Interaction of Transducin with Light-activated Rhodopsin Protects It from Proteolytic Digestion by Trypsin*

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The trypsic cleavage pattern of transducin (Gt) in solution was compared with that in the presence of phospholipid vesicles, rod outer segment (ROS) membranes kept in the dark, or ROS membranes containing light-activated rhodopsin, metarhodopsin II (Rh*). When Gt was in the high affinity complex with Rh*, the α subunit was almost completely protected from proteolysis. The protection of α at Arg204 was complete, while Arg310 was substantially protected. The cleavage of βγ at Lys18 was protected in the presence of phospholipid vesicles, ROS membranes kept in the dark, or ROS membranes containing Rh*. The cleavage of βγ was slower in the presence of ROS membranes or phospholipid vesicles. When the Rh*Gt complex was incubated with guanyl-5′-yl thiophosphate, a guanine nucleotide analog known to release the high affinity interaction between G and Rh*, the protection at Arg310 and Arg204 was diminished. From our results, we propose that Rh* either physically blocks access of trypsin to Arg204 and Arg310 or maintains the heterotrimer in such a conformation that these cleavage sites are not available. Since Arg204 is involved in the switch interface with βγ (Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311-319), it may be that βγ is implicated in protecting this cleavage site in the receptor-bound, stabilized heterotrimer. Arg310 is not near the βγ subunit, thus we believe that the high affinity binding of Gt to Rh* physically or sterically blocks access of trypsin to this site. Thus, Arg310, only a few angstroms away from the carboxyl terminus of α, which is known to directly bind to Rh*, is likely to also be a part of the Rh* binding site. This is in agreement with other studies and has implications for the mechanism by which receptors catalyze GDP release from G proteins.

The protection of Lys18 in the presence of phospholipid vesicles suggests that the amino-terminal region is in contact with the membrane, consistent with the crystal structure of the heterotrimer (Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311-319).

Certain extracellular signals, including hormones, neurotransmitters, neuromodulators, chemokines, odorants, and light, activate a class of receptors that initiate cellular effects via activation of heterotrimeric G proteins. Agonist binding to the G protein-coupled receptors leads to conformational changes that promote a tighter interaction with specific G proteins, catalysis of GDP release, and subsequent G protein activation. In the absence of guanine nucleotides, agonist binding to the receptor is stabilized by the bound G protein. The structural basis of the ternary complex among agonist, receptor, and heterotrimeric G protein is an active area of study. Extensive mutagenesis experiments, as well as peptide competition investigations for a variety of G protein-coupled receptors, have led to an understanding that the second and third cytoplasmic loops and, in some circumstances, the putative fourth loop, as well as portions of α helices VI and VII, are important in recognition of cognate G proteins (1-3). It has been shown that the heterotrimeric G protein, rather than just the α or βγ subunits, is required for the interaction, but studies pointing out the importance of specific regions are thus far limited to the α subunits (4-7).

The crystal structures of the active (GTPγS) and GDP AlF4−-bound (8-10) and inactive (GDP-bound) (11, 12) forms of the α subunits of transducin (Gt) and Gt have been reported. Analysis of the two crystal forms has established the nature of the conformational change induced by the exchange of GTP for GDP and the switch mechanism by which the presence or absence of the γ-phosphate defines the active or inactive state of the α subunit (9, 11). The high resolution crystal structures of the heterotrimeric G proteins, Gt and G, provide a fundamental context for understanding how a heterotrimer interacts with the membrane and with activated receptors (13, 14). The molecular mechanisms involved in the conformational changes of the α subunit and the nucleotide exchange induced by the heterotrimeric G protein interacting with activated receptors are of great interest. Denker et al. (15) suggested that agonist binding to the receptor initiates the exchange of nucleotide from Gt by movement of the α subunit carboxyl-terminal region. The guanine ring of GDP interacts with the residues TCAT, 30 residues from the carboxyl terminus, in a loop at the amino terminus of the final α-helix (α5). The receptor-stimulated movement of α-helix 5 may allow for the release of GDP. Consistent with this suggestion, disturbing the interactions between the conserved TCAT region of the α subunit and the guanine ring of GDP has been shown to decrease GDP affinity.

