Activation of the β₂-Adrenergic Receptor-Goₐ Complex Leads to Rapid Depalmitoylation and Inhibition of Repalmitoylation of Both the Receptor and Goₐ*

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Palmitoylation is unique among lipid modifications in that it is reversible. In recent years, dynamic palmitoylation of G protein α subunits and of their cognate receptors has attracted considerable attention. However, very little is known concerning the acylation/deacylation cycle of the proteins in relation to their activity status. In particular, the relative contribution of the activation and desensitization of the signaling unit to the regulation of the receptors and G proteins palmitoylation state is unknown. To address this issue, we took advantage of the fact that a fusion protein composed of the stimulatory α subunit of trimeric G protein (Goα) covalently attached to the β₂-adrenergic receptor (β₂AR) as a carboxyl-terminal extension (β₂AR-Goα) can be stimulated by agonists but does not undergo rapid inactivation, desensitization, or internalization. When expressed in SF9 cells, both the receptor and the Goα moieties of the fusion protein were found to be palmitoylated via thioester linkage. Stimulation with the β₂-adrenergic agonist isoproterenol led to a rapid depalmitoylation of both the β₂AR and Goα and inhibited repalmitoylation. The extent of depalmitoylation induced by a series of agonists was correlated (0.99) with their intrinsic efficacy to stimulate the adenyl cyclase activity. However, forskolin-stimulated cAMP production did not affect the palmitoylation state of β₂AR-Goα, indicating that the agonist-promoted depalmitoylation is linked to conformational changes and not to second messenger generation. Given that, upon activation, the fusion protein mimics the activated receptor-G protein complex but cannot undergo desensitization, the data demonstrate that early steps in the activation process lead to the depalmitoylation of both receptor and G protein and that palmitoylation requires later events that cannot be accommodated by the activated fusion protein.

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Palmitoylation is a post-translational modification that is limited to a small subset of cellular proteins among which proteins involved in signal transduction are prevalent (1). This thioesterification of cysteine residues by palmitate distinguishes itself from other lipid modifications such as prenylation and myristoylation by its reversibility. Indeed, in contrast to myristoyl and prenyl moieties that are added co-translationally and generally remain attached to the proteins until the protein gets degraded, the protein-bound palmitate is added post-translationally and turns over more rapidly than the protein itself (2–4). Moreover, the palmitoylation state of several proteins has been shown to be dynamically regulated. In particular, biological regulation of the palmitoylation state of heterotrimeric G proteins and of their cognate receptors has been demonstrated (5–12).

Activation of Goₐ through receptor stimulation, following direct activation with aluminum fluoride and cholera toxin or as a result of site-directed mutagenesis, has been shown to lead to an increased incorporation of [3H]palmitate into Goₐ during pulse labeling experiments. Because pulse-chase labeling experiments clearly indicated that stimulation increased the depalmitoylation rate, the enhanced incorporation was attributed to an accelerated turnover rate of the Goₐ-bound palmitate (10–12). Interestingly, Jones et al. (8) found that, despite the increased turnover rate, activation of Goₐ did not significantly affect its stoichiometry of palmitoylation, thus challenging the notion that stimulation ultimately favors the depalmitoylation reaction (12). Agonist stimulation of the β₂-adrenergic receptor (β₂AR) has also been shown to increase the amount of covalently attached [3H]palmitate (5) as a result of an increased turnover rate of the receptor-bound palmitate (7). A similar agonist-promoted increase in the turnover rate of receptor-bound palmitate was observed for the α₂₃AR (13), the D₂-dopamine receptor (14), and the m₃-muscarinic receptor (9).

Biologically regulated changes in the palmitoylation state of either receptors or G proteins may have important functional consequences. For example, mutations that prevent palmitoylation of various Go subunits have been found to inhibit their association with the plasma membrane and thus their signal-

¶¶¶ The abbreviations used are: Goₐ, stimulatory α subunit of trimeric G protein; β₁AR, β₁-adrenergic receptor; SF9, Spodoptera frugiperda; G protein, guanine nucleotide-binding protein; β₂AR-Goₐ, fusion protein linking Goₐ and a histidine hexamere to the β₂AR carboxyl terminus and amino terminus, respectively; β₂AR-Thr-Goₐ, fusion protein linking the β₂AR tagged at its amino and carboxyl termini by the Flag epitope and histidine hexamere, respectively, and Goₐ through an engineered thrombin cleavage site; PAGE, polyacrylamide gel electrophoresis; [125I]CYP, radio labeled iodoacetylindol. 31014 This paper is available on line at http://www.jbc.org
ing function (15–18), suggesting that biological modulation of the G protein palmitoylation state could regulate their signaling properties. Palmitoylation of Goα has also been reported to increase its affinity for Gβγ (19). For receptors, abolition of palmitoylation by site-directed mutagenesis has been shown to either decrease coupling to G proteins (9, 20–23), affect receptor internalization (24–26), or modulate receptor phosphoryla-
tion by regulatory kinases (5, 27, 28).

