Interaction Sites of the COOH-terminal Region of the γ Subunit of cGMP Phosphodiesterase with the GTP-bound α Subunit of Transducin*

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In photoreceptor cells, visual transduction occurs through photoexcitation of rhodopsin, GTP activation of the α subunit of transducin, and interaction between GTP-bound transducin α subunit and the inhibitory γ subunit of phosphodiesterase. The γ subunit of phosphodiesterase, in turn, accelerates the hydrolysis of GTP on the α subunit of transducin. Within the COOH-terminal residues (46–87) of the phosphodiesterase γ subunit, Trp-70 has been implicated in phosphodiesterase activation, transducin α subunit-phosphodiesterase γ subunit interaction, and the GTP hydrolysis accelerating activity. We have derivatized the phosphodiesterase γ subunit with a reversible photoactivatable reagent, [125I]-N-[3-iodo-4-azidophenylpropionamido-S-(2-thiopyridyl)]cysteine ([125I]ACTP), at cysteine (Cys-68). A light-dependent, cross-linked complex of guanosine 5′-(γ-thio)triphosphate-bound transducin α subunit and ACTP-derivatized phosphodiesterase γ subunit formed after photolysis of a 1:1 stoichiometric complex of the two proteins. The specificity of complex formation between the transducin α subunit and the phosphodiesterase γ subunit was demonstrated by specific protection by the C68A mutant of the phosphodiesterase unit, which is critical for GTP hydrolysis accelerating triphosphate-bound transducin α subunit.

Heterotrimeric GTP-dependent proteins (G-proteins) mediate transduction of various signals from cell surface seven helical transmembrane receptors to their intracellular effector targets. In vertebrate photoreceptor cells, photoexcited rhodopsin activates a visual transduction cascade via the α subunit of the rod G-protein, transducin (αt), by catalyzing GDP-GTP exchange. The GTP-bound form of transducin, αtGTP, dissociates from rhodopsin and the βγ subunit of transducin and activates cGMP phosphodiesterase (PDE) by binding the inhibitory subunit, PDE γ (Pγ), thus releasing the catalytic activity of PDE ββ subunits (1–3). The activated PDE hydrolyzes cGMP, which is crucial in closing cation-specific channels, and the resulting hyperpolarized rod cell regulates neural signal transduction (4–7). The activated PDE is turned off by activation of the GTPase activity of α subunit of transducin, resulting in the hydrolysis of GTP to GDP. Unlike small G-proteins, such as Ras, which rely on the GTPase-activating protein (GAP) to accelerate the hydrolysis of GTP, heterotrimeric GTP-dependent proteins contain a “built-in” GAP-like domain in the α subunits (8). It is clear, however, that some effectors involved in heterotrimeric G-protein pathways (such as rod cell cGMP phosphodiesterase and phospholipase C β1) accelerate the intrinsic GTPase activity of the interacting α subunits (Gor and Goq, respectively) (9, 10).

The interaction between the αtGTP and PDE γ subunits is crucial for PDE activation and αtGTP hydrolysis. Recent data have indicated that the central region of Pγ (mainly residues Pγ 24–45) is involved in the interaction between αt and Pγ, whereas the COOH-terminal region (Pγ 46–87) is involved in determining both the interaction with the Pαβ catalytic subunits and the GAP activity of Pγ (11–16). The COOH-terminal region of Pγ has been shown to bind to the αt 293–314 sequence (17). A study of the Pγ mutants, W70F and W70A, indicated that Trp-70 was crucial for Pγ binding to αt as well as the GTPase accelerating action of Pγ on αt (18, 19).

Recently, Hamm and colleagues have shown that the 11 COOH-terminal most residues of Pγ (76–87) are intimately involved in the

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¶ The abbreviations used are: αt, α subunit of transducin; PDE, phosphodiesterase; GAP, GTPase-activating protein; GTP-S, guanosine 5′-(γ-thio)triphosphate; Pγ, γ subunit of phosphodiesterase; ACTP, N-[3-iodo-4-azidophenylpropionamido-S-(2-thiopyridyl)]cysteine; TPCk, N-tosyl-1-phelanylalanine chloromethyl ketone; NEM, N-ethylmaleimide; βMe, 2-mercaptoethanol; PVDF, polyvinylidene difluoride; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; HPLC, high performance liquid chromatography; AIPPS, 3-iodo-4-azidophenylpropyl succinimide; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethy]glycine.
GTP-bound Transducin α Subunit/Phosphodiesterase γ Subunit Interaction

EXPERIMENTAL PROCEDURES

Chemical
GTP, GTPγS, soybean trypsin inhibitor, and N-ethylmaleimide (NEM) were products of Sigma; TPCK-treated trypsin and clostripain (sequencing grade) were purchased from Promega; Mono S column and blue Sepharose CL-6B were obtained from Pharmacia Biotech Inc.; BNPS-skatole was obtained from Pierce; hydroxylamine hydrochloride was purchased from Fisher; C4 reversed-phase HPLC column was obtained from Vydac Corp.

