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Receptor-mediated changes at the myristoylated amino terminus of $G\alpha_{i1}$ proteins[†]

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Abstract

GPCRs catalyze nucleotide release in heterotrimeric G proteins, the slow step in G protein activation. $G_{i/o}$ family proteins are permanently, cotranslationally myristoylated at the extreme amino terminus. While myristoylation of the amino terminus has long been known to participate in anchoring G_i proteins to the membrane, the role of myristoylation in regards to interaction with activated receptors is not known. Previous studies have characterized activation-dependent changes in the amino terminus of $G\alpha$ proteins in solution [Medkova, M. (2002) *Biochemistry* 41, 9963-9972; Preininger, A.M. (2003) *Biochemistry* 42, 7931-7941], but changes in the environment of specific residues within the $G\alpha_{i1}$ amino terminus during receptor-mediated G_i activation has not been reported. Using site-specific fluorescent labeling of individual residues along a stretch of the $G\alpha_{i1}$ amino terminus, we found specific changes in the environment of these residues upon interaction with activated receptor and following GTP γ S binding. These changes map to a distinct surface of the amino-terminal helix opposite the $G\beta\gamma$ binding interface. The receptor-dependent fluorescent changes are consistent with a myristoylated amino terminus in close proximity to the membrane and/or receptor. Myristoylation affects both the rate and intensity of receptor activation-dependent changes detected at several residues along the amino terminus (with no significant effect on the rate of receptor-mediated GTP γ S binding). This work demonstrates that the myristoylated amino terminus of $G\alpha_{i1}$ proteins undergoes receptor-mediated changes during the dynamic process of G protein signaling.

GPCRs act as GEFs for heterotrimeric G proteins, catalyzing nucleotide exchange on $G\alpha$ subunits, which is the rate-limiting step in G protein activation. Agonist-mediated activation of GPCRs is coupled to GDP release by a mechanism which can be probed using biophysical techniques. While crystal structures for a number of G proteins and their respective subunits (1-7), as well as those of the prototypical GPCR, rhodopsin (8,9), and more recently the β_2 -adrenergic receptor (10), reveal much of what we know about the structure and function of these proteins, there is currently no high-resolution structure of an activated receptor in complex with its cognate G protein. Thus, biophysical and biochemical studies provide valuable insights regarding the structural determinants and dynamics of G protein activation. A functional heterotrimer is known to be essential for receptor-mediated G protein activation; the amino-terminal region of $G\alpha$ directly participates in heterotrimer formation, facilitating high affinity $G\alpha$ - $\beta\gamma$ binding (11,12).

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The amino terminus of $G\alpha$ (as well as the C terminus (13,14) and $\alpha 4$ - $\beta 6$ loop (15)) have been implicated in receptor-mediated interactions in a number of biochemical studies. One of the first reports of such an interaction arose from the observation that a 15-residue peptide encompassing amino-terminal residues 8-23 of $G\alpha_t$ competitively inhibited rhodopsin- G_t interaction but alone did not stabilize metarhodopsin II formation (16). More recently, activated rhodopsin was found to crosslink to residues 19-28 spanning the amino-terminal region of native $G\alpha_t$ (17). The amino terminus of $G\alpha_q$ has also been implicated in the selectivity of receptor coupling, as addition of amino-terminal $G\alpha_q$ -specific residues to the amino terminus of $G\alpha_{i/o}$ proteins conferred the ability of this chimera to couple to G_q -coupled receptors, as measured by phosphatidylinositol hydrolysis (18). These studies indicate a specific interaction between the amino terminus of $G\alpha$ proteins and activated receptors.

$G\alpha_{i/o}$ family members (including $G\alpha_t$) are permanently, cotranslationally modified by myristate (consisting of 14 carbons) at the amino-terminal glycine residue, increasing hydrophobicity of this region. Myristoylation enhances $G\alpha$ - $\beta\gamma$ subunit association (19), but it is not essential for this association. *In vitro*, myristoylation of $G\alpha$ and farnesylation of the γ subunit of $G\beta\gamma$ are functionally redundant in facilitating coupling to activated receptors (20), as the ability of an unmyristoylated $G\alpha$ protein (in complex with $G\beta\gamma$) to undergo receptor-mediated GTP γ S binding was dependent on farnesylation of the $G\gamma$ subunit. A recent study on the functional significance of myristoylation which was conducted in transgenic mice revealed a role for myristoylation in deactivation of $G\alpha_t$ subunits. In mice expressing a myristoylation deficient form of $G\alpha_t$, the fraction of unmyristoylated proteins properly localized to rod outer segments (ROS) were found to be activation competent, with the remainder of $G\alpha_t$ mislocalized to inner compartments (21). The properly localized (unmyristoylated) $G\alpha_t$ fraction in the ROS was able to bind $G\beta\gamma$ and undergo receptor-mediated GTP γ S binding similar to native $G\alpha_t$, but had defects in deactivational processes such as GTPase activity and PDE-mediated GAP activity (21). These data point to a role for myristoylation beyond that of simple membrane localization and $G\beta\gamma$ binding.

Using biochemical and biophysical studies of purified, fluorescently labeled myristoylated (and unmyristoylated $G\alpha_{i1}$ proteins) and membrane bound receptors, we demonstrate here that specific amino-terminal residues of $G\alpha_{i1}$ proteins undergo receptor-mediated changes during the dynamic process of G protein activation. To examine changes at individual positions along the amino terminus, residues of interest in the amino terminus of a $G\alpha_{i1}$ protein lacking solvent-exposed cysteines ($G\alpha_{i1}$ Hexa I) were mutated to cysteine, followed by thiol-directed fluorescent labeling. The receptor-mediated environmental changes in individual amino-terminal $G\alpha_{i1}$ residues reported here, and the effect of myristoylation on those changes, together shed light on receptor-mediated changes that are communicated through the amino terminus of $G\alpha_{i1}$ proteins.

Materials and Methods

Materials

GDP and GTP γ S were purchased from Sigma-Aldrich (Milwaukee, WI). BODIPY® 630 methyl bromide (8-Bromomethyl-4,4-difluoro-3,5-bis-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene, MW 449, (BD⁶³⁰) and Alexa Fluor® 594 C₅-maleimide, (A1) were purchased from Invitrogen (Madison, WI). All other reagents and chemicals were of the highest available purity.