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The abbreviations used are: GTPγS, guanosine 5′-O-(thiotriphosphate); Gt, the G protein of rod outer segment, transducin; Gt, a G protein coupled to the inhibition of adenyl cyclase; Gα, a G protein present predominantly in brain; αβγ, the α and βγ subunits of transducin; α subunit of Gt; ROS, rod outer segment; Rh, rhodopsin; Rh*, metarhodopsin II; PL, phospholipid vesicles; PC, phosphatidylcholine; GDPβS, guanyl-5′-yl thiophosphate; PBS, phosphate-buffered saline; TLCK, 1-chloro-3-tosylamido-7-aminoo-2-heptanone hydrochloride; TPCK, 1-1-tosylamido-2-phenylethyl chloromethyl ketone; MOPS, 3-(N-morpholino)propanesulfonic acid.
It has been known for many years that the presence of G proteins increases agonist affinity to receptors, while binding of either GDP or GTP to the G protein complex disrupts the high affinity agonist binding state (18, 19). Thus, one of the primary ways of measuring the ternary complex formed by the agonist, receptor, and G protein, is by analyzing the agonist affinity for the receptor. G protein binding to the light-activated rhodopsin (Rh) receptor induces a high affinity state that can be measured either as decreased ability to remove the G protein from the membrane (20) or as stabilization of the active signaling state of rhodopsin, metarhodopsin II (Rh*) (21, 22). The high affinity state is induced by Rh*-catalyzed loss of GDP, leading to an empty guanine nucleotide binding pocket (23). The addition of either GTP or GDP can promote the loss of the high affinity state, measured by centrifugation (20, 24, 25) or decay of Rh* (26, 27). The mechanisms involved in initiating the allosteric modulation of the agonist binding site on the receptor upon guanine nucleotide binding to the G protein are still unknown.

The studies presented here investigate the following areas: 1) determining the regions on Gt, besides those already implicated, involved in its high affinity interaction with Rh*; 2) elucidation of the conformational changes of Gt induced by the tight interaction with Rh*; and 3) resolving how the conformational changes induced by guanine nucleotide binding to the α subunit decrease the affinity of Gt for Rh* and cause Rh* decay. The tryptic digestion pattern of Gt is well known (28, 29). The GTP-induced conformational switch leads to a changed proteolytic digestion at Arg204, in α-helix 2 (28, 30). A proteolytic site at the amino terminus of the αt subunit is partially protected by the presence of the βγ subunit (31). In this work, we examine whether the high affinity interaction between Gt, and activated rhodopsin (Rh*) affects the αt and βt subunit proteolytic digestion pattern. The effects of phospholipid vesicles (PL), rod outer segment (ROS) membranes kept in the dark, or ROS membranes containing Rh* on tryptic digestion patterns of Gt were investigated. In particular, we focused on the changes in the tryptic digestion pattern and time course of the αt subunit induced by Rh* interaction. Our results indicate that the presence of ROS membranes containing Rh*, Rh*, or phospholipid vesicles each uniquely affects Gt proteolysis but that interaction with Rh* has the most dramatic effects.

EXPERIMENTAL PROCEDURES

Materials—TPCK-treated trypsin was purchased from Worthington. GDFPS and TLCK were products of Boehringer Mannheim. The LumiGLO substrate kit and peroxidase-labeled antibodies to rabbit or mouse IgG were purchased from Kirkegaard and Perry Laboratories, Inc. Fluorescent and standard molecular weight markers were obtained from Sigma. All other chemicals and reagents were of the highest purity available.

Antibodies—Monoclonal antibody 4A was prepared and purified as described by Hamm and Bownds (32) and Witt et al. (33). The α subunit antisera 116, 1398, and 8645 were a generous gift of Dr. D. Manning (Department of Biochemistry, Bayle College of Medicine, Dallas, TX). Antisera 116 and 1198 were prepared essentially as described by Kim et al. (38) and resuspended in buffer A (10 mM MOPS, pH 7.5, 200 mM NaCl, 2 mM MgCl2) containing 0.1 mM EDTA. The phospholipid vesicles were prepared as described (39). In the final vesicle preparation, the total phospholipid concentration was 4.9 mM, which corresponded to that present in ROS membranes containing 82 μM rhodopsin.