Radiochemistry of [125I]ACTP

The radiochemistry of 5 Ci/mmol [125I]ACTP was accomplished by the reaction at room temperature for 12 h of carrier-free (2200 Ci/mmol) [125I]-iodo-4-azido-phenylpropionamido-S(2-thiophenyldiyl) cysteine ([125I]ACTP), which was derivatized as a mixed disulfide to Py at Cys-68. A proposed interaction region of the Cys-68 to Trp-70 portion of Pγ was purchased from Fisher; C4 reversed-phase HPLC column was as described previously (12, 24). Briefly, cells suspended in 50 mM Tris HCl, pH 7.6, containing protease inhibitors (phenylmethane sulfonyl fluoride, TPCK, leupeptin, aprotinin, and pepstatin A) were used within 1 week.

Preparation of atGTPγS and atGDP

Holotransducin was prepared from frozen dark-adapted bovine retinas (from J. A. & W. L. Lawson Co.) by published procedures (21). The holotransducin was then loaded onto a blue Sepharose CL-6B column, and the αGTP and βγ were purified according to Yamazaki et al. (22). The αGTP-γS subunit was released from the bovine rod outer segment membranes using GTP-γS and purified on blue Sepharose CL-6B, as described by the method of Kroll et al. (23). The purity of the α and βγ subunits was determined by SDS-polyacrylamide gel electrophoresis (PAGE). Staining with Coomassie Blue demonstrated that the subunits were more than 95% pure. The purified proteins were stored at −80 °C and used within 1 month.

Preparation of [125I]ACTP and Assessment of GTPase Activating Activity

Recombinant Py, which was overexpressed in Escherichia coli strain BL21 DE3, was purified from a Mono S column and C4 reversed-phase HPLC column, as described previously (12, 24). Briefly, cells suspended in 50 mM Tris-Cl, pH 7.6, containing protease inhibitors (phenylmethylsulfonyl fluoride, TPCK, leupeptin, aprotinin, and peptatin A) were broken using a French press and centrifuged at 20,000 rpm (−50,000 × g) for 20 min. The supernatant was loaded onto a Mono S column and eluted with 50 mM Tris-Cl to 1 M NaCl gradient (−40% NaCl) using an fast protein liquid chromatography system. The Py-enriched fractions were loaded onto a C4 reversed-phase HPLC column and eluted with a 30–60% acetonitrile gradient with 0.1% trifluoroacetic acid throughout over a 40-min period (Py eluted at approximately 40% acetonitrile). The fractions containing Py were pooled and lyophilized with a speed vacuum system and stored at −80 °C for further use.

Derivatization of Py was carried out by incubating Py with 5 Ci/mmol [125I]ACTP (1:2 molar ratio) in a solution of 120 mM NaCl, 10 mM HEPES, 6 mM MgCl2, pH 7.6, at room temperature overnight. The reaction mixture was chromatographed through a C4 reversed-phase HPLC column and eluted with an acetonitrile/H2O gradient in 0.1% trifluoroacetic acid (30–60% acetonitrile over 40 min with a flow rate of 0.5 ml/min). The [125I]ACTP and unlabeled Py eluted at approximately 35 and 41% acetonitrile, respectively, whereas Py-ACTP eluted at approximately 45% acetonitrile. The purity of the elution fractions was determined by 15% SDS-PAGE and autoradiography of the wet gel. The Py-[125I]ACTP was estimated to be greater than 99% radiopure. On occasion, a repeat of this HPLC procedure was necessary. The Py-[125I]ACTP fractions were collected, lyophilized, stored at −20 °C, and used within 1 week.