Preparation of fluorescently-labeled G proteins

In order to examine changes in specific residues using thiol-directed labels, we used a parent $G\alpha_{i1}$ in which solvent-exposed cysteines were conservatively replaced by site-directed mutagenesis, and then this construct was used as a basis for site-specific cysteine mutagenesis within the amino terminus. Construction, expression, purification and fluorescent labeling of $G\alpha_{i1}$ and $G\alpha_{i1}$ Hexa I proteins (unmyristoylated and myristoylated) were performed as described previously (22,23), with minor modifications. Briefly, the parent $G\alpha_{i1}$ Hexa I lacking solvent-exposed cysteines was generated with an expression vector encoding rat $G\alpha_{i1}$ with six amino acid substitutions at solvent-exposed cysteines (C3S-C66A-C214S-C305S-C325A-C351I) and a hexahistidine tag between amino acid residues M119 and T120. This construct served as the template for introducing individual cysteine substitutions at sites of interest using the QuickChange system (Stratagene, La Jolla, CA) as previously described; DNA sequencing confirmed all mutations. The mutant constructs are referred to as $G\alpha_{i1}$ Hexa I, followed by the site of cysteine substitution, and followed by M if the protein is expressed as a myristoylated protein. For example, Hexa I-13CM refers to the $G\alpha_{i1}$ Hexa I parent protein containing a cysteine substitution at the 13th residue and expressed with N-myristoyl transferase to obtain myristoylated protein, whereas Hexa I-13C is the same protein expressed in the absence of the N-myristoyl transferase vector to produce the unmyristoylated counterpart. The mutant constructs were expressed in *E. coli* BL21-Gold (DE3) with or without a N-myristoyl transferase (NMT) vector pbb131, which also encodes kanamycin resistance for selection of myristoylated proteins (generously provided by M. Linder, Washington University), and purified as detailed previously (23). Coomassie staining of urea SDS PAGE gels (24) demonstrate $G\alpha_{i1}$ Hexa I proteins are fully myristoylated (23) when co-expressed with the NMT vector. Purified proteins were stored at -80°C in buffer A containing 50 mM Tris, 100 mM NaCl, 2 mM MgCl_2 , 10 μM GDP, pH 7.5, and with 10% (v/v) glycerol; wild-type $G\alpha_{i1}$ containing native, solvent-exposed cysteines was additionally supplemented with 5 mM β -mercaptoethanol or 1 mM DTT prior to storage at -80°C . After purification, all proteins used in this study were greater than 85% pure, as estimated by Coomassie staining of SDS-polyacrylamide gels.

$G\alpha_{i1}$ Hexa I proteins were labeled as described previously (22,23) using A1 probe (Invitrogen, WI) with a labeling time of 1 hour. Extensive washing with buffer A in a 10 kDa molecular weight concentrator separated labeled protein from unbound probe, followed by gel filtration/spin purification of labeled proteins, resulting in a labeling efficiency between 0.25-0.6 $\text{mol}_{\text{label}}/\text{mol}_{\text{protein}}$ for all A1-labeled Hexa I proteins (measured by comparing label to protein concentration, $\epsilon_{588\text{nm}} = 96,000 \text{ cm}^{-1}\cdot\text{M}^{-1}$ for the A1 probe and protein concentrations as determined by the Bradford assay). All labeled $G\alpha_{i1}$ Hexa I proteins used in these assays demonstrated $\geq 40\%$ increase in intrinsic tryptophan emission upon AlF_4^- activation after labeling, as expected for properly folded and functional $G\alpha$ proteins (25). This is consistent with previous results demonstrating amino-terminally labeled $G\alpha_{i1}$ Hexa I proteins are activation competent and retain the ability to bind to $G\beta_1\gamma_1$ (22,23). $G\beta_1\gamma_1$ (native, bovine, containing multiple solvent-exposed cysteines) was labeled as described above with the thiol-reactive probe BD^{630} (Invitrogen, Madison, WI), resulting in a labeling efficiency of $>0.85 \text{ mol}_{\text{label}}/\text{mol}_{\text{protein}}$, using $\epsilon_{635\text{nm}} = 73,000 \text{ cm}^{-1}\cdot\text{M}^{-1}$.

Preparation of ROS, detergent-solubilized rhodopsin and $G\beta_1\gamma_1$

Urea-washed rod outer segment membranes (ROS) containing dark-adapted rhodopsin were prepared as previously described (26) and stored protected from light at -80°C in a buffer containing 10 mM MOPS, 200 mM NaCl, 2 mM MgCl_2 , 1 mM dithiothreitol (DTT) and 100 μM phenylmethylsulfonyl fluoride (PMSF). ROS detergent solubilization for supplementary experiments was performed as follows (42): under dim red light, urea-washed dark adapted ROS membranes were solubilized in 50mM Tris, pH 7.5, containing

100 mM NaCl and dodecylmaltoside (DM) to a final concentration of 0.5% at 4 °C with gentle agitation for 15 minutes. Insoluble material was removed by centrifugation at $20,000 \times g$ for 1 hr at 4 °C and solubilized rhodopsin concentration in the supernatant was determined using $\epsilon_{500} = 40,600 \text{ cm}^{-1}\text{M}^{-1}$. DM-solubilized dark rhodopsin thus obtained was substituted for ROS membranes in the assay of receptor-mediated fluorescence intensity changes upon light activation in labeled proteins as described below. These supplementary assays (conducted with soluble receptors) employed a final concentration of 750 nM DM-solubilized rhodopsin, 0.01% DM and 200 nM A1-labeled $\text{G}\alpha_{i1}$ Hexa I protein in complex with $\text{G}\beta_1\gamma_1$ in assay buffer, to determine the ability of solubilized rhodopsin to mediate light dependent changes in emission from amino-terminally labeled $\text{G}\alpha_{i1}$ Hexa I proteins in complex with native $\text{G}\beta_1\gamma_1$. $\text{G}\beta_1\gamma_1$ (hereafter referred to as $\text{G}\beta\gamma$) was purified from bovine retina as described previously (26) and stored in a buffer containing 10 mM Tris, 100 mM NaCl, 5 mM β -mercaptoethanol (BME), pH 7.5, and with 10% (v/v) glycerol.

Intrinsic tryptophan fluorescence

To monitor the ability of $\text{G}\alpha_{i1}$ Hexa I proteins to undergo activation, both before and after labeling, intrinsic tryptophan (Trp^{207} in $\text{G}\alpha_i$, analogous to Trp^{211} in $\text{G}\alpha_i$) fluorescence was measured as previously described (25). Briefly, labeled proteins (100 nM) in buffer A (50 mM Tris, 100 mM NaCl, 1 mM MgCl_2 , pH 7.5) containing 10 μM GDP were monitored by excitation at 280 nm and emission at 340 nm before and after the addition of AlF_4^- (10 mM NaF and 50 μM AlCl_3) using a Varian Cary Eclipse spectrofluorometer. Properly folded and functional $\text{G}\alpha$ subunits demonstrate a $\geq 40\%$ increase in intrinsic tryptophan emission upon AlF_4^- activation, in comparison to emission in the basal, GDP-bound state (25).

Binding of labeled $\text{G}\alpha_{i1}$ Hexa I proteins to $\text{G}\beta\gamma$

Subunit binding was measured in buffer A (supplemented with 10 μM GDP) using increasing amounts of $\text{G}\beta\gamma$ and 10 nM A1-labeled unmyristoylated $\text{G}\alpha_{i1}$ Hexa I-3C (as previously described for fluorescently-labeled Hexa I proteins (22)), with excitation/emission (ex/em) 570/616 nm. Changes in maximal emission (compared to basal emission in the absence of $\text{G}\beta\gamma$) were plotted as a function of increasing $\text{G}\beta\gamma$ concentration and fitted using a sigmoidal dose-response model using Prism 4.0 (Graphpad Software). For myristoylated proteins, the binding affinity was determined by FRET between labeled subunits (23), which are relatively solvent-protected and immobile even in the absence of $\text{G}\beta\gamma$ proteins (23), preventing efficient detection of binding by the relatively simpler protocol used for unmyristoylated proteins. Binding between A1-labeled myristoylated $\text{G}\alpha_{i1}$ Hexa I-3CM (10 nM) and increasing amounts of $\text{G}\beta\gamma$ -BD⁶³⁰ in buffer A was measured as a decrease in donor emission as a result of FRET, with ex/em 570/612 nm, less emission changes in the absence of labeled $\text{G}\alpha$ subunits, and fitted to a sigmoidal dose-response curve with Prism 4.0 (GraphPad Software).

