed α_1 , although the majority of the radioactivity was in the 15 kDa peptide (Figure 2d).

Since it has been quantitatively determined that mercuric acetate cleaves greater than 99 % of the thioglycosidic bond (Figure 1), the label observed in the 15 kDa fragment is the result of cross-linking within this domain. In contrast, radiolabelling of the 14 kDa peptide must have occurred as a result of interaction between the C-terminus and the N-terminus of α_1 . Since the BNPS-skatole cleavage was incomplete, 50–60 % as assessed by densitometry scanning, the 24 and 25 kDa peptides and the α subunit can also be seen by Coomassie Blue staining (Figure 2d). This yield of cleavage with BNPS-skatole is consistent with other BNPS-skatole-treated proteins [40]. Thus the 24 and 25 kDa peptides were radiolabelled because they contain the 14 and 15 kDa peptides respectively (Figures 2c and 2d). No radiolabel was transferred into the 9 kDa BNPS-skatole peptide. These results suggest that the C-terminal domain encompassing Cys 347 is folded in such a way that it primarily interacts within the 15 kDa BNPS-skatole C-terminal domain and, secondarily, within the 14 kDa N-terminal domain.

CNBr cleavage was used to further identify the domains of interaction within α_1 . The photolabelled α subunit was treated with CNBr, the peptides were resolved by SDS/PAGE [22] and blotted on to PVDF membrane (results not shown). Cleavage of α_1 produced multiple radioactive bands. Only one of the bands, which had the majority of the radiolabel and an apparent molecular mass of 5 kDa, could be sequenced. Edman degradation of this peptide produced the amino acid sequence HESLHLFNSI(C)N, which corresponds with positions 240–251 of α_1 as predicted from the cDNA sequence (Figure 3). This

Figure 5 Scheme for CNBr cleavage of γ_i

The amino acid sequence of γ_i has methionine (M) residues at positions 16 and 30 (a). CNBr cleavage would yield peptides of 16, 14, and 43 amino acids (b). Incomplete cleavage would yield peptides of 30 and 57 amino acids or approx. 3 and 5.6 kDa respectively (c). Purified γ_i , cross-linked to an α subunit (α - γ) and treated with mercuric acetate, was cleaved with CNBr. The peptides were resolved by SDS/16.5:3%-PAGE [23] and blotted on to PVDF membrane (d). The autoradiogram from the PVDF membrane is shown and the γ -subunit peptides are indicated on the right.

result is consistent with photocross-linking within the 15 kDa BNPS-skatole fragment of α_i (Figure 3b).

Intermolecular interactions of the C-terminal domain of α_i

The α_i dimers and trimers (see [15]) were purified by SDS/PAGE, desalted and treated with mercuric acetate before BNPS-skatole cleavage. Radiolabel from the α_i dimers was found to be transferred into both the 14 and 15 kDa BNPS-skatole fragments (Figure 4, lane c). Thus α_i dimers interact predominantly via the C-terminus of one α_i molecule and the N-terminus of another α_i molecule. When α_i subunits from trimeric α_i oligomers were analysed with BNPS-skatole, the α_i subunits showed radiolabel photoinserted predominately into the 9 kDa BNPS-skatole fragment, although some radiolabel was transferred to the 15 kDa fragment (Figure 4, lane d). These data suggest that the α_i trimers were associated, at least in part, from the C-terminus of one monomer, which contained the ADP-ribosylation site, to the 9 kDa BNPS-skatole fragment of another monomer.

Identification of α - γ -interacting domains of holo-transducin

The presence of intermolecular photocross-linking between α and γ subunits (i.e. α - γ) in pure holo-transducin has been

reported previously [15]. The domain of the γ subunit, which was radiolabelled because of the transfer of [32 P]2-azido-ADP-ribose from Cys³⁴⁷ of α_i , would indicate sites of interaction between the α and γ subunits. The amino acid sequence of the γ subunit, obtained from the cDNA, shows that there are two methionine residues at positions 16 and 30 [41]. A complete CNBr digest of the γ subunit would produce three peptides (Figure 5b). Using CNBr as a cleavage method would thus localize the radiolabelled domain that interacts with [32 P]2-azido-ADP-ribose and the C-terminus of α_i .