Measurement of single-turnover GTPase activity of transducin was performed as described (25). Briefly, photoreceptor membranes free of endogenous PDE but retaining endogenous transducin (20 μM rhodopsin) were preincubated with Py or Py-[125I]ACTP (0.3 μM), and the reaction was started by adding 0.2 μM [γ-32P]GTP and terminated by addition of perchloric acid. Inorganic phosphate was measured by liquid scintillation. The rates of the single-turnover GTPase reaction were calculated from single exponential fits of the data.

Interaction of at and [125I]ACTP-derivatized Py and Photolysis Condition

The cross-linked α-[125I]ACTP-Pγ band was excised from the gel and eluted using a Bio-Rad 422 electric elutor. The cross-linked dimer was cleaved to free Py and Pγ-[125I]ACTP by 2% 2-mercaptoethanol (β-Me) by reaction at room temperature for 10 min, and the two proteins were separated by 12% PAGE. The 125I-labeled α was eluted from the gel slice with water by incubation at 4 °C overnight. The elated α was desalted by passing the sample (0.2 ml) through a 3-ml Sephadex G50 column, washing with 0.8 ml of water, and eluting with 1 ml of water.

BNPS-skatole Cleavage—The 125I-labeled α subunit (10–50 μg) was dissolved in 10 μl of water. Solutions of 1.3 mg/ml BNPS-skatole acetic acid solution was added, and the solution was incubated at 47 °C for 2 h (26, 27). The 15-kDa BNPS-skatole fragment was purified by SDS-PAGE, eluted from the gel, and desalted on Sephadex G25 by the same procedure as described above.

Clostripain Cleavage of the BNPS-skatole Fragment—This cleavage was performed in a solution containing 20 mM Tris, 1 mM CaCl2, and 5 mM β-Me, pH 7.8, with an enzyme/protein ratio of 1:5 to 1:10 at 37 °C (28).

Hydroxylamine Cleavage—This method was carried out with 2 mM hydroxylamine in 0.2 M K2CO3, pH 10.5, at 42 °C for 3 h (29, 30).

Trypsin and Clostripain Cleavage—Trypsin and clostripain cleavages of native αGTPγS were performed according to published procedures (31, 32).

Following electrophoresis, the proteins or peptides were transferred to PVDF (polyvinylidene difluoride) (Bio-Rad, 0.2 μm) in a buffer of 10 mM CAPS, 20% methanol, pH 11, at 5 mA for 60 min using a Hoefer trans-blot apparatus.

Analytical Methods

Automatic amino-terminal protein sequencing was performed using an ABI, model 470A, gas-phase sequencer equipped with an on-line model 120A phenylthiohydantoin analyzer. Microsequencing and radiosequencing were performed at the Supramolecular Structure Facility, Michigan State University. SDS-PAGE (10, 12, and 15%) was performed by the method of Laemmli (33). Tricine 16.5% polyacrylamide electrophoresis was performed, as described by Schagger and von Jagow (34). Protein concentration was determined by the Coomassie Blue binding method (35) using bovine serum albumin as a standard or spectrophotometrically at 280 nm using a molar extinction coefficient of 7100 for Py (24). Densitometric scans of the Coomassie Blue-stained gels and autoradiography were performed on a model SL-504XL Zeineh Sonolaser Scanning Densitometer. Computer modeling was performed on Unix Silicon Graphics using the Insight II (version 2.3.0) Program of Biosym Technologies in the laboratory of Dr. Robert Fillingame, Department of Biomolecular Chemistry, University of Wisconsin Medical School.

RESULTS

Specific Interaction between Py-[125I]ACTP and atGTPγS—The γ subunit of PDE contains a single cysteine residue, Cys-68. This provides an opportunity to introduce a photoactivatable reversible probe into Py in order to determine the interaction between at and the COOH-terminal region of Py. An additional feature of the Cys-68 position is that it is two residues away from the crucial Trp-70, which has been shown to be involved in Py binding to at and for the GAP activity of Py.
Incubating $^{125}$I-ACTP with Py at room temperature overnight in the dark in a 1:2 molar ratio (Py/$^{125}$I-ACTP) produced approximately a 50% derivatization of Py (data not shown). A C4 reversed-phase HPLC column was used successfully to separate Py-$^{125}$I-ACTP from free $^{125}$I-ACTP and Py with a final yield of ~40%. The purity of Py-$^{125}$I-ACTP, as determined by Coomassie Blue staining and wet gel autoradiography of an SDS-polyacrylamide gel, was found to be more than 99%. A second HPLC was often performed to be certain that there was no contamination from free $^{125}$I-ACTP. Analysis by SDS-PAGE of fractions from the HPLC column is shown in Fig. 1a. Autoradiography demonstrates the high purity of the Py-$^{125}$I-ACTP (fractions 3–5) and clear separation from contaminants (fractions 7–10). Following purification of Py-$^{125}$I-ACTP, the ability of the derivatized protein to accelerate α-GTP hydrolysis was compared with nonderivatized Py. The transducin GTPase assay showed that derivatized Py has essentially the same ability to accelerate transducin GTPase as nonderivatized Py. The GTPase assay showed that Py-$^{125}$I-ACTP (~0.372 s$^{-1}$) had essentially identical activity to Py (~0.313 s$^{-1}$) (Fig. 1b). These data are consistent with previous observations that substitution of Cys-68 on Py with alanine or derivatization with a fluorescent probe did not alter the interaction of Py with αt (17, 19).