Despite these potentially important roles, very little is known concerning the mechanism that regulates palmitoylation of these proteins. Both enzymatic (29–32) and nonenzymatic (33, 34) acylation reactions have been proposed for Goα, whereas an enzyme that can catalyze the depalmitoylation of Ga proteins has recently been identified (35). However, the mechanisms by which activation of the signaling pathway could control the acylation/deacylation cycle remain unknown.

Analysis of the effects of stimulation on the palmitoylation status of receptors and G proteins is complicated by several factors (for a review, see Ref. 36). These include the fact that, following the initial conformational changes and protein–protein interactions that are promoted by receptor stimulation, multiple processes that limit the extent of the activation and contribute to signal termination come rapidly into play. It follows that it is difficult to temporally distinguish between the early events that lead to activation from the ones involved in rapid desensitization of the signaling system. This is an important problem because these two sets of events could theoretically have opposite effects on the palmitoylation reaction. Indeed, on a time scale that is virtually indistinguishable from that of the activation of the G proteins, stimulation of the receptors leads to their progressive functional inactivation. This desensitization results largely from agonist-promoted phosphorylation, uncoupling, and internalization of the receptors (37, 38). Internalization of the G proteins has also been suggested to contribute to desensitization of the signaling unit (39–43).

In an effort to distinguish between the effects of activation and desensitization on receptor and G protein palmitoylation, we took advantage of a β2AR-Goα fusion protein that can be activated but not desensitized, internalized, or down-regulated (44, 45). The pharmacological properties of such receptor-G protein fusion constructs have recently attracted considerable attention and many of their properties have been recently reviewed (46, 47). The agonist-bound β2AR-Goα fusion protein presumably mimics an early intermediate in the normal activation cycle. Also of interest to the present study is the fact that complete physical dissociation between the receptor and Goα, which normally follows the initial stimulatory interaction, is not permitted in the fusion protein. These features of the fusion protein allow study of the effects of early activation events on the palmitoylation state of the receptor and G protein independently of those resulting from the inactivation processes. Furthermore, the use of fusion protein restricts the analysis to those receptors and G proteins that did physically interact in the course of the experiment. We report that stimulation of β2AR-Goα with β-adrenergic agonists promotes rapid depalmitoylation and inhibits palmitoylation of both the receptor and the Goα subunit. This contrasts with the facilitated repalmitoylation that is observed when the two proteins are expressed individually and suggests that early events in the activation process lead to the depalmitoylation of the two proteins, whereas later deactivation mechanisms, that do not occur for the fusion protein, are required for the repalmitoylation reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Grace’s insect medium, lactalbumin, yeastolate, penicil-

lin, streptomycin, glutamine, fungizone, pluronic acid, and phosphate-

buffered saline were from Life Technologies, Inc. Fetal bovine serum

**was obtained from Immunonuc. [125I]CYP, [α-32P]ATP, [γ-32P]ATP,

[αH]AMP, and (9,10)-[3H]palmitate were purchased from M
del. Al-

prenoi, isoproterenol, dichloroisoproterenol, ATP, GTP, CAMP, for-
solin, isobutylmethylxanthine, phosphonoxypruvate, bovine serum albumin, myokinase, cyanogen bromide, and anti-FlagTM M2 were ob-

tained from Sigma. Pyruvate kinase and Gentamicin were from Calbio-

chem. IC118551 was from Tocris. Benzamidine, soybean trypsin inhib-

itor, leupeptin, and n-dodecyl-β-D-maltoside was from Alexis Corp.