Bovine ROS membranes were prepared as described previously (29). ROs membranes stripped with 4 M urea (urea-washed ROS membranes) were prepared as described (40). Aliquots of both ROS membrane preparations were stored in the dark at −80 °C until needed. Rhodopsin concentration was determined by measuring the absorbance at 500 nm for all samples. The absorbance of solubilized ROS membranes was measured at 500 nm before and after bleaching. Gt was extracted from ROS membranes as described previously (29) and stored in 40% glycerol at −20 °C. Gt concentration was determined by the Coomassie Blue binding method (41), using bovine serum albumin as a standard (Pierce).

Proteolysis of Gt—Limited tryptic digestion of Gt in the absence and presence of urea-washed ROS membranes were performed as described (40). Aliquots of both ROS membrane preparations were stored in the dark at −80 °C until needed. Rhodopsin concentration was determined by measuring the absorbance at 500 nm for all samples. The absorbance of solubilized ROS membranes suspensions at 500 nm before and after bleaching. Gt was extracted from ROS membranes as described previously (29) and stored in 40% glycerol at −20 °C. Gt concentration was determined by the Coomassie Blue binding method (41), using bovine serum albumin as a standard (Pierce).

RESULTS

The recent determination of the high resolution crystal structure of the heterotrimeric G protein, Gt, provided some indication of the orientation of the molecule with respect to the membrane and the visual receptor rhodopsin. To further understand the structural basis of the high affinity interaction between rhodopsin and Gt, we determined the effect of the rhodopsin-Gt complex formation on the accessibility of tryptic cleavage sites on the αt and βt subunits.

The sequential appearance of proteolytic fragments during the time course of Gt-limited tryptic digestion has been widely studied, and the origin of these fragments is well characterized in several laboratories (16, 17).
(28–30) (Fig. 1). As indicated, the $\alpha_t$ subunit has three regions that are readily available for limited trypsin digestion: Lys$^{18}$, Arg$^{204}$, and Arg$^{215}$. Five transient proteolytic fragments of the $\alpha_t$ subunit appear (a$_{38}$, a$_{44}$, a$_{22}$, a$_{23}$, and a$_{12}$), while the final fragments are a$_{21}$, a$_{12}$, a$_{4}$, and a$_{2}$ (29). The $\beta_t$ subunit is rapidly converted to two stable fragments ($\beta_{23}$ and $\beta_{14}$) (Fig. 1), while the $\gamma_t$ subunit remains intact (28). Under non-denaturing conditions, the two tryptic fragments of the $\beta_t$ subunit and the $\gamma_t$ subunit remain tightly associated (28).

Purified Gt was incubated with PL, ROS membranes containing inactive (Rh), or light-activated rhodopsin (Rh*), and after centrifugation to remove any soluble Gt, the membrane fractions were incubated with trypsin. The rate of cleavage was followed for 2 h. Potentially, interaction of Gt with membranes or Rh* could either protect or increase accessibility of known cleavage sites or expose new cleavage sites.

The time course of limited digestion of Gt in the presence and absence of urea-washed ROS membranes is shown in Fig. 2. When soluble Gt was digested with trypsin, the sequential appearance of proteolytic fragments that migrated in the Coomassie Blue-stained polyacrylamide gels with apparent molecular weights of 38, 34, 32, 23, 21, 14, and 12 kDa was evident (Fig. 2A). According to our previous study (29) (Fig. 1) these peptide bands corresponded to five $\alpha_t$ (a$_{38}$, a$_{44}$, a$_{22}$, a$_{23}$, and a$_{12}$) and two $\beta_t$ fragments (b$_{23}$ and b$_{14}$) A dramatically different result was obtained when Gt was incubated with ROS membranes containing Rh* and allowed to form a high affinity complex. In this complex, the $\alpha_t$ subunit remained relatively uncleaved during the 2 h of digestion time course (Fig. 2B). To determine whether this protection occurred specifically because of the high affinity interaction with Rh*, parallel experiments were performed using Gt bound to ROS membranes kept in the dark (Fig. 2D) or Gt-Rh* incubated with GDP$\beta$S, which dissociates the high affinity complex (26, 27) (Fig. 2C). When Gt was in the presence of inactive Rh, the time course of tryptic digestion of $\alpha_t$ was much more similar to that observed for soluble Gt, while in the presence of Rh* and GDP$\beta$S, the tryptic pattern was intermediate. Since rhodopsin was also partially cleaved by trypsin, the proteolytic digestion pattern of the $\alpha_t$ subunit was difficult to analyze in the Coomassie Blue. A, time course of Gt limited trypsic digestion; B, time course of Gt limited trypsic digestion in the presence of Rh*; C, time course of Gt limited trypsic digestion in the presence of Rh* plus GDP$\beta$S; D, time course of Gt limited trypsic digestion in the presence of Rh. Molecular weight standards are indicated, as are the size and the origin of the fragments. Gt control at time 0.