Based on the previous determination of the stoichiometry of native αt-GTP and Py (36), the αt-GTPγS was mixed with Py-$^{125}$I-ACTP at a 1:1 molar ratio. Following photolysis, the sample was treated with 4 mM NEM (to remove free sulfhydrys and prevent disulfide exchange) and 2% SDS (to terminate the reaction). When the reaction mixture was analyzed by 15% SDS-PAGE, a cross-link between αt and Py produced a band at 49 kDa that could be observed by both Coomassie Blue staining (Fig. 2, lane 2) and autoradiography (Fig. 2, lane 6). This cross-link was not observed in the dark control (Fig. 2, lanes 1 and 4). Light-dependent formation of the cross-linked 49-kDa heterodimer was completely protectable by preincubating αt-GTPγS with excess C68A Py, which has been shown to retain binding and functional GAP activity (Fig. 2, lanes 4 and 8) (19). The use of C68A Py as a protector (which does not contain cysteine) eliminated the complication of disulfide bond exchange from Py-$^{125}$I-ACTP to C68A Py. In some experiments, residual βγ subunits of transducin contaminants of the αt-GTPγS provided a useful internal control for the specificity of the Py-$^{125}$I-ACTP binding. No cross-linkage between βγ subunits of transducin contaminants of the αt-GTPγS was preincubated with (over αt-GTPγS) for 5 min, followed by a stoichiometric addition of Py-$^{125}$I-ACTP. Following incubation for 30 min at room temperature, conditions represented in lanes 1 and 3 were maintained in the dark, whereas conditions represented in lanes 2 and 4 were photolyzed. Following the addition of 4 mM NEM and 2% SDS, the samples were analyzed by a 15% PAGE.
Cleavage Scheme

![Diagram showing cleavage schemes for αt.]

**Fig. 3. Summary of the cleavage schemes for αt.** Since αt contains two tryptophan residues at positions 127 and 207, the cleavage of αt with BNPS-skatole yielded three complete peptides with apparent molecular masses of 14, 9, and 15 kDa. Only the 15-kDa peptide, sequence 208 to COOH terminus, was radioiodine-labeled (Fig. 4). This 15-kDa peptide was further cleaved with clostripain to produce three peptides with apparent molecular masses of 12, 10, and 6 kDa. Microsequencing of the 12-kDa peptide indicated that this peptide started with its NH2 terminus at Met-239 and likely extended to the COOH-terminal end. Radiosequencing of the peptide showed radioiodine release from His-244. Since the α subunit of transducin contains one Asn-Gly bond at position 287–288, cleavage of the 33-kDa fragment at Met-239 and likely extended to the COOH terminus of the molecule. The NH2-terminal sequence that was obtained for the 6-kDa fragment is a close to the histidine 244 side chain of the α helix. Additional cleavage strategies were pursued to investigate whether multiple reaction sites on αt could be determined. The presence of more than one insertion site would be useful to obtain a three-dimensional reconstruction of the interaction of the Cys-68 region of Py with αt.