Receptor-mediated fluorescence intensity changes in labeled proteins

Labeled $\text{G}\alpha_{i1}$ Hexa I proteins were reconstituted with $\text{G}\beta\gamma$ at 4 °C prior to addition of excess dark ROS, which was then transferred to a cuvette containing buffer A, with final concentrations of 400 nM $\text{G}\alpha$ protein, 800 nM $\text{G}\beta\gamma$, and 2 μM rhodopsin. All manipulations involving dark rhodopsin were performed in the dark or under dim red light to prevent its activation. The emission (dark spectra) from the cuvette containing dark ROS and A1-labeled subunits in complex with $\text{G}\beta\gamma$ was scanned between 580-750 nm (5 nm bandpass) with excitation at 575 nm (2.5nm bandpass) at 21 °C using a Varian Cary Eclipse. After collecting dark spectrum, cuvettes were subjected to light activation, and light spectra were collected in duplicate 4 minutes after the photoactivation of rhodopsin by a Vivitar electronic flash assembly. Finally, GTP γ S spectra were collected in duplicate 4 minutes after addition of GTP γ S (40 μM) to the light activated sample in the cuvette. While duplicate

readings were averaged for the light and GTP γ S spectra, the dark spectrum was collected only once prior to light activation in order to avoid spurious activation of rhodopsin.

Time-dependent changes in fluorescently labeled proteins

The rate of amino-terminal changes in A1-labeled G α_{i1} Hexa I residues upon light activation of rhodopsin was measured by monitoring the increase in emission at 617 nm (excitation 575 nm) of indicated labeled G α_{i1} Hexa I subunits reconstituted with G $\beta\gamma$ (200 nM each) in the presence of dark-adapted rhodopsin (200 nM) at 21 °C both before (basal) and within seconds after activation by light, and monitored over time. The data were plotted as emission increase compared to basal emission prior to receptor activation, and the rate was determined by plotting the data as a percent of maximal and fitting the data to an exponential association curve using Prism 4.0. The rate of changes in A1-labeled G α_{i1} residues upon GTP γ S binding was measured in a similar manner. The emission at 617 nm (excitation 575 nm) was monitored at 21 °C both before and after addition of GTP γ S (10 μ M) to indicated labeled G α_{i1} Hexa I subunits complexed with G $\beta\gamma$ (200 nM each) in the presence of light-activated rhodopsin (200 nM) for 30 minutes. Receptor-free nucleotide exchange (basal exchange) in the absence of rhodopsin in reconstituted heterotrimers is performed essentially as described above to obtain GTP γ S spectrum, with emission monitored at 617 nm over a period of 45 minutes, to examine changes in environment of the labeled residue in the absence of ROS membranes. The data (average of 3 or more independent experiments) were normalized to the level of fluorescence before GTP γ S addition (set to 1.0), and the rate was determined by fitting the data to an exponential dissociation curve using Prism 4.0.

Receptor-mediated nucleotide exchange in unlabeled proteins

The rate of receptor-catalyzed nucleotide exchange was measured by monitoring the time-dependent increase in the Trp²¹¹ fluorescence (ex/em 290/340 nm) of G α_{i1} proteins reconstituted with G $\beta\gamma$ (200 nM each) in the presence of 100 nM rhodopsin (light activated) at 21 °C following the addition of GTP γ S (10 μ M). The data (average of 3 or more independent experiments) were normalized to the baseline (0%) and the fluorescence maximum (100%). The exchange rate was determined by fitting the data to an exponential association curve using Prism 4.0 (GraphPad Software).

Rhodopsin binding assay

The ability of labeled myristoylated and unmyristoylated G α_{i1} proteins to bind rhodopsin in urea-washed ROS membranes was determined by adding aliquots of the indicated labeled G α_i Hexa I proteins (5 μ M) preincubated with an excess of G $\beta\gamma$ (10 μ M) to rhodopsin (50 μ M) in a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl and 1 mM MgCl₂ in the dark, after light activation, and with addition of GTP γ S (40 μ M) to a light activated sample. Following 30 minutes incubation at 4 °C, the membranes in each sample were pelleted by centrifugation at 20,000 $\times g$ for 1 hour, and supernatants removed from pellets. For the dark samples, reactions were foil-protected from light until removal of supernatant, which was performed under dim red light. The isolated supernatant and pellet fractions were boiled, resolved by SDS-PAGE, visualized with Coomassie blue and quantified by densitometry using BioRad Multimager. Each treatment (dark, light, and light + GTP γ S) was quantified by comparison of amount of 40 kDa G α_{i1} proteins in either pellet or supernatant to the sum total of G α in both fractions, as identified by co-migration with G α_{i1} standard, and expressed as a percentage of the total G α present in each given sample. Data are average of at least 3 independent experiments.

Results

Labeling of $G\alpha_{i1}$ Hexa I proteins

In order to measure receptor-mediated changes in the amino terminus of $G\alpha_{i1}$ proteins, in the presence and absence of myristoylation, we labeled a number of individual amino-terminal $G\alpha_{i1}$ Hexa I proteins at specific side chain positions (figure 1A). A $G\alpha_{i1}$ Hexa I parent protein lacking solvent-exposed cysteines was used as a template for introduction of cysteine residues at positions indicated in figure 1A, expressed as both myristoylated and unmyristoylated proteins. Both wild-type $G\alpha_{i1}$ and $G\alpha_{i1}$ Hexa I proteins, in complex with $G\beta_1\gamma_1$ ($G\beta\gamma$), can productively interact with rhodopsin (as seen in receptor-mediated GTPase and $^{35}\text{[S]}$ -GTP γ S binding (22, 27, 28)).

Prior to using fluorescently-labeled $G\alpha_{i1}$ proteins as reporters of $G\alpha$ environmental changes in an experimental protocol involving rhodopsin, it was necessary to select a fluorescent probe with spectral characteristics that do not overlap those of rhodopsin. Rhodopsin and its photoactivated metarhodopsin intermediates MI, MII, and MIII exhibit absorbance spectra which can interfere with many commonly used fluorescent probes. Highly conjugated probes with excitation maxima in the far red range (where rhodopsin and its intermediates are not affected) were therefore sought for this study. We examined three highly conjugated fluorescent probes with spectral characteristics in the far red range: BODIPY⁵⁹⁵, Atto⁶¹⁰, and Alexa⁵⁹⁵ (A1). Both Atto⁶¹⁰ and A1 probes effectively labeled desired residues with an efficiency of labeling between 0.25-0.6 mol_{label}/mol_{protein}, while BODIPY labeling of $G\alpha$ subunits was much less efficient (0.1 mol_{label}/mol_{protein}), likely due to poor solubility of this probe in buffer conditions required to maintain optimal activity of $G\alpha$ subunits. The $G\alpha_{i1}$ Hexa I proteins successfully labeled with the Atto⁶¹⁰ fluorescent probe underwent significant photobleaching under our assay conditions, in contrast to $G\alpha_{i1}$ Hexa I proteins labeled with A1, which were relatively photostable. Although not all fluorescent molecules are environmentally sensitive, the A1 probe reports changes in the polarity of its environment, as free, unreactive A1 probe alone demonstrated an increased emission intensity in methanol as compared to its emission in aqueous buffer (figure 1B).