In the presence of urea-washed ROS membranes, the fragments originating from the cleavage of the $\beta_t$ subunit were visible in the gels (Fig. 2). Although under all three conditions a small amount of $\beta_{23}$ and $\beta_{14}$ fragments was present during the time course, it appeared that there was less of each fragment throughout the time course (Fig. 2). This was different from the digestion time course of soluble Gt, in which the two fragments were generated immediately. Thus, it would appear that the presence of membranes of any kind partially protects the $\beta_t$ subunit from tryptic cleavage at Arg$^{219}$. To examine the presence of $\alpha_t$ fragments more clearly, im-
To quantitate the data, we analyzed the immunoblots by densitometry (Fig. 4). Both the disappearance of $\alpha_t$ and the appearance of the $\alpha_{34}$, $\alpha_{32}$, and $\alpha_{21}$ fragments were measured. Fig. 4 shows clearly that the $\alpha_t$ subunit was protected in the presence of Rh*.$^a$ This implies that all three major proteolytic sites of $\alpha_t$ are protected when $G_t$ is in high affinity interaction with Rh*.$^a$ (Table I).

In the presence of PL, there was an increased generation of $\alpha_{34}$ (Fig. 4B) and decreased generation of the $\alpha_{32}$ fragment (Fig. 4C). This suggests that interaction of $G_t$ with phospholipids leads to protection of the cleavage site at Lys$^{38}$ (see also Fig. 5). In the presence of Rh, there was also a decreased production of the $\alpha_{32}$ fragment (Fig. 4C) but no compensating increase of $\alpha_{34}$ (Fig. 4B). In fact, there was a decreased production of the $\alpha_{34}$ fragment. This suggests that Rh is capable of weakly protecting the cleavage site at Arg$^{304}$. In the presence of either PL or Rh, the production of $\alpha_{21}$ was increased with respect to $G_t$ in solution (Fig. 4D). It thus appears that proteolytic cleavage at Arg$^{304}$ is accelerated when Lys$^{38}$ is protected.

GDP/$\beta\gamma$S is known to reverse the high affinity state between $G_t$ and Rh* (26, 27). The data (Fig. 4, Table I) show that the high affinity interaction with Rh* was needed for its protection of proteolytic sites on $\alpha_t$, because there was a partial reversal of the protection of Arg$^{310}$ and Arg$^{304}$. Comparison of the amounts of $\alpha_{34}$ and $\alpha_{32}$ shows that even in the low affinity state, the cleavage site at Lys$^{38}$ was protected, consistent with the ability of PL or Rh to protect this site.

To examine in more detail the ability of phospholipids to protect the cleavage site at Lys$^{38}$, we studied the appearance of the transient $\alpha_{34}$ fragment in the presence or absence of PL. Fig. 5 shows that PL completely inhibited the production of the $\alpha_{34}$ fragment. In addition, in the presence of PL, the formation of $\alpha_{32}$ was decreased at the expense of an increased formation of the $\alpha_{34}$ fragment.

Table I summarizes the findings, while the locations of the various cleavage sites on the $G_t$ heterotrimer are shown in the stereoview in Fig. 6. It is interesting to note that all $\alpha_t$ tryptic cleavage sites are located on the same side of the molecule (Fig. 6), in close proximity to the receptor, $\beta\gamma$ subunit, and effector binding regions. This observation may suggest that protein regions that are involved in protein-protein interaction are flexible.

**DISCUSSION**

The studies described here provide insight into the regions of a G protein that bind to an activated receptor. The accessibility of $G_t$ to tryptic proteolysis is changed dramatically when it is in high affinity interaction with Rh*. We have dissected the effects of phospholipids, ROS membranes containing inactive Rh, and the low affinity interaction of GDP/$\beta\gamma$S-bound $G_t$ with Rh* on the availability of the tryptic cleavage sites.