**Recombinant Baculoviruses Construction**—The recombinant c-Myc-

β2AR baculovirus was generated by subcloning the cDNA of a c-Myc-

tagged human β2AR (5) into the pVL13Z recombinant plasmid (In-

tegron, Inc.). pBacPAK-pBacHis-β2AR-Goα was constructed by inserting the sequences encoding the human β2AR and Goα in the pBacPAK-3HIS vector under the control of the polyhedrin promoter. The recombinant baculovirus containing the β2AR-Goα fusion construct was generated as described previously (53). Following infection of Sf9 cells with the appropriate viruses, expression of β2AR and β2AR-Goα was assessed by radioligand binding assays and Western blot analysis.

**Cell Culture, Metabolic Labeling, and Membrane Preparations**—Sf9 cells were cultured in Grace’s supplemented media containing 10% fetal bovine serum, 0.001% plasminogen in spinner flasks (Bellco Glass) at 27 °C in the presence or absence of β-adrenergic ligands for various periods of time as described previously (7). In some experiments, labeling was allowed to proceed for 45 min before β-adrenergic ligands were added. Labeling was stopped by chilling the reaction on ice. Cells were centrifuged at 500 × g for 5 min at 4 °C, rinsed twice with ice-cold PBS and resuspended in 20 ml of an ice-cold lysis buffer containing 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, and the amount of lipids was assayed by thin-layer chromatography. Lipid extraction was allowed to proceed for 45 min at 4 °C, and solubilized receptors were purified as described below.

**Receptor Affinity Purification**—Alprenolol-Sepharose affinity purification matrix was synthesized according to the method of Benovic et al. (54). This matrix was used to purify the Sf9-derived β2AR, β2AR-Goα, and β2AR-Thr-Goα as described previously (5). The affinity purified preparations were concentrated using Centriprep and Centricon car-

tridges (Amicon), and the amount of β2AR, β2AR-Goα, or β2AR-Thr-

Goα in each sample was determined by 125I]CYP soluble radioligand bind-

ing assay as described elsewhere (55).

**Hydroxylamine Treatment, Chemical and Enzymatic Cleavages**—For hydroxylamine treatment, purified β2AR or β2AR-Goα was mixed to an equal volume of 1 mM Tris, pH 7.0, containing or not 1 mM NH2OH and incubated overnight at 4 °C. Cytidine monophosphate (dNTP) cleavage was carried out following a protocol described by Luo et al. (56). Briefly, affinity purified β2AR-Goα was separated by SDS-PAGE in nonreducing condition. The proteins were then transferred electrothermally to nitrocellulose membrane. The band corresponding to β2AR-Goα, which normally follows the initial stimulatory interaction, is not permitted in the fusion protein. These features of the fusion protein allow study of the effects of early activation events on the palmitoylation state of the receptor and G protein independently of those resulting from the inactivation processes. Furthermore, the use of fusion protein restricts the analysis to those receptors and G proteins that did physically interact in the course of the experiment. We report that stimulation of β2AR-Goα with β-adrenergic agonists promotes rapid depalmitoylation and inhibits palmitoylation of both the receptor and the Goα subunit. This contrasts with the facilitated repalmitoylation that is observed when the two proteins are expressed individually and suggests that early events in the activation process lead to the depalmitoylation of the two proteins, whereas later deactivation mechanisms, that do not occur for the fusion protein, are required for the repalmitoylation reaction.
Western blot analyses were performed, aliquots of the samples were loaded in parallel gels and transferred onto nitrocellulose membranes. The β2AR-Thr-Gαs and β2AR moieties of the fusion protein were first visualized using the Flag M2 antibody (dilution 1:10,000) and the Renaissance chemiluminescence reagent plus (Mandel). The same membrane was then stripped using 100 mM glycine, pH 2.2 (57), and reprobed, using a rabbit polyclonal antibody against the carboxy-terminal portion of Gαs subunit (dilution 1:8, 500) (a generous gift of Dr. A. D. Strosberg, Institut Cochin de Génétique Moléculaire, Paris), to reveal the β2AR-Thr-Gαs and Gαs moiety of the fusion protein.

**Radioligand Binding Assay—**Sf9 cells infected with the recombinant β2AR or β2AR-Gαs baculoviruses were harvested and rinsed twice with ice-cold phosphate-buffered saline, and the membranes were prepared according to Mouillac et al. (5). Membrane suspensions were added to obtain a concentration of 2–10 µg/ml in a final volume of 500 µl of 75 mM Tris, 12.5 mM MgCl2, 2 mM EDTA containing a saturating concentration (250 pm) of the radiolabeled β-adrenergic antagonist 3H]IP. Nonspecific binding was determined as the residual binding observed in the presence of 100 µM alprenolol. Binding reactions carried out at room temperature for 90 min were stopped by rapid filtration over glass-fiber filters.