**Cleavage of Radioiodinated αt with Hydroxylamine—**Since there is a single Asn-Gly bond in αt (Asn-287-Gly-288), the radioiodinated αt was bisected into two fragments with hydroxylamine. As expected, two major cleavage fragments, a 33-kDa peptide, which contained the majority of the αt sequence, including the main portion of the BNPS-skatole cleavable GTPase domain, and a 6-kDa fragment, which contained the entire α helix, the β strand, and the COOH-terminal α helix were obtained (see scheme in Fig. 3 and Fig. 6a, left). A minor fragment at approximately 18 kDa was also observed as a result of alternate cleavage at an asparagine-serine linkage. Both major fragments were found to be radioactive, as seen in the autoradiogram (Fig. 6a, right), indicating that the probe inserted into more than one site on αt in the GTPase domain. Since the insertion site on the 33-kDa fragment had been identified previously as His-244, as described in Fig. 5, these data indicated that the additional site(s) of insertion were in the α/βα/αβ portion of the α subunit. NH2-terminal microsequencing of the smaller 6-kDa fragment (Fig. 6) confirmed that the hydroxylamine cleavage had indeed occurred at Asn-287, and based on apparent size, this fragment was likely to extend to the COOH terminus of the molecule. The NH2-terminal sequence that was obtained for the 6-kDa fragment is shown through the five residues at the bottom of Fig. 6. This sequence precisely matched the sequence of the α helix/loop leading into the β strand. Radiosequencing of this smaller fragment showed 125I release at the 21st and 23rd cycle, which corresponded to Met-308 and Arg-310 (Fig. 6b). Further clostripain and trypsin cleavages of label-transferred radioiodi-
Trp-70 sequence of Pmolecular modeling and triangulation to locate the Cys-68–from the azido moiety to the disulfide bond. Identification of approximately 9–12 Å, which is estimated to be the distance from P helices, as determined by computer modeling (Fig. 7). The a

Thus, we have defined a region of Pγ Cys 68 interaction with α-GTPγS which is between the exposed face of the α4 and α9 helices, as determined by computer modeling (Fig. 7). The distance from Pγ Cys-68 to His-244, Met-308, and Arg-310 of αt is approximately 9–12 Å, which is estimated to be the distance from the azido moiety to the disulfide bond. Identification of three insertion sites on αt provided the opportunity to utilize molecular modeling and triangulation to locate the Cys-68–Trp-70 sequence of Pγ on the αt structure using the crystal structure coordinates of the α subunit of transducin (38).

DISCUSSION

The interaction between αt and Pγ is important for both cGMP PDE activation and αt-GTP hydrolysis. Several investigators have shown that αt-GTPγS is physically bound to Pγ, thus activating PDE by removing the inhibitory constraint of

![FIG. 5. Identification of the photoinsertion site of Pγ-[125I]ACTP on the clostripain-cleaved 15-kDa BNPS-skatole fragment of αt. Following reversal of cross-linked αt-[125I]ACTP-Pγ with β-Me, [125I]αt-GTPγS was separated on a 12% PAGE gel, eluted with water, and cleaved with BNPS-skatole, as described under “Experimental Procedures.” The 15-kDa BNPS-skatole cleavage fragment (208–350 sequence of αt) was further eluted from the gel with water and then cleaved with clostripain, as described under “Experimental Procedures.” αt autoradiogram of PVDF-transferred clostripain cleavage fragments from the 15-kDa BNPS-skatole fragment. A major radiolabeled polypeptide from clostripain cleavage (12-kDa band) was obtained. αt, chemical and radiochemical sequencing of the 12-kDa polypeptide. The 12-kDa peptide was chemically sequenced and shown to contain NH2-terminal Met-239 and would likely extend to the COOH-terminal end of the molecule. The first 10 letters represent the actual sequence obtained from NH2-terminal microsequencing. Radiosequencing indicated radioiodine release on the 6th cycle, which corresponds to His-244.

![FIG. 6. Identification of radiolabeled photoinsertion sites on αt using hydroxylamine cleavage. αt PVDF-transferred hydroxylamine cleaved peptides from radiolabeled αt. On the left is the Coomassie Blue staining and on the right is the resulting autoradiogram. Hydroxylamine cleavage of αt-GTPγS produced two major bands (33 and 6 kDa). Both polypeptides were radioactive. αt, chemical and radiochemical sequencing of the 6-kDa peptide. NH2-terminal microsequencing of the smaller 6-kDa fragment confirmed that the peptide started at glycine and likely extended to the COOH-terminal end. The first five letters shown in bold represent the actual microsequencing result. Radiosequencing indicated radioiodine release at the 21st and 23rd cycle, which corresponds to Met-308 and Arg-310, respectively.