Labeling amino-terminal residues of $G\alpha_{i1}$ Hexa I proteins with the A1 probe does not perturb the ability of these proteins to undergo activation-dependent changes, nor does it prevent binding to $G\beta\gamma$ subunits, consistent with previous studies using a variety of fluorescent probes (22,23). For example, in figure 1C, activation-dependent changes induced by the transition state mimetic, AlF_4^- result in a $\geq 40\%$ increase in intrinsic tryptophan fluorescence, a prerequisite for use of the labeled proteins in these studies. $G\beta\gamma$ binds labeled $G\alpha_{i1}$ Hexa I proteins; the dose-dependent changes in emission from protein labeled at the third residue upon binding to $G\beta\gamma$ (figure 1D) resulted in an apparent K_d of 30 nM and 230 nM for myristoylated (1D, top) and unmyristoylated (1D, bottom) proteins, respectively. These values are consistent with previously published values for the interaction between fluorescently labeled $G\alpha_{i1}$ Hexa I subunits and $G\beta\gamma$ (22,23), and the relatively higher affinity of $G\beta\gamma$ for myristoylated subunits is consistent with the known myristoylation-dependent enhancement of subunit affinity (29).

Amino terminus reports receptor-mediated changes in environment upon activation and GTP γ S binding

After confirming that labeled $G\alpha_{i1}$ Hexa I proteins are properly folded and functional, we sought to determine whether these proteins could report interactions with activated, membrane-bound receptors, using an experimental protocol depicted in figure 2A. *In vivo*, rhodopsin is coupled to G_t activation. Both $G\alpha_i$ (and G_i family member $G\alpha_o$) functionally couple to rhodopsin *in vitro*, and both undergo cotranslational myristoylation, but unlike

$G\alpha_t$, $G\alpha_i$ expresses well in *E. coli*. Both myristoylated and unmyristoylated $G\alpha_{i1}$ subunits, in complex with native, acylated $G\beta_1\gamma_1$ ($G\beta\gamma$) subunits, are known to bind to rhodopsin upon light activation, and to be released from the membrane upon subsequent GTP binding (14, 20); the goal of these studies was to examine changes in specific amino-terminal residues during this process. Therefore, emission of a fluorescently labeled $G\alpha_{i1}$ Hexa I protein (reconstituted with $G\beta\gamma$) was first scanned in the presence of ROS membranes containing dark-adapted rhodopsin, resulting in the basal (dark) emission spectrum to which later changes were then compared. Emission was scanned again after light activation (catalyzing GDP release and generating the empty pocket state), and finally after addition of GTP γ S to the cuvette, which leads to dissociation from the receptor, enabling both $G\alpha$ and $G\beta\gamma$ to interact with their respective downstream effectors. Normalizing emission from labeled G protein to that obtained in the basal (dark) states eliminates effects associated with protein-lipid interaction which occur independently of receptor activation.

Given the fact that rhodopsin is light sensitive and demonstrates multiple absorbance maxima, the probe selected for these studies must exhibit spectral characteristics in the far red range, to prevent overlap with rhodopsin. The majority of rhodopsin absorbance (and that of its photo-intermediates) occurs at wavelengths below 500 nm. Studies by Imamoto et al. demonstrate that the A1 probe is compatible with assays involving rhodopsin (30). In control experiments to confirm this, rhodopsin was scanned from 580-750 nm (in the absence of labeled protein) in the dark, after light activation, and upon addition of GTP γ S, with excitation at 575 nm. Rhodopsin does not substantially contribute to the overall emission signal, as seen in supplementary figure S1A and resulting difference spectra (inset, figure 2B). As a further control, we also measured emission changes from free probe (made unreactive by treatment with DTT) in the presence of ROS membranes, in the absence of G protein. The free, unreactive A1 probe in the presence of dark ROS showed no evidence of light- or GTP γ S- dependent changes under our experimental conditions (supplementary figure S1B).

Using the protocol depicted in figure 2A, we examined the emission spectra of myristoylated (figure 2B) and unmyristoylated (figure 2C) $G\alpha_{i1}$ Hexa I proteins labeled at the third residue in complex with $G\beta\gamma$, first in the presence of inactive rhodopsin (black traces) and again upon receptor activation (red traces). Both myristoylated and unmyristoylated labeled proteins demonstrated robust changes in their environment upon receptor activation, relative to their respective environments in the inactive state. Upon receptor-mediated GTP γ S binding, only the unmyristoylated protein demonstrated a decrease in fluorescence, corresponding to a relatively more solvent-exposed (aqueous) environment for the unmyristoylated amino terminus upon GTP γ S binding (figure 2C, green). The myristoylation-dependent difference in environment upon GTP γ S binding is not due to the presence of membrane lipids in the experiment, as this difference is still evident in a membrane-free, receptor-free environment (figure 2D) as a result of basal (unstimulated) nucleotide exchange. Furthermore, we confirmed the ability of this myristoylated protein to undergo GTP γ S-dependent changes in solution by intrinsic Trp²¹¹ fluorescence, which was found to increase in a manner similar to wild type upon addition of GTP γ S (not shown). Therefore, there is a similar degree of solvent protection in both the heterotrimeric and the GTP γ S-bound state for myristoylated protein labeled at the third residue (figures 2B and D). Together these results are consistent with a myristoylation-dependent solvent protection of the extreme amino terminus upon GTP γ S binding.

Comparison of light- and GTP-dependent changes in specific amino-terminal residues of $G\alpha_{i1}$ proteins

We next examined relative differences in emission of 9 individually labeled amino-terminal residues during the G protein cycle in myristoylated and unmyristoylated proteins, which

reflect changes in the probe's environment upon activation and deactivation. Comparison of the emission of each one of the individually labeled proteins upon receptor activation and GTP γ S binding (figures 3A-B) paints a picture of the relative changes in environment for each residue as the G protein moves from the inactive, heterotrimeric, dark state (obtained in the presence of dark adapted ROS), to the receptor-bound empty pocket (light) state, and finally to the GTP γ S-bound state. Although myristoylation confers a greater degree of membrane association for heterotrimeric G α subunits in the dark (figure 3C-D), both myristoylated and unmyristoylated subunits efficiently translocate to membrane fractions upon light activation. Because introduction of membranes to buffer a cuvette containing soluble G proteins can induce a small variation in signal due to scatter, we set the basal emission of the labeled proteins in the presence of dark adapted ROS to 1.0; all subsequent changes were normalized to basal. This protocol accounts for contributions to basal emission from membrane association of myristoylated proteins in the dark, as well as small variations in labeling efficiencies at different residues.