**Adenylyl Cyclase Assay—**Adenylyl cyclase activity was determined in membrane preparations according to the method of Salomon et al. (58). Activities were determined in the presence or absence of the following activators: 1 µM isoproterenol, 10 µM alprenolol, 10 µM dichloroisoproterenol, or 100 µM forskolin. Data were expressed as picomoles of cAMP produced per min per mg of protein. Protein concentrations were measured by the method of Bradford (Bio-Rad) using bovine serum albumin as standard (59).

**RESULTS AND DISCUSSION**

**Functional Characteristics of the β2AR-Gαs Fusion—**Fig. 1 illustrates the two fusion protein constructs between β2AR and Gαs (β2AR-Gαs and β2AR-Thr-Gαs) that were used in the present study. Infection of Sf9 cells with recombinant baculoviruses encoding either of the two β2AR-Gαs fusion proteins conferred both β2AR binding (data not shown) and β-adrenergic-stimulated adenylyl cyclase activities (Fig. 2) confirming that, as observed in mammalian systems (44, 45, 48), the fusion proteins are synthesized, translocated to the plasma membranes, and functional. Also in agreement with what was observed in mammalian systems, sustained stimulation of β2AR-Gαs with an agonist does not promote any desensitization of the β-adrenergic-stimulated adenylyl cyclase activity. This is in sharp contrast with the rapid desensitization observed in Sf9 cells expressing the wild type β2AR. Indeed, as seen in Fig. 2, pretreatment of β2AR expressing cells with 1 µM isoproterenol for 30 min reduced the isoproterenol-stimulated adenylyl cyclase activity by 23% without significantly affecting the basal activity, thus leading to a desensitization of 40% of the net agonist-stimulated adenylyl cyclase activity. The same treatment was without effect on the isoproterenol-stimulated adenylyl cyclase activity in cells expressing the β2AR-Gαs fusion protein. Agonist pre-treatment for as long as 24 h was also unable to promote any desensitization of the fusion protein. This characteristic of β2AR-Gαs was interpreted by Bertin et al. (44) as an indication that the covalent complex can become activated but does not enter the deactivation path upon agonist stimulation. This contention is also supported by the observation that the formation of the nucleotide-sensitive high affinity state for agonist can be readily observed for β2AR-Gαs in both mammalian (44) and Sf9 (49, 50) cells but that no agonist-promoted internalization or down-regulation was observed upon sustained stimulation (Ref. 44, and data not shown).

It follows that β2AR-Gαs provides a convenient model to study the early events involved in the activation of the β2AR-Gs complex without the confounding effects of the regulatory events leading to deactivation. This may be particularly important when considering that, following their initial activating interaction, the receptor and Gαs may be targeted to distinct cellular compartments upon dissociation. Therefore, β2AR-Gαs allows study of the early events linked to the activation of a single G protein by a unique receptor molecule at equimolar ratio in a common cellular compartment.

**Palmitoylation of β2AR-Gαs—**Based on the premises described above, we undertook study of the dynamics of β2AR-Gαs palmitoylation and the effect of agonist activation on the palmitoylation state of this complex. As shown in Fig. 3, metabolic labeling of Sf9 cells expressing either β2AR or β2AR-Gαs led to the incorporation of [3H]palmitate in the two proteins. The major radiolabeled bands of ~45 and ~96 kDa obtained following alprenolol-Sepharose affinity purification corresponded to the expected molecular masses for the β2AR and β2AR-Gαs, respectively, when expressed in Sf9 cells (5). Molecular species with identical electrophoretical mobilities were detected in Western blot analysis using anti-β2AR and anti-αs antibodies (data not shown), thus confirming the identity of the fusion protein. In Fig. 3A, identical numbers of β2AR and of β2AR-Gαs, as assessed by radioligand binding, were loaded but densitometric analysis revealed around 1.7 times higher [3H]palmitate incorporation into the β2AR-Gαs than β2AR, consistent with the fact that two palmitoylation sites are present in the fusion protein as compared with only one in the receptor. Fig. 3B illustrates
the sensitivity of the labeling to hydroxylamine treatment, indicating that the [3H]palmitate was covalently attached to both β2AR and β2AR-Gs via thioester bonds.