It is well known that heterotrimeric G proteins are membrane-associated even in the absence of activated receptors. This association is thought to be mediated mainly by interactions of fatty acyl and prenyl groups of $\alpha$ and $\gamma$ subunits with membranes (42, 43). All $G_t$ protein $\alpha$ subunits are modified at or near their amino termini by covalent attachment of the fatty acid myristate and/or palmitate, while the $\gamma$ subunits are prenylated at a cysteine residue located in their carboxyl termini (43). The $\alpha_t$ subunit is heterogenously modified at its amino terminus by myristate and three other less hydrophobic fatty acids (44, 45), but it does not contain palmitate. The presence of membranes is important for $\alpha_t$-$\beta\gamma$ subunit interaction (46), so it might be expected that their presence affects the proteolytic cleavage pattern of $G_t$. In fact, we found that both phospholipid vesicles and ROS membranes protect the cleavage site in the amino terminus of $\alpha_t$ at Lys$^{38}$.
Numerous biochemical (29, 31, 47, 48) and mutational (49, 50) studies have implicated the amino terminus of G protein \( \alpha \) subunits in the interaction with \( \beta \) subunits. The resolution of the crystal structure of heterotrimeric \( G_t \) (14) has confirmed that the amino-terminal helix of the \( \alpha_t \) subunit is involved in forming a binding site for the \( \beta_t \) subunit. Numerous potential proteolytic cleavage sites are located in this amino-terminal helix of \( \alpha_t \) (29). Navon and Fung (31) have shown that the presence of \( \beta_t \) reduces the cleavage rate of the \( \alpha_t \) subunit at Glu21 by Staphylococcus aureus V8 protease. The chymotryptic cleavages sites at Leu15 and Leu19 are partially protected (51) by the presence of \( \beta_t \) that directly contacts these residues (14). It is likely that in the heterotrimeric \( G_t \), the tryptic cleavage site at Lys18 is also partially protected. The presence of membranes of any kind determines an increase of the protection. The effect may be direct or the consequence of a conformational change of the amino-terminal \( \alpha \)-helix of \( \alpha_t \) induced by the concomitant interaction with \( \beta_t \) and membranes. Overall, the protection suggests that this region is in contact with the membrane, consistent with the crystal structure of the heterotrimer (14).

### Table I

| Condition           | Lys\(^{18}\) | Arg\(^{264}\) | Arg\(^{110}\) |
|---------------------|--------------|--------------|--------------|
| \( G_t \)           | +            | +            | +            |
| \( G_t + \text{Rh}^* \) | +/−          | +/−          | +/−          |
| \( G_t + \text{Rh}^* + \text{GDP} \beta \text{S} \) | ++          | −             | +/−          |
| \( G_t + \text{Rh} \)          | ++          | −             | +/−          |
| \( G_t + \text{PL} \)            | ++          | −             | −            |

*+++, complete protection; +++, protection; +, mild protection; +/−, partial protection; −, no protection.*