Since the A1 probe demonstrates decreased emission intensity in a more aqueous environment and increased intensity in a more hydrophobic environment (figure 1B), the receptor-mediated increases in emission upon light activation from the labeled proteins (figure 3A-B) likely represent increases in the hydrophobicity of the environment detected by the fluorescent probe. The intensities exhibited for the labeled proteins are shown relative to that detected for each one in the inactive, heterotrimeric state (as measured in the presence of ROS membranes containing dark-adapted rhodopsin, figure 3A-B, black bars, and figure 2A, left panel). The light-activated state shown by the red bars in figure 3 effectively represents the receptor-bound, empty pocket conformation depicted in figure 2A, center panel. Light activation is accompanied by increased emission intensity at nearly all positions examined along the amino-terminal helix (figures 3A-B, red bars), as compared to their inactive, heterotrimeric states (black bars). Light-dependent changes in fluorescence were accompanied by enhanced membrane association (where the relative percentage of G α in the pelleted fraction exceeds the percentage of G α in the soluble fraction) for both myristoylated and unmyristoylated proteins (figures 3C-D), consistent with activation-mediated binding of labeled proteins to membrane-bound receptors. This localization is reversed by addition of GTP γ S (figures 3A-B, green bars) to the light-activated samples, indicating increased solubility upon GTP γ S binding.

The light-dependent changes in fluorescence ranged from 25-40% increase in emission over basal upon light activation of receptor in myristoylated proteins labeled at the 3rd, 13th, 17th, and 21st residue (figure 4A). Myristoylation-dependent differences in emission intensity were statistically significant at several positions (figure 4A). These changes (and those related to receptor-mediated GTP γ S binding) are eliminated when labeled G proteins are boiled prior to use in the assay (10 minutes at 95 °C). The emission changes in G proteins which occur after flashing light on the system are also ablated when heat denatured ROS membranes (10 minutes at 95 °C) are substituted for ROS membranes kept at 4 °C in the experiment (in conjunction with properly folded G-proteins). The lack of light-dependent changes exhibited using after heat-denaturation of either G proteins or rhodopsin suggest the changes observed are directly related to the proper folding and functioning of these proteins.

While enhanced membrane localization of myristoylated proteins might be predicted to mediate the increases in intensity upon light activation, it is important to keep in mind that these changes are relative to their environment in the dark state. Both myristoylated and unmyristoylated proteins efficiently translocate to membrane fractions upon light activation, with the myristoylated proteins demonstrating a significantly higher degree of membrane association in the dark state than unmyristoylated (figure 3C-D), which results in a smaller relative difference between the dark versus light membrane localization for myristoylated

proteins, and likewise a larger difference between these two states for the unmyristoylated protein. If membrane localization was solely responsible for light-dependent changes in emission, then the myristoylated proteins would be predicted to show a smaller difference in emission upon light activation than the unmyristoylated protein. However, this is not the case. This argues against membrane translocation as the cause of the myristoylation-dependent enhancement of emission we observe upon receptor activation.

Although this study is focused on the relationship between membrane-bound receptors and amino terminal residues of $G\alpha_{i1}$ proteins, and although detergents would be expected to diminish interactions between membrane bound receptors and myristoylated proteins, we investigated the role of membrane lipids in this system using a detergent solubilized form of rhodopsin and labeled, myristoylated $G\alpha_{i1}$ Hexa I proteins. The labeled proteins retained the ability to report changes in emission upon light activation of receptors, albeit to a slightly lesser extent to that seen using intact ROS membranes, which were eliminated by heating the soluble rhodopsin preparation for 10 minutes at 95 °C prior to use in these experiments (supplemental figure 2).

We further characterized the time-dependence of the increase in emission upon light activation from myristoylated and unmyristoylated proteins labeled at the 21st residue (figure 4C), which differed significantly upon receptor activation (figure 4A). The rate of the light-dependent change is considerably faster for the myristoylated protein (0.023 sec⁻¹), in comparison to its unmyristoylated counterpart (0.012 sec⁻¹). These results suggest myristoylation can enhance both the rate and intensity of receptor-catalyzed changes in fluorescence emission seen upon light activation.

Finally, binding of GTP γ S increased the solvent exposure of nearly all of the residues along the amino terminus (figures 3A-B, green bars versus red bars) as compared to their environment in the light activated state. Myristoylation dramatically inhibited the GTP γ S-mediated change in solvent exposure at position 3, and had a more subtle effect at residues 10 and 13 (figure 4B). We characterized the time-dependence of the decrease in emission intensity upon GTP γ S binding at the 13th residue (figure 4D), which was significantly altered by myristoylation as seen in figure 4B. The rate of change in emission which accompanies GTP γ S binding in the presence of light-activated receptor shown in figure 4D was nearly the same for myristoylated and unmyristoylated labeled proteins (0.018 sec⁻¹ and 0.022 sec⁻¹, respectively) a similarity which is confirmed using unlabeled proteins, described below. Therefore, myristoylation-dependent differences in emission intensity which accompanies GTP γ S binding does not necessarily result in differences in the rates of GTP γ S binding from the labeled proteins.

Myristoylation does not enhance receptor-mediated nucleotide exchange of $G\alpha_{i1}$ Hexa I proteins as measured by intrinsic Trp²¹¹ fluorescence in unlabeled proteins

Since we detected no significant differences in the rates of GTP γ S binding in labeled proteins (despite some differences in intensity), we confirmed this finding by comparison of these rates in unlabeled proteins (figure 5). This was accomplished by taking advantage of the fact that the intrinsic tryptophan fluorescence emission (reported by Trp²¹¹ located in the Switch II region) increases upon GTP γ S binding, and the time dependence of this increase can be measured in the presence of light-activated rhodopsin (14) which is able to catalyze nucleotide exchange in G_i (as well as G_t) proteins. $G\alpha_{i1}$ Hexa I- $\beta\gamma$ complex is known to undergo a slightly higher rate of nucleotide exchange than wild-type $G\alpha_{i1}\beta\gamma$ (14), shown in figure 5B for comparison. This effect is due to conservative replacement of native solvent-exposed cysteines, a prerequisite to generation of the cysteine-depleted $G\alpha_{i1}$ Hexa I parent protein, previously shown to have functional activities similar to wild-type $G\alpha_{i1}$ (22,23). Receptor-mediated nucleotide exchange rates for myristoylated and unmyristoylated

proteins reveal no significant differences in the rates of nucleotide exchange for myristoylated proteins and unmyristoylated $G\alpha_{i1}$ Hexa I proteins (figures 5A-B), similar to results obtained with labeled $G\alpha_{i1}$ Hexa I proteins. Furthermore, the slightly higher (but statistically indistinct) rate of receptor-mediated nucleotide exchange in unmyristoylated proteins is not likely a result of increased basal exchange, as the exchange rate of unmyristoylated $G\alpha_{i1}$ Hexa I -3C (figure 2D) is 0.007 sec^{-1} , which is one-third the receptor-catalyzed rate (figure 5B). Therefore it is unlikely that receptor-independent activation of $G\alpha$ is a major contributor to exchange rates in these unmyristoylated proteins. The ability of unmyristoylated proteins to undergo efficient receptor-mediated nucleotide exchange also confirms that myristoylation is not a requirement for productive interaction with membrane-bound receptors, consistent with results from these and other published studies (both *in vitro* and *in vivo*) (20,21).