**Agonist-promoted Depalmitoylation of β2AR-Gs**—To assess the effect of receptor stimulation on the dynamics of palmitoylation, pulse labeling experiments were carried out in the presence or absence of agonists for periods varying between 5 and 60 min. In the absence of agonist, incorporation of palmitate into β2AR-Gs increased almost linearly for the first 30 min of labeling and remains stable thereafter (Fig. 4). The presence of isoproterenol during the labeling period greatly inhibited the incorporation of [3H]palmitate in the fusion protein. This unexpected result contrasts sharply with the agonist-promoted increase in palmitate incorporation observed on β2AR and Gs when these proteins are expressed individually (5, 7, 10–12).

For the β2AR and Gs expressed separately, the increase in palmitate turnover was linked to a faster rate of depalmitoylation upon agonist stimulation (7, 12). The apparent increase in [3H]palmitate incorporation was thus attributed to a concomitant acceleration of the repalmitoylation reaction. It follows that the agonist-promoted reduction of [3H]palmitate incorporation into the β2AR-Gs fusion protein could result from
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a slower depalmitoylation or reflect an inhibition of the repalmitoylation reaction. To distinguish between these two hypotheses, cells were metabolically labeled with [3H]palmitate in the absence of agonist. Following a 45-min pulse period, corresponding to the period required to attain steady state labeling, isoproterenol was added or not in the continued presence of [3H]palmitate and incubated for an additional 5 or 15 min. As seen in Fig. 5, incubation with isoproterenol rapidly reduced the extent of β2AR-Gαs palmitoylation, thus suggesting that agonist stimulation promotes its rapid depalmitoylation and that repalmitoylation of the active complex cannot occur. This is in sharp contrast with the increased repalmitoylation that is observed when identical treatment is carried out in cells expressing the wild type β2AR as an individual protein (Fig. 5B).

Because the β2AR-Gαs fusion protein can be activated but that later processes of inactivation such as G protein dissociation, desensitization, or internalization do not occur, it could be hypothesized that early events leading to activation of the receptor-G protein complex promote depalmitoylation but that later processes are required for repalmitoylation. The fact that the depalmitoylation and repalmitoylation reactions may occur with very similar kinetics when the β2AR and Gαs are expressed as individual proteins may explain why Jones et al. (8) did not observe any change in the stoichiometry of palmitoylation of Gαs upon activation despite the universally observed increase in the turnover rate of the Gαs-bound palmitate. The stabilization of the activated receptor-G protein complex using β2AR-Gαs allowed isolation of the effects that resulted solely from the activation process.

The effect of β-adrenergic ligands of various levels of intrinsic activity was then assessed on the palmitoylation of β2AR-Gαs. As shown in Fig. 6, the addition of all ligands caused a significant reduction in the incorporation of the labeled fatty acid into β2AR-Gαs. Interestingly, the extent of the decrease in labeling was directly correlated (r² = 0.992) to the intrinsic activity of the compounds toward β2AR-Gαs as assessed in a membrane adenyl cyclase assay (Fig. 6B). However, direct stimulation of cAMP production by forskolin did not affect the palmitoylation of β2AR-Gαs (Fig. 6C), thus suggesting that the agonist-promoted depalmitoylation is linked to conformational changes imposed by the agonists and not to second messenger generation.

[3H]Palmitate Incorporation into the β2AR and Gαs Moieties of the Fusion Protein—Cysteine 341 of β2AR and cysteine 3 of Gαs, corresponding to position 358 and 428 in β2AR-Gαs, respectively, represent the confirmed palmitoylation sites of these two proteins (20, 29). In the experiments described above, palmitoylation of β2AR-Gαs was studied as a whole with no specific consideration of the individual palmitoylation sites. To determine whether the two sites were indeed palmitoylated and to assess if agonist treatment had similar effects on the palmitoylation state of the two proteins, we took advantage of another fusion protein construct in which a thrombin cleavage site was engineered between the receptor and Gαs (β2AR-Thr-2AR-Gαs, see Fig. 1). As a control, thrombin treatment was performed on wild type β2AR without any effect on the palmitoylation state nor the integrity of the receptor (data not shown). Fig. 7A shows that thrombin treatment of the purified fusion protein, following metabolic labeling, generated two labeled proteins corresponding to the expected mobility for β2AR and Gαs, indicating that the two proteins were palmitoylated within the fusion construct. The identity of the cleaved frag-