Py subunit (39–43). Pγ has been shown to have binding sites for both αt and PDEαβ (15, 17, 44, 45). Mutational experiments have revealed that there are two major functional regions in Pγ; that is, a central polycationic region encompassing residues 24–45 containing a binding site for αt and PDEαβ and a COOH-terminal region, 46–87, which contains both the inhibitory region of Pγ that binds to PDEβ and the functional region that accelerates the GTPase activity of α subunit of transducin (11–15, 24, 44, 46). Among the Py 24–45 residues, Lys-41, Lys-44, Lys-45, Arg-24, and Arg-33, as well as other hydrophilic amino acids, appear to be involved in binding to αt (15, 47). Considerable evidence has been obtained supporting the thesis that the COOH-terminal region of Pγ also contains binding sites for αt, especially within residues 53–76 (15, 16). The COOH-terminal 11 residues of Pγ, 77–87, are crucial for interaction with PDEβ or GTPase accelerating activity but less important for αt binding (16, 24).

Strategies to investigate protein-protein interactions involving transfer of a radiolabeled moiety from a “donor” protein (in this case Pγ) to a “receiver” protein (in this case αt-GTPγS) has been used successfully in our laboratory (20, 37) for intramolecular and intermolecular domain analysis of holotransducin. The particular advantage of the [125I]ACTP (20) reagent is the capability of introducing a reversible disulfide bond, allowing
ylamine cleavage result, as shown in Fig. 6 and illustrated in the derivatized 24–45 fragment of P. NH2-terminal microsequencing and radiosequencing identified the region between the insertion sites; red follows: red, His-244, Met-308, and Arg-310 of at-GTPγS are photoinsertion sites; pink, Asn-297, Val-301, and Glu-305 are residues on at-GTPγS, which have been identified to be involved in the interaction with Py by Spickofsky et al. (54); yellow, Cys-210 and Cys-250 of at-GTPγS and Cys-68 of Py; white, Trp-70 of Py; green, Pro-69 of Py. White indicates the residues from at, whereas yellow indicates the residues from Py. The estimated distances from Cys-68 of Py to His-244, Met-308, and Arg-310 assume a 9–12-Å probe length.

“label transfer” from the donor protein to the receiver protein. As discussed previously, it was possible to use the entire Py subunit in these experiments since only one cysteine exists (Cys-68) in the molecule. Another important requirement of the [125I]ACTP-derivatized Py is that this molecule must be functionally active. This property is clearly shown in Fig. 1b, with comparison with native Py. The ACTP probe was further useful in these experiments, since this reagent provided multiple insertion sites at His-244, Met-308, and Arg-310, thus demonstrating that the Cys-68 sulphydryl is located in a bridging region between the α/βk and the αk region of at.

The specificity of interaction between the derivatized Py and αt was demonstrated by the protection of covalent cross-linking by the C68A mutant of Py (Fig. 2), as well as the need for native at-GTPγS. The αt-GDP under these same experimental conditions was able to interact weakly with Py, producing approximately 25% of the light-dependent cross-link that was observed with the at-GTPγS (data not shown). This is in agreement with the observation of Hamm and colleagues for a photoprobe-derivatized 24–45 fragment of Py and its interaction with αt-GDP (48). Taken together, these data are consistent with that of Kutuzov and Pfister (49), which indicated an interaction between the GDP-bound form of αt and the Py effector subunit.

The data from experiments shown in Figs. 5 and 6 define the location of the transferred photoprobe from Py to αt-GTPγS. NH2-terminal microsequencing and radiolabeling identified the location of insertion into the 15-kDa BNPS-skatole fragment, which yields a smaller clostripain fragment of 12 kDa at His-244 in the middle of the αk helix (see Fig. 5b). The hydroxylamine cleavage result, as shown in Fig. 6 and illustrated in the scheme in Fig. 3, demonstrated that photoprobe insertion occurred in at on both “sides” of the single Asn-Gly bond at Asn-287 and Gly-288 at the entry into the αk helix, since both the 33-kDa and the 6-kDa fragments were radiolabeled (see Fig. 6a). Labeling of the 33-kDa segment was consistent with location of label in the 15-kDa BNPS-skatole fragment and the 12-kDa clostripain fragment (Fig. 5a). Labeling of the 6-kDa hydroxylamine fragment (Fig. 6) was shown to be split between Met-308 and Arg-310, which further located specific labeling at the end of αhelix and the loop leading to βk on at (Fig. 6b). Interestingly, the 306–310 region, which encompasses the residues that we have identified, were also identified by Hamm and colleagues (48) using the central 24–45 sequence of Py as a probe. A possible explanation of these results is summarized below with the use of molecular modeling (see Figs. 7 and 8).