An experiment was carried out in which pure holo-transducin was ADP-ribosylated with [32 P]2-azido-NAD⁺. Holo-transducin was photolysed and then treated with mercuric acetate, thus cleaving the covalent thioglycosidic bond between the α - γ complex resulting in the transfer of [32 P]2-azido-ADP-ribose from the α to the γ subunit. The radiolabelled γ subunits were purified by SDS/16.5:3%-PAGE [23]. Radioactive γ subunits were identified by autoradiography prior to Coomassie Blue staining and excised from the gel and eluted as described in the Materials and methods section. The radiolabelled γ subunit in the eluant was subjected to CNBr cleavage and the resultant peptides were resolved by SDS/16.5:6%-PAGE, blotted directly on to PVDF membrane and stained with Coomassie Blue [30].

Three radiolabelled peptides were observed by autoradiography of the PVDF membrane (Figure 5d). On the basis of the predicted molecular size of the CNBr peptides (Figures 5b and 5c), the following peptides were observed: (i) a 6.2 kDa peptide which is consistent with a farnesylated γ_i subunit [42], (ii) a minor radioactive peptide with an apparent molecular weight of 5.6 kDa and (iii) a major radioactive peptide with an apparent molecular mass of 4.3 kDa. A limited amino acid sequence, Leu-Val-Ser, was obtained by Edman degradation of the 4.3 kDa fragment. This amino acid sequence is consistent with CNBr cleavage at methionine-30, suggesting the radiolabelled 4.3 kDa fragment is from the C-terminal half of the γ subunit (Figure 5b). Thus the C-terminus of the γ subunit in the pure holo-transducin heterotrimer interacts with the C-terminus of α_i .

DISCUSSION

Purified γ_i , which was [^{32}P]2-azido-ADP-ribosylated at the C-terminus, was internally photocross-linked within the α subunit. The photoinserted radiolabel was localized to both the N- and C-terminal domains of α_i . Photoinsertion into the 14 kDa BNPS-skatole fragment of α_i , which represents the N-terminus, could only occur if there was a physical association between the C- and the N-terminus of α_i or that the domains were in close proximity. A similar conclusion was obtained when Cys³⁴⁷ was derivatized with etheno-NAD⁺ [13].

CNBr was used to locate peptides which were radiolabelled as a result of label transfer within α_i , and the only peptide for which sequence could be obtained coincided with positions 240–251 of α_i . This result further localized the photoinsertion site of [^{32}P]2-azido-ADP-ribose between residues 240 and the C-terminus. These data are consistent with the tertiary model derived from the crystal structure of α_i [9–11].

The labelling of an intramolecular domain within α_i is consistent with photocross-linking experiments using the thiol reagent, [^{125}I]ACTP, which also selectively derivatized Cys³⁴⁷ [18]. The photoinsertion site for [^{125}I]ACTP was determined to be on the 12 kDa tryptic peptide of α_i [43], which contains amino acids 204–310 and is localized to the N-terminus of the 15 kDa

Figure 6 Model for G-protein and receptor interacting domains

The subunits, $\alpha\beta\gamma$, of the heterotrimeric G-protein, G_q , are shown in relation to the receptor. The subunits have been divided into N- (N) and C-terminal (C) domains. The C-terminus of α_i contains Cys³⁴⁷, which is the ADP-ribosylation site. The N- and C-terminal domains of α_i are folded towards the middle of the molecule. In addition, the carboxyl-terminus is in close proximity with: (i) the N-terminus of α_i , (ii) a domain within the C-terminus, (iii) the C-terminus of γ_i , (iv) the receptor and (v) the N-terminal domain of the β subunit [3]. Dhanasekaran et al. [18] have shown the close proximity of Cys³⁴⁷ to the middle of the α_i polypeptide. Bubis and Khorana [16] have shown that the N-terminus of the β subunit and the C-terminus of the β subunit are in close proximity.