Changes in fluorescence emission intensity are accompanied by shifts in emission maxima of labeled proteins upon receptor activation and GTP γ S binding

Closer examination of the emission maxima (em_{max}) of the free A1 probe in aqueous buffer solution versus emission in a more hydrophobic environment such as methanol (figure 1B) reveals a small but reliable shift of the em_{max} to lower wavelengths (blue-shifted emission) in a less aqueous environment, when plotted as percent of each of their respective maximum intensities (figure 6, top panel). The em_{max} of an equimolar amount of free A1 probe shifted from 617 nm in aqueous buffer to 609 nm in methanol, a change of 8 nm. The emission of A1-labeled proteins also demonstrated a small but detectable blue shift upon receptor activation, on the order of 1-4 nm, which are reversed by GTP γ S binding (figure 6). Closer examination of the magnitude of these shifts reveals a relatively greater blue shift for proteins labeled at the 3rd, 13th, 17th, and 21st residues. While this probe reports more robust changes in overall emission intensity than in the relative smaller shifts seen in emission maxima, the intensity changes (figure 3A-B) are generally consistent with blue shifts seen upon light activation, which are reversed by GTP γ S binding (figure 6).

Discussion

Biophysical studies have broadened our understanding of receptor-mediated G protein activation, and in combination with crystal structures, provide powerful tools to probe G protein signaling. The most complete crystallographic views of $G\alpha$ amino-terminal structure have been obtained with unmyristoylated $G\alpha$ proteins in complex with $G\beta\gamma$, which shows the $G\alpha$ amino-terminal α -helix making extensive contacts with $G\beta\gamma$ (figure 1A). Since isolated $G\alpha$ proteins are unstable in the nucleotide-free state, and since there is no high resolution structure of an activated receptor in complex with a G protein, biophysical studies offer unique insights into the conformation of receptor-bound, empty pocket state of $G\alpha$ subunits. Our results demonstrate that residues all along the amino-terminal helix of $G\alpha_{i1}$ Hexa I proteins undergo specific receptor-mediated changes upon activation. These changes map to a distinct surface of the amino-terminal helix, suggesting intimate involvement of the amino terminus of $G\alpha$ in changes that accompany receptor-mediated G protein activation processes. While these data do not rule out contributions from membrane lipids in receptor-mediated amino-terminal environmental changes observed in $G\alpha_{i1}$ Hexa I proteins, such contributions are secondary to receptor activation, as heat denaturation of rhodopsin (as well as heat denaturation of soluble rhodopsin) eliminates these changes, and because these changes persist in the presence of detergent-solubilized receptors.

The fluorescent probe linked to individual amino-terminal $G\alpha_{i1}$ Hexa I residues reports changes in its environment during signaling. The current data, including observations that the amino terminus can crosslink to activated receptors (17) and the ability of amino-terminal peptides to compete with $G\alpha_t$ for stabilization of activated rhodopsin (16) are

altogether consistent with a specific interaction between amino terminal residues of $G\alpha$ with activated receptors. These interactions result in changes in the environment of individually labeled residues.

In the heterotrimeric (basal) state, this environment is depicted in dark spectra, which are obtained in the presence of membrane-bound, inactive receptor. The light spectra, obtained after light activation of receptor and before $GTP\gamma S$ addition, generates the receptor-bound, empty pocket conformation. The light-activated emission spectra reflect changes in the environment of the probe linked to individual amino-terminal residues that occur upon receptor-mediated G protein activation. Myristoylation enhanced not only the intensity, but also the rate of the light-mediated changes in emission upon light activation. The myristoylation-dependent increase in the rate of light-dependent emission changes may be due to enhanced G protein binding to ROS in the dark state, as increased G protein membrane localization prior to light activation would reduce the factors affecting the rate of light activation from a 3-dimensional to a 2-dimensional diffusion issue.

The increases in intensities upon light activation, accompanied by blue-shifted emission maxima, together indicate a less aqueous-accessible environment upon receptor activation. Furthermore, several of the residues which demonstrate both the greatest emission shifts and intensity changes upon receptor activation (residues 13, 17 and 21) map to a distinct face of the $G\alpha$ amino-terminal α -helix (figure 7A) located opposite the $G\beta\gamma$ binding interface (figure 7B). The native residues at positions 13, 17, and 21 (V13, K17, and R21) in $G\alpha_i$ proteins would be predicted to promote membrane interactions through hydrophobic and electrostatic interactions, which may augment interactions of G proteins with membrane bound receptors *in vivo*. Despite the fact that mutation to cysteine and addition of the A1 probe did not perturb the ability of the labeled proteins to undergo activation-dependent changes, to bind $G\beta\gamma$, to exchange nucleotide or to report activation-dependent changes, it is not unreasonable to expect that unlabeled, native proteins may interact even better with membrane bound receptors than the labeled proteins used in this study. Nevertheless, since heat denaturation of the labeled proteins eliminated their ability to detect changes in their environment, as did heat denaturation of the rhodopsin preparations, this suggests the changes we observe are a result of G protein-receptor coupling between functional proteins.

Receptor activation induces release of GDP and binding of GTP, thus lowering the affinity of activated $G\alpha$ subunits for receptors. This is reflected by a decrease in emission for nearly all of the A1-labeled proteins upon $GTP\gamma S$ binding relative to the light-activated state, and this decrease is generally more pronounced for unmyristoylated proteins, suggesting myristoylation reduces the aqueous exposure of amino-terminal residues in the $GTP\gamma S$ -bound state. This was unrelated to the presence of membrane lipids, as a similar decrease in emission was noted after basal (uncatalyzed) nucleotide exchange occurred in solution (figure 2D). The relatively greater degree of aqueous exposure for several of the unmyristoylated proteins compared to their myristoylated counterparts upon $GTP\gamma S$ binding is consistent with previous EPR studies of unmyristoylated $G\alpha_{i1}$ Hexa I proteins, which show a dramatic transition towards disorder at the amino terminus upon addition of $GTP\gamma S$ to the receptor:G protein complex (22). Solution studies of GDP-bound myristoylated $G\alpha_i$ Hexa I proteins reveal a relatively immobilized EPR spectra for individual residues all along the amino terminus, even in the absence of $G\beta\gamma$ (23), unlike the more mobile amino-terminal residues seen in unmyristoylated $G\alpha_{i1}$ Hexa I proteins (22). Previous fluorescence studies of these isolated, labeled, myristoylated proteins in solution indicated that the amino terminus in the activated, GDP-AlF₄⁻ bound state is in a more hydrophobic environment than the inactive, GDP-bound state (23). A solvent protection of the myristoylated amino terminus via an (unidentified) intramolecular binding site would explain the relatively similar

recovery we observe of myristoylated and unmyristoylated GTP γ S-bound protein in the soluble fraction after centrifugation.