We proposed that when the COOH-terminal region of Py binds to αt, Cys-68 is placed within the exposed face of αt between the α helix and the αk helix in such a way as to allow a 9–12-Å radius of interaction of the ACTP probe, which reacts with His-244, Met-308, or Arg-310. This was strongly supported by the recent data from Erickson et al. (50). Using a resonance transfer method, Erickson et al. (50) found the distance be-
using synthetic peptides as probes, Rarick et al. (51) found that a 22-amino acid peptide, corresponding to residues 293–314 of P, interacts with the αβγ subunit of Gαt and the interaction is that the central region of Pγ (residues 24–45 of Pγ) interacts with the αβγ subunit, and possibly αβγ loops of αt, whereas residues 69–77 of Pγ interact with the α4 helix, αβγ loop and that Pγ residues 63–68 may interact with the α3 helix (Fig. 8).

Previous suggestions have been made that the interaction mechanism of P with αt occurs with the initial binding of αt 293–314 at the COOH-terminal region of Pγ, resulting in a lowered binding affinity at a second site, at 250–275, with Pγ 24–45 (17, 52). Based on our experiments, an alternative mechanism that is appealing is that when the binding of the central basic region (24–45) of Pγ to αt (αβγ, αβγ, and possibly αβγ) occurs, the interaction of the COOH-terminal of Pγ to αt is reduced, which was supported by the recent study from Yamazaki et al. (56). Upont receptor activation of GTP binding to αt and dissociation of the βγ subunits of transducin, the COOH terminus of Pγ binds tightly to the ααγ helix regions of αt and accelerates GTP hydrolysis by a mechanism that is yet to be determined.