BNPS-skatole fragment. The recently resolved crystal structures of α_i [9–11] and α_{11} [12] also demonstrate the proximity of the N- and C-domains within α subunits. A model which summarizes the results obtained from radiolabel transfer experiments with transducin is shown in Figure 6. The C-terminus of α_i interacts with amino acids localized between positions 252 and 350 of the linear sequence of the α_i polypeptide. In addition, the N-terminus of α_i was shown to be in close proximity with the C-terminus of α_i . The C-terminus of α_i is also in close proximity to the C-terminal domain of γ_i . In our cross-linking experiments there was no evidence for labelling the β_i subunit, even though this subunit is four times the size of γ_i , which indicates a specific orientation of subunits within heterotrimeric G-proteins.

The structural orientation of cross-linked α_i dimers suggests that the C-terminus of one α_i molecule interacts with the N-terminus of another α_i . These results suggested that the C-terminal domain of the α subunits may be involved in intermolecular cross-linking between α subunits, providing physical evidence for the oligomeric nature of α subunits. Such an oligomeric organization of $G\alpha$ has been observed by chemical cross-linking as well as by other methods [15,44–46]. The functional significance for the oligomeric organization of $G\alpha$ subunits has been proposed as a means to amplify an extracellular signal where one activated receptor molecule could activate many G-protein molecules [47–50]. Our results presented here identify the domains of α subunits involved in forming such oligomeric complexes. The fact that dimerization and trimerization of α subunits is consistently observed suggests a specific pattern of interaction between monomers of α subunits.

Additional evidence for a structural as well as a functional role for the ADP-ribosyl moiety attached to Cys³⁴⁷ is the observation that the azide moiety inserted into a segment of α_i that is defined by the CNBr peptide which begins with His²⁴⁰. This sequence is located in the $\alpha 3$ helix [9], which has been shown to be part of the effector binding domain for the cyclic GMP phosphodiesterase γ subunit [10] and has been implicated as one of the domains of α_s , which interacts with adenylyl cyclase [10]. The helical structures which define this region of α_i also include the $\alpha 4$ helix shown by Artemyev et al. [51] to interact with the central core sequence of cyclic GMP phosphodiesterase γ (residues 24–45) and the sequence of α_i (residues 250–275), which has been shown by Cunnick et al. [52] to compete with α_i binding to cyclic GMP phosphodiesterase γ . It is therefore likely, on the basis of the present results, that the ADP-ribose moiety folds the C-terminus of α_i such that a productive interaction between rhodopsin and α_i cannot occur and that the adenine ring impairs the effector interaction with the cyclic GMP phosphodiesterase γ subunit. This putative 'double-barrelled' effect of the ADP-ribosyl moiety effectively ensures that coupling between receptor and effector is impaired and would be predicted to be a general inhibitory mechanism by which ADP-ribosylation of heterotrimeric G-proteins, which is catalysed by pertussis toxin, prevents the propagation of signal transduction.

In summary, we have successfully developed a method to study the structure of heterotrimeric G-proteins. Our results confirm the utility of this approach as a means to study G-protein structure. The domains which we have found to interact with the C-terminus of ADP-ribosylated α_i include the γ subunit and the N- and C-termini of α_i . Our studies localize a contiguous region containing amino acids 240–308 in proximity to Cys³⁴⁷ of α_i and, on the basis of label transfer to the 14 kDa BNPS-skatole fragment, we can conclude that Cys³⁴⁷ is proximal to the N-terminal domain containing amino acids 1–127. In fact, the crystal structure of α_i , as well as the structure deduced from mutational studies, support this model [9,10,53].

This work was supported by National Institutes of Health grant GM 33138, and R.R.V. was an Advanced Predoctoral Fellow of the Pharmaceutical Manufacturers' Association Foundation.

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