Numerous biochemical (29, 31, 47, 48) and mutational (49, 50) studies have implicated the amino terminus of G protein \( \alpha \) subunits in the interaction with \( \beta \) subunits. The resolution of the crystal structure of heterotrimeric \( G_t \) (14) has confirmed that the amino-terminal helix of the \( \alpha_t \) subunit is involved in forming a binding site for the \( \beta_t \) subunit. Numerous potential proteolytic cleavage sites are located in this amino-terminal helix of \( \alpha_t \) (29). Navon and Fung (31) have shown that the presence of \( \beta_t \) reduces the cleavage rate of the \( \alpha_t \) subunit at Glu21 by Staphylococcus aureus V8 protease. The chymotryptic cleavage sites at Leu15 and Leu19 are partially protected (51) by the presence of \( \beta_t \) that directly contacts these residues (14). It is likely that in the heterotrimeric \( G_t \), the tryptic cleavage site at Lys18 is also partially protected. The presence of membranes of any kind determines an increase of the protection. The effect may be direct or the consequence of a conformational change of the amino-terminal \( \alpha \)-helix of \( \alpha_t \) induced by the concomitant interaction with \( \beta_t \) and membranes. Overall, the protection suggests that this region is in contact with the membrane, consistent with the crystal structure of the heterotrimer (14).
Our results indicate that in the presence of Rh* the $\alpha_t$ subunit of heterotrimeric $G_t$ is almost completely protected from tryptic hydrolysis. In particular, the cleavage site at Arg$^{310}$ is not available for digestion. We cannot determine whether this protection is the consequence of Rh* binding to that region of $\alpha_t$ or a change of $\alpha_t$ conformation upon Rh* binding. However, the former possibility is in agreement with previous observations. Data reported by Hamm et al. (4) indicate that the $\alpha_t$ residues 311–323 participate with the carboxyl-terminal residues 340–350 to form the binding site for Rh*. Resolution of the crystal structure of the $\alpha_t$ subunit in the GTP$\gamma$S and GDP-bound forms (8, 11) has shown that $\alpha_t$ residues 320–323 (TCAT) contribute directly to nucleoside binding through a van der Waals contact with the guanine ring. A receptor-stimulated movement of $\alpha$-helix 5 may allow for the release of GDP. Consistent with this suggestion, disturbing the interactions between the conserved TCAT region ($\beta_6$–$\alpha_5$ loop) of the $\alpha$ subunit and the guanine ring of GDP has been shown to decrease GDP affinity (16, 17). The crystal structures of $\alpha_t$-GDP and heterotrimeric $G_t$ also show that Arg$^{310}$ is located in a surface-exposed loop, the $\alpha_4$/$\beta_6$ region, which is spatially close to the carboxyl-terminal residues, 340–350 (Fig. 6). The activated receptor could trigger the release of bound guanine nucleotide by interacting with this region in concert with the COOH-terminal region. Binding of GDP$\beta$S interacting through the guanine ring with the $\beta_6$–$\alpha_5$ loop could decrease the affinity of the $\alpha_t$ subunit for Rh* via $\beta_6$ or $\alpha_5$. This effect is demonstrated here by the partial reversal of the protective effect of Rh* at Arg$^{310}$ by GDP$\beta$S (Table I). Both guanine nucleoside diphosphate and triphosphate are able to modulate the interaction of the $\alpha_t$ subunit with Rh* and cause Rh* decay as reported by several observations (26, 27). The mechanism of this communication between the Rh* and nucleotide binding sites is not known.

In the presence of dark-adapted rhodopsin, the $\alpha_t$ cleavage site at Arg$^{310}$ is not completely available for tryptic digestion as indicated by the production of lower amounts of the 34-kDa fragment. A larger amount of the fragment is generated when $G_t$ is digested in the presence of phospholipid vesicles. These observations suggest that there is a low affinity interaction of this region with inactive Rh.

Our data also indicate that in the presence of Rh* another tryptic cleavage site of $\alpha_t$, Arg$^{204}$, is less available to digestion.

In the presence of GDP$\beta$S, the accessibility to this cleavage site is partially restored. Arg$^{204}$ is located in the switch II region, residues 198–215 (8), and is protected from tryptic hydrolysis in the $\alpha_t$-GTP$\gamma$S complex but not in the $\alpha_t$-GDP complex (28). The switch II region undergoes a distinct transition from a distorted helical structure with the Arg$^{204}$ side chain in a solvent-exposed conformation when $\alpha_t$ is in the GDP-bound form to a well ordered helix ($\alpha_2$-helix) interacting with residues from the $\alpha_3$-helix and switch III in the $\alpha_t$-GTP$\gamma$S complex (9). Structural studies have shown that this region forms an important part of the $\beta_\gamma$ contact interface (14) (Fig. 6). In the presence of Rh*, protection of the cleavage site of $\alpha_t$ at Arg$^{204}$ can result from a direct interaction of Rh* with this region or as a consequence of a conformational change induced by the activated receptor. Alternatively, Rh* can directly or indirectly cooperate with $\beta_\gamma$ to determine this protection. In the presence of GDP$\beta$S, this protective effect is reduced. Thus, after release of GDP as a consequence of $\alpha_t$ activation by Rh*, the binding affinity of the switch II region for $\beta_\gamma$ may increase, and the accessibility of the protease to Arg$^{204}$ is almost completely blocked. Our results suggest that in the “empty pocket” state the $\alpha_t$ subunit forms a tight complex with $\beta_\gamma$ and Rh*.

In conclusion, using limited tryptic digestion as an experimental approach, we have defined the regions of $G_t$ involved in the interaction with the activated receptor. In the presence of Rh*, two major cleavage sites of the $\alpha_t$ subunit, Arg$^{204}$ and Arg$^{310}$, are protected, while the cleavage rate at Lys$^{186}$ is substantially decreased in the presence of either ROS membranes or PL.

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