A number of proteins are known to utilize myristoyl-switching mechanisms to regulate signaling, including recoverin, PKA catalytic subunit, MARCKS and ARF, with ARF's myristoyl switch triggered by GTP binding. Membrane lipids affect the nucleotide binding of a full-length ARF, but not an amino-terminally truncated (unmyristoylated) Arf protein, and a myristoylated amino-terminal peptide derived from the ARF protein also inhibited ARF activities (44). Myristoylation of G α proteins may serve an analogous switching function in heterotrimeric G $_i$ proteins, mediating association with membrane-bound receptors (in concert with acylation of G γ). Myristoylation may regulate solubility of individual G α -GTP subunits as they dissociate from membrane-bound receptors by protecting the myristoylated amino terminus of the GTP-bound subunit from the aqueous solvent through an intramolecular binding site on the surface of the protein. The current study reveals a myristoylation-dependent solvent protection for the extreme amino terminus of G α_{i1} after receptor-mediated GTP γ S binding. Further work to unambiguously identify a potential intramolecular binding site for the myristoylated amino terminus of G α_{i1} is currently underway.

There is a vast amount of data supporting GPCR dimerization/oligomerization, both *in vitro* and *in vivo* (reviewed in (31), (32)). Although the current work is not aimed at addressing the question of receptor:G protein stoichiometry, it is interesting to consider a role for the myristoylated amino terminus in receptor dimerization. A dimeric receptor provides a plausible explanation for the ability of an activated receptor to crosslink to spans of residues in both the carboxy- and amino-terminus of G α_t (17,33) with chemical and photo-activatable crosslinkers. These regions are separated by as much as 28 Å, as seen in the crystal structure of inactive G $_t$ heterotrimer (PDB file 1GOT). The carboxy-terminal eighth helix of rhodopsin (often referred to as the fourth cytoplasmic loop of rhodopsin) is also known to be involved in transducin coupling through both its amino and carboxyl terminus, as seen in peptide studies and site-directed mutagenesis of this region (34-36).

Structural studies have confirmed that the length of an entire heterotrimeric G protein is sufficient to span a receptor dimer, using crystallographic constraints from G proteins, rhodopsin and the recently solved β -adrenergic receptor (2,10,33). Results from atomic force microscopy support a dimeric model of rhodopsin (33), and are also consistent with higher order structures, which is not unlikely *in vivo*, given the density of receptors in the rod outer segment. The existence of GPCR dimers is supported by results from small angle neutron scattering which suggested that the leukotriene B4 receptor-1, BLT1 (a member of the rhodopsin family of GPCRs) assembles with G $_i$ proteins in a 2:1 receptor:G protein ratio upon agonist activation, using receptors stabilized in detergent solution (37). Recent reports suggest rhodopsin dimerization may play roles distinct from activation *in vivo*, as monomeric rhodopsin is sufficient for G protein activation *in vitro* (43).

While little has been published on the functional coupling of specific residues in the amino terminus of G α proteins to activated receptors, the carboxy-terminus of G α proteins has been more extensively characterized in terms of GPCR interactions. Recent work shows that GPCR activation is coupled to a rotation and translation of the α -5 helix in the carboxy terminus of G α_{i1} Hexa I proteins, which may aid in GDP release, as elimination of this movement by a flexible linker significantly impairs receptor-mediated nucleotide exchange (14). Crystal structures of isolated G α subunits (unmyristoylated) place residues within the carboxy terminus (residue 345) and the last resolved residues in the amino terminus (residue 33) into close proximity with each other. The amino-terminal portion of G α (and its proximity to the carboxy terminus) has also been implicated in the specificity of receptor

coupling in functional assays using a chimeric approach. The region which links the amino-terminal α -helix to the first β -sheet of the GTPase domain (residues 31-33 in $G\alpha_i$) is proximal to the carboxy terminus in $G\alpha$ crystal structures, and this linker was shown to be of particular importance to the specificity of receptor coupling, using a $G\alpha_{15/16}$ chimera (38). This is consistent with the idea that the amino and carboxy termini work together in concert to affect nucleotide release upon receptor activation.

The lever arm model of receptor-mediated nucleotide release proposed by Bourne et al. (39) implicates a receptor-mediated amino-terminal conformational change in $G\alpha$ which rotates $G\alpha$ away from $G\beta\gamma$, thus distorting the nucleotide binding site and opening an exit route for GDP release. However, the functional redundancy of farnesylation of $G\gamma$ and myristoylation $G\alpha$ in receptor-mediated G protein activation does not support this lever-arm model of G-protein activation. Furthermore, although our data are consistent with a role for the amino terminus of $G\alpha_{i1}$ in receptor-mediated G protein activation, the data do not directly address the contribution from $G\beta\gamma$. Nevertheless, the current work provides convincing evidence for the intimate contact between the amino terminus of $G\alpha_{i1}$ and activated, membrane bound receptors, indicated by the receptor-mediated change in the environment of the amino terminus. More intensive studies detailing changes in the spatial relationship between $G\alpha$, $G\beta\gamma$ and receptor (and the order of such changes) leading to nucleotide release must be undertaken to fully elucidate the complete mechanism of receptor-mediated nucleotide release leading to G protein activation.

Altogether this study identifies specific receptor-mediated changes occurring along the amino terminus, many of which are distinctly affected by myristoylation, and a myristoylation-dependent solvent protection of the extreme amino terminus of $G\alpha_{i1}$ proteins after receptor-mediated G protein activation. This work paints a picture of a conformationally adaptable myristoylated amino-terminal region of $G\alpha$ proteins. Myristoylation-dependent variations in the environment and conformation of amino-terminal residues of $G\alpha_{i1}$ proteins upon G protein activation may play a role regulating interactions with $G\beta\gamma$, membrane-bound receptors and soluble effectors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GPCR	G protein coupled receptor
G protein	heterotrimer consisting of $G\alpha_i\beta_1\gamma_1$
GEF	guanine nucleotide exchange factor
N-terminus	amino terminus
Hexa I	$G\alpha_{i1}$ isoform lacking 6 solvent-exposed cysteines
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
G _t	G protein of the rod outer segment, transducin
G _i	family of G proteins coupled to inhibition of adenylyl cyclase
G α	α subunits of G proteins
EPR	electron paramagnetic resonance
G $\beta\gamma$	G $\beta_1\gamma_1$ -subunit derived from native G _t
ROS	membranes from rod outer segments containing rhodopsin
SDS	sodium dodecyl sulfate
PMSF	phenylmethanesulfonyl fluoride
A1	Alexa
BD	Bodipy
DTT	dithiothreitol
NMT	N-myristoyl transferase
MES	2-(N-morpholino)ethanesulfonic acid
Tris	tris(hydroxymethyl) aminomethane
FRET	fluorescence resonance energy transfer
MI	metarhodopsin I
MII	metarhodopsin II
MIII	metarhodopsin III
AU	arbitrary units
SEM	standard error of mean