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REFERENCES

1. Bennett, N., and Clerc, A. (1989) Biochemistry 28, 7418–7424
2. Wensel, T., and Stryer, L. (1990) Biochemistry 29, 2155–2161
3. Rens-Domiano, S., and Hamm, H. E. (1995) FASEB J. 9, 1059–1066
4. Stryer, L. (1991) J. Biol. Chem. 266, 10711–10714
5. Johnson, G. L., Dhanasekaran, N., Gupta, S. K., Lowndes, J. M., Vaillancourt, R. R., and Ruohola, A. E. (1991) J. Cell. Biochem. 47, 136–146
6. Bourne, H. R., and Stryer, L. (1992) Nature 355, 541–543
7. Hepler, J. R., and Gilman, A. G. (1992) Trends Biochem. Sci. 17, 383–387
8. Markby, D. W., Orurust, R., and Bourne, H. R. (1993) Science 262, 1985–1991
9. Arshavsky, V. Y., and Bownds, M. D. (1995) Nature 375, 416–417
10. Berstein, G., Blank, J. L., Jou, D. Y., Eaton, J. H., Rhee, S. G., and Ross, E. M. (1992) Cell 70, 411–418
11. Morrison, D. F., Cunnick, J. M., Oppert, B., and Takemoto, D. J. (1989) J. Biol. Chem. 264, 11671–11681
12. Brown, R. L., and Stryer, L. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4922–4927
13. Lipkin, V. M., Dumler, I. L., Muradov, K. G., Artemyev, N. O., and Eitingon, R. N. (1998) FEBS Lett. 234, 287–290
14. Lipkin, V. M., Bondarenko, V. A., Zagranichnik, V. E., Dobrynina, L. N., Muradov, K. G., and Natocin, M. Y. (1998) Biochem. Biophys. Acta 1176, 250–256
15. Brown, R. L. (1992) Biochemistry 31, 5918–5925
16. Skiba, N. P., Artemyev, N. O., and Hamm, H. E. (1995) J. Biol. Chem. 270, 4130–41325
17. Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P., and Hamm, H. E. (1992) J. Biol. Chem. 267, 25067–25072
18. Otto-Bruc, A., Antonny, R., Vuang, T. M., Chardin, P., and Chabre, M. (1993) Biochemistry 32, 8636–8645
19. Slepak, V. Z., Artemyev, N. O., Zha, Y., Dunke, C. L., Sabacan, L., Sondek, J., Hamm, H. E., Bownds, M. D., and Arshavsky, V. Y. (1995) J. Biol. Chem. 270, 14319–14324
20. Dhanasekaran, N., Wessling-Resnick, M., Kelleher, D. J., Johnson, G. L., and Ruohola, A. E. (1988) J. Biol. Chem. 263, 17942–17950
21. Wessling-Resnick, M., and Johnson, G. L. (1987) J. Biol. Chem. 262, 9316–9323
22. Krell, S., Phillips, W. J., and Cerione, R. A. (1989) J. Biol. Chem. 264, 4490–4497
23. Arshavsky, V. Y., Dunke, C. L., Zha, Y., Artemyev, N. O., Skiba, N. P., Hamm, H. E., and Bownds, M. D. (1994) J. Biol. Chem. 269, 18882–18887
24. Arshavsky, V. Yu., Gray-Keller, M. P., and Bownds, M. D. (1991) J. Biol. Chem. 266, 15850–15857
25. Fontana, A. (1970) Methods Enzymol. 25, 419–423
26. Crimmings, D. L., McCourt, D. W., Thoma, R. S., and Scott, M. G. (1990) Anal. Biochem. 187, 27–38
27. Mitchell, W. (1977) Methods Enzymol. 47, 165–170
28. Bernstein, P., and Ballan, G. (1977) Methods Enzymol. 47, 133–145
29. Steinman, H. M., Naik, V. R., Abernethy, J. L., and Hill, R. L. (1974) J. Biol. Chem. 249, 7326–7335
30. Fung, B. K. R., and Nash, C. B. (1983) J. Biol. Chem. 258, 10503–10510
31. Mazzoni, M. R., Malinski, J. A., and Hamm, H. E. (1991) J. Biol. Chem. 266, 14072–14081
32. Laemmli, U. K. (1970) Nature (Lond.) 227, 686–688
34. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
36. Fung, B. K. K, Young, J. H., Yamane, H. K., and Griswold-Prenner, I. (1990) Biochemistry 29, 2657–2664
37. Vaillancourt, R. R., Dhanasekaran, N., and Ruoho, A. E. (1995) Biochem. J. 311, 987–993
38. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) Nature 366, 654–663
39. Yamanaki, A., Stein, P. J., Chernoff, N., and Bitensky, M. W. (1983) J. Biol. Chem. 258, 8188–8194
40. Deterre, P., Bigay, J., Robert, M., Pfister, C., Kuhn, H., and Chartre, M. (1986) Proteins Struct. Funct. Genet. 1, 188–193
41. Deterre, P., Bigay, J., Forguet, F., Robert, M., and Chartre, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2424–2428
42. Wensel, T., and Stryer, L. (1986) Proteins Struct. Funct. Genet. 1, 90–99
43. Fung, B. K. K., and Griswold-Prenner, I. (1989) Biochemistry 28, 3133–3137
44. Morrison, D. F., Rider, M. A., and Takemoto, D. J. (1987) FEBS Lett. 222, 266–270
45. Whalen, M. M., and Bitensky, M. W. (1989) Biochem. J. 259, 13–19
46. Takemoto, D. J., Hurt, D., Oppert, B., and Cunnick, J. (1992) Biochem. J. 281, 637–643
47. Oppert, B., Gonzalez, K., Hurt, D., Cunnick, J., and Takemoto, D. (1991) Biochem. Biophys. Res. Commun. 181, 306–309
48. Artemyev, N. O., Mills, J. S., Thornburg, K. R., Knapp, D. R., Schey, K. L., and Hamm, H. E. (1993) J. Biol. Chem. 268, 25611–25615
49. Kutuzov, M., and Pfister, C. (1994) Eur. J. Biochem. 220, 963–971
50. Erickson, J. W. Mittal, R., and Cerione, R. A. (1995) Biochemistry 34, 8693–8700
51. Rarick, H. M., Artemyev, N. O., and Hamm, H. E. (1992) Science 256, 1031–1033
52. Cunnick, J., Twamley, C., Uдовichenко, И., Gonzalez, K., and Takemoto, D. J. (1994) Biochem. J. 297, 87–91
53. Chou, P. Y., and Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251–276
54. Spickofsky, N., Robichon, A., Danho, W., Fry, D., Greeley, D., Gravers, B., Madison, V., and Margolskee, R. F. (1994) Nature 1, 771–781
55. Arkinstall, S., Chabert, C., Maundrel, K., and Peitsch, M. (1995) FEBS Lett. 364, 45–50
56. Skiba, N. P., Rae, H., and Hamm, H. E. (1996) J. Biol. Chem. 271, 413–424