FIG. 7. Thrombin cleavage of β2AR-Thr-Gαs. Sf9 cells were infected with a baculovirus encoding the β2AR-Thr-Gαs fusion protein. Metabolic labeling with [3H]palmitate was then carried out for 45 min before adding 1 μM isoproterenol (ISO) or not (control) (CTL) for an additional 15 min. The fusion protein was purified by alprenolol-Sepharose affinity chromatography and treated (+) or not (−) with thrombin (Thr, 10 NIH units/ml) for 30 min. The reactions were resolved under nonreducing conditions (panel A) or mildly reducing (10 mM dithiothreitol) conditions (panel B) by SDS-PAGE containing 6 M urea. 1.2 pmol of receptor, as assessed by [125I]CYP soluble radioligand binding, were loaded in each lane in both panels A and B. Relative incorporation of [3H]palmitate into the fusion β2AR-Thr-Gαs or the β2AR and the Gαs moieties were estimated by densitometric analysis of the fluorograms. The fluorograms shown are representative of four independent experiments. In each case, parallel SDS-PAGE were carried out for Western blot analysis, as described under “Experimental Procedures,” to confirm the identity of each protein.

FIG. 8. CNBr cleavage of β2AR-Gαs. Panel A, expected sizes of the 19 fragments obtained after complete CNBr cleavage of the fusion protein, using the program PROLYSIS. Because the two palmitoylation sites are located on the carboxyl-terminal portion of the β2AR moiety and the amino-terminal portion of the Gαs moiety, two distinct palmitoylated fragments of 16.4 and 7.2 kDa and corresponding to the β2AR (*) and the Gαs (**) -derived peptides, respectively, should be produced. Panel B, infected Sf9 cells expressing β2AR-Gαs were labeled for 45 min with [3H]palmitate and then treated with the vehicle (CTL, lane 1) or with isoproterenol (ISO, lane 2) for 15 min in the continued presence of labeled palmitate. The radiolabeled fusion protein was purified, resolved by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Bands corresponding to the fusion protein were then treated with CNBr for 3 h. Generated peptides were separated by SDS-PAGE. The fluorogram shown is representative of two independent experiments.
ments was further confirmed by Western blot analysis using the anti-αs antibody to detect Goα and the anti-Flag M2 antibody to detect the Flag epitope-bearing β2AR. The apparently higher [3H]palmitate incorporation observed into the β2AR band when compared with Goα most likely reflects the presence of some background labeling observed in this region of the gel even in the absence of thrombin.

As previously observed for β2AR-Gαs, the presence of isoproterenol during the metabolic labeling induced a significant reduction of the [3H]palmitate incorporation into β2AR-Goαs. Thrombin cleavage revealed that the overall decrease in the radiolabeling of the fusion protein was the consequence of a reduction of [3H]palmitate incorporation into both the receptor and Goαs. The agonist-induced Goαs depalmitoylation that we observed could be mediated, in part, by the receptor-promoter dissociation of βγ subunits from the activated fusion protein. Indeed, as reported by Iiri et al. (19), βγ did protect GDP-bound αs but not αs-GTP[S] from depalmitoylation by a recombinant esterase. Because, nonreducing SDS-PAGE conditions could lead to aggregation of some proteins, including the receptor, reducing conditions were also used. As shown in Fig. 7B, identical results were obtained when receptor and Goαs were resolved under mildly reducing conditions (10 mm dithiothreitol) that diminished aggregation and promoted only partial chemical depalmitoylation.

Depalmitoylation of both receptor and Goαs and the effect of isoproterenol on the two proteins was further confirmed using CNBr hydrolysis of the [3H]palmitoylated β2AR-Goαs construct. The primary sequence of β2AR-Goαs containing 18 methionines, complete cleavage should generate 19 fragments (Fig. 8A). Given that the two palmitoylation sites are located on two distinct fragments, two peptides distinguishable by their size are expected to be [3H]palmitoylated. Calculated masses for the expected palmitoylated fragments are 16.4 and 7.2 kDa corresponding to the β2AR- and the Goαs-derived peptides, respectively. As shown in Fig. 8B, CNBr treatment yielded two peptides of the expected electrophoretic mobility, confirming that both the receptor and Goαs were palmitoylated within the fusion protein. The difference in the labeling intensity of the two bands most likely results from quantitatively different elution of some background labeling observed in this region of the gel even in the absence of thrombin.