ex/em	excitation/emission
em _{max}	emission maxima

1D

Figure 1. Labeling of $G\alpha_{i1}$ Hexa I protein with A1 probe

(A) Location of individual Cys mutations required for subsequent thiol-directed labeling of $G\alpha_{i1}$ Hexa I proteins. G_i structure from pdb file 1GP2 (40), rendered with Chimera (41) program. Amino terminus of G_i heterotrimer, with $G\beta\gamma$ in light grey, $G\alpha GDP$ in blue, and residues selected for labeling in red. (B) A1 probe (inset) reports changes in environment; dissolution of 10 nM A1 probe in methanol (purple) is accompanied by increased emission intensity, as compared to the same concentration of probe in aqueous buffer (teal), each scanned in triplicate ($\pm$ SEM). (C) A minimum of 40% increase in intrinsic Trp²¹¹ fluorescence (ex/em 280/340 nm) is seen upon AlF_4^- activation of all properly folded and functional $G\alpha$ proteins, shown here for $G\alpha_{i1}$ Hexa I fluorescently labeled at the third residue, in both myristoylated (blue) and unmyristoylated (pink) proteins. (D) A1-labeled $G\alpha_{i1}$ Hexa I subunits bind $G\beta\gamma$. Addition of increasing amounts of $G\beta\gamma$ subunits to A1-labeled $G\alpha_{i1}$ Hexa I subunits labeled at the third residue in a myristoylated (top) or unmyristoylated (bottom) protein results in a dose-dependent change in emission from labeled $G\alpha$ protein upon subunit association, less background in the absence of $G\alpha$ protein (% change over basal, average of 3 independent measurements, $\pm$ SEM).

2A.

2B

2C

2D

Figure 2. Receptor-mediated and activation-dependent changes in A1-labeled $G\alpha_{11}$ Hexa I proteins

(A) Schematic of G protein activation by membrane-bound receptor. (B-C) Activation-dependent changes (representative spectra) in the presence of rhodopsin. $G\alpha_{11}$ Hexa I proteins (B, myristoylated; C, unmyristoylated) are fluorescently labeled at the third residue, reconstituted with $G\beta\gamma$, and emission sequentially scanned (excitation 575 nm), first in the presence of dark-adapted rhodopsin (black trace), then after light activation (red), and finally upon addition of $GTP\gamma S$ (green). Inset to B: control difference spectra of rhodopsin alone (dark grey, light activated - dark state; light grey, light activated state - after addition of $GTP\gamma S$ to ROS) in the absence of G protein (raw data shown in supplementary figure S1A).

(D) Changes in emission from labeled proteins reconstituted with G $\beta\gamma$ (ex/em 575/615) upon basal GTP γ S binding in the absence of ROS membranes containing rhodopsin. Emission changes are monitored over 45 minutes to allow for full exchange under uncatalyzed conditions. Experiments were performed in triplicate and normalized to emission prior to GTP γ S addition (set to 1.0).

3A

3B

3C

3D

Figure 3. Amino-terminal residues of G α_{II} Hexa I proteins report specific receptor-dependent changes in environment

(A,B) $G\alpha_{11}$ Hexa I proteins (myristoylated, A; unmyristoylated, B) are fluorescently labeled at the indicated residue, reconstituted with $G\beta\gamma$, and emission sequentially scanned (excitation 575 nm), first in the presence of dark-adapted rhodopsin (black bars, normalized to 1.0), after light activation (red bars), and finally upon addition of $GTP\gamma S$ (green bars). Results from 3 or more independent experiments ($\pm$ SEM), each performed in duplicate. (C) Rhodopsin binding assay: representative SDS-PAGE (Coomassie stain) analysis of $G\alpha_{11}$ Hexa I proteins labeled at the 13th residue, reconstituted with excess of $G\beta\gamma$ prior to binding to ROS in the dark (D), light (L), and after light activation followed by addition of $GTP\gamma S$ (G). S denotes supernatant fraction and P denotes fraction from pellet after centrifugation. (D) Quantitation of membrane binding experiments, average of three independent experiments, ($\pm$ SEM, *** $p < 0.0001$). Asterisks demonstrate a significant increase in membrane association for myristoylated $G\alpha$ in complex with $G\beta\gamma$ in the presence of dark rhodopsin.

Figure 4A **Figure 4B** **4C** **4D** **Figure 4. Comparison of receptor-mediated changes in myristoylated and unmyristoylated labeled proteins**

Direct comparison of light-dependent intensity changes (A) and GTP γ S-dependent intensity changes (B) from figure 3A-B (* $p < 0.01$; ** $p < 0.001$; p*** < 0.0001). (C-D) Time-dependence of receptor-mediated changes in emission of myristoylated and unmyristoylated proteins (ex/em 575/615 nm, average of 3 or more independent experiments). (C) Time-dependent change upon light activation for protein labeled at the 21st residue relative to GDP-bound, inactive state (set to 0) and measured immediately after light activation. (D) Time dependence of GTP γ S-mediated changes in emission of myristoylated and unmyristoylated protein labeled at the 13th residue in the presence of light-activated receptor immediately after addition of GTP γ S as described in materials and methods, compared to level of emission in the light-activated state (set to 1.0).

Fig. 5. Comparison of receptor-mediated changes in unlabeled proteins reported by intrinsic Trp²¹¹ fluorescence

(A) Emission of $G\alpha_{i1}$ Hexa I 17C (unmyristoylated, grey) and 17CM (myristoylated, black) in complex with $G\beta\gamma$ is monitored (ex/em 290/340) after addition of GTP γ S in the presence of activated rhodopsin, compared to the level prior to addition of GTP γ S, as described in materials and methods. (B) Receptor-mediated exchange rates measured for each individual $G\alpha_{i1}$ Hexa I mutant protein. The exchange rates were determined by fitting each data set to an exponential association curve. Data represent the average of 4-6 independent experiments ($\pm$ SEM).

Figure 6. The A1 probe reports environmentally-dependent changes in emission maxima (Top panel): Free, unreactive, A1 probe (10 nM) undergoes an 8 nm blue shift in methanol (black trace) versus aqueous buffer (grey), with respective intensities normalized to 100%. (Bottom panel): intensity changes for labeled G α_{11} Hexa I proteins are accompanied by a shift in emission maxima (in nm, average of 3 independent measurements) upon receptor activation and GTP γ S binding. The third column (D-L) is the net blue shift for each labeled protein upon light activation, obtained by subtraction of $em_{maxdark} - em_{maxlight}$ (in nm).

Figure 7. Receptor-mediated changes map to a distinct surface of the $G\alpha_i$ amino-terminal helix
 (A) Helical wheel depiction of ordered residues in amino terminus of $G\alpha_i$ as seen in G_i heterotrimer, file 1GP2 (39), with disordered extreme amino-terminal residues shown with dotted line. Residues colored according to receptor-mediated blue shifts in $\epsilon_{m\max}$ of myristoylated protein: red, greater than 2 nm blue shift; purple, 1-2 nm; teal, less than 1 nm.
 (B) G_i heterotrimer featuring the face of G_i implicated in binding to membrane bound receptor (structure from pdb file 1GP2 (39), rendered with Chimera (40) program). $G\alpha$ shown in yellow, $G\beta$ in green, $G\gamma$ in pink, with the last ordered residues in C terminus of $G\alpha$ (residue 347) and $G\gamma$ (residue 61) labeled as CT α and CT γ , respectively, and colored teal. Red: residues 13, 17 and 21 in the amino-terminal helix of $G\alpha$ subunit.