Mapping of the Mastoparan-binding Site on G Proteins

CROSS-LINKING OF $[^{125}I$-Tyr$^3$,Cys$^1$]MASTOPARAN TO G$_s$.

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Mastoparan (MP) activates GTP-binding regulatory proteins (G proteins) by promoting GDP/GTP exchange through a mechanism similar to that of G protein-coupled receptors (Higashijima, T., Burnier, J., and Ross, E. M. (1990) J. Biol. Chem. 265, 14176-14180). [Tyr$^3$, Cys$^1$]MP was shown to have regulatory activity similar to that of mastoparan when assayed in the presence of diithreitol (DTT). Activation by [Tyr$^3$, Cys$^1$]MP in the absence of DTT was complex in its kinetics, concentration dependence, and dependence on detergents. $[^{125}I$-Tyr$^3$,Cys$^1$]MP bound covalently to the a subunit of G proteins. Cross-linking was blocked by mastoparan or [Tyr$^3$, Cys$^1$]MP. Cross-linking was enhanced by the addition of $\beta\gamma$ subunits, but no cross-linking to $\beta\gamma$ subunits was observed. Cross-linking was inhibited by incubation of G, with guanosine 5'-O-(thiotriphosphate) and Mg$^{2+}$ and was reversed by incubation with DTT or 2-mercaptoethanol. Stoichiometry of labeling was consistent with the cross-linking of one molecule of $[^{125}I$-Tyr$^3$,Cys$^1$]MP/a subunit, and CNBr hydrolysis of the [Tyr$^3$,Cys$^1$]MP-a adduct yielded one major labeled peptide fragment of ~6 kDa. Amino acid sequencing of this CNBr fragment prepared from recombiant a showed that cross-linking occurred at Cys$^3$. No $\alpha$, sequence was obtained from the same fragment prepared from bovine brain a, which is blocked by a myristoyl group at Gly$^2$. Regulation of G, by MP was eliminated by tryptic proteolysis of the amino-terminal region. These observations suggest that the amino-terminal domain of G protein $\alpha$ subunits contributes to the mastoparan-binding site, which may also be the receptor-binding site, and is involved in regulation of nucleotide exchange.

G protein$^1$-mediated signaling depends on selective mutual recognition between G proteins and receptors, each of which are members of large families of homologous proteins. G proteins are activated by binding GTP, and activation is terminated when the bound GTP is hydrolyzed to GDP.

Receptors promote the activation of G proteins by catalyzing the replacement of GDP by GTP (see Refs. 1 and 2 for review). G proteins are heterotrimers that are bound to the inner face of the plasma membrane via the $\beta$ and $\gamma$ subunits, which are shared among different G proteins. The unique $\alpha$ subunits, which bind GTP and activate effector proteins, determine the identity of G protein trimers and are the primary determinants of selectivity for receptors (see Ref. 1 for review). G protein-coupled receptors are integral plasma membrane proteins. They are based on a core of seven membrane-spanning helices oriented such that the amino terminus is extracellular and the carboxyl terminus is cytoplasmic (see Ref. 3 for review). Because G protein $\alpha$ subunits and G protein-coupled receptors are each families of homologous proteins, it is likely that the structure of the receptor-G protein interface is conserved, with only enough variability to allow receptors to selectively activate some G proteins, but not others.

Mastoparan is a peptide toxin that promotes GDP/GTP exchange on G proteins through a mechanism that is virtually identical to that of agonist-liganded receptors. Mastoparan catalyzes GDP/GTP exchange at submicromolar Mg$^{2+}$ concentrations and does not alter the rate of hydrolysis of bound GTP. Its action is blocked by pertussis toxin-catalyzed ADP-ribosylation of the G protein $\alpha$ subunit and is enhanced by the presence of $\beta\gamma$ subunits (4, 5). Mastoparan also competes with receptors for binding to G proteins (5). Mastoparan forms an amphiphilic $\alpha$ helix when it binds to phospholipid membranes and presents four positive charges to the aqueous face (6). The sites on the second and third cytoplasmic loops of receptors that are thought to interact with G proteins are also positively charged and near the membrane surface (Refs. 7–12; see Ref. 3 for review). Based on these data, it seems likely that mastoparan mimics the G protein-binding domain on receptors and binds to G protein $\alpha$ subunits at their receptor-binding sites (5).

Mastoparan binds and modulates the activities of several proteins other than G proteins, most notably calmodulin (13); and at high concentrations, it exerts nonspecific effects on membranes. We are currently designing mastoparan analogs that are more specific for G proteins relative to other cellular sites and that are selective among related G proteins. However, before mastoparan can be safely used as a model for receptors, it is necessary to demonstrate the existence of a discrete mastoparan-binding site on G protein $\alpha$ subunits. Because even the most potent mastoparan congeners act at concentrations >100 nM, it is unlikely that equilibrium binding measurements can readily establish the existence of such a site. This report describes the cross-linking of a mastoparan analog to a unique site on the $\alpha$ subunit of G, through a disulfide bridge. Several aspects of cross-linking correlate well

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$^1$ The abbreviations used are: G protein, GTP-binding regulatory protein; MP, mastoparan; [I-Tyr$^3$,Cys$^1$]MP, [monooiodo-Tyr$^3$,Cys$^1$] mastoparan; DTT, diithreitol; NEM, N-ethylmaleimide; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; GTPyS, guanosine 5'-O-(thiotriphosphate); Hes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PVDF, polyvinylidenedifluoride; HPLC, high pressure liquid chromatography.

$^+$ H. Mukai and T. Higashijima, unpublished data.
with regulation by mastoparan. It is likely that the site of cross-linking forms part of a specific mastoparan-binding site, which may be the receptor-binding site as well.

**EXPERIMENTAL PROCEDURES**

**Peptides**—Mastoparan and [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP were synthesized by solid-phase methods and were purified by HPLC using a 20–60% acetonitrile gradient in 0.1% trifluoroacetic acid (5). Purity was >97% according to analytical C<sub>18</sub> HPLC. Radiodination of [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP was performed using chloramine T. The free sulfhydryl group was first blocked by incubation of 2 nmol of peptide with 50 nmol of 0.5% dithiobis(nitrobenzoic acid). Carrier-free Na<sup>125</sup>I (1 mCi, 380 pmol) and 5 μl of 0.4 mg/ml chloramine T were added (58-μl total volume), and the mixture was incubated at 23 °C for 1 min. The reaction was stopped by adding 5 μl of 5 mg/ml sodium metabisulfite, and the nitrobenzoic acid group was removed by incubation with 1 μmol of DTT at 30 °C for 30 min. Unreacted [125I] and nitrobenzoic acid-DTT complex were removed on a 1-ml column of Amberlite IRA-410 (acetate form). Peptide was eluted with 10 ml of 10% acetic acid. The eluate was lyophilized, and monoiodinated peptide was purified by C<sub>18</sub> HPLC (6-mm inner diameter, 25 cm) using a linear gradient of acetonitrile (0% for solvent A, 95% for solvent B) in 0.1% trifluoroacetic acid. The original monoiodinated, and diiodinated peptides were eluted at 45.4, 47.6, and 48.9% solvent B, respectively. Nonradioactive [1-Tyr<sup>3</sup>,Cys<sup>18</sup>]MP was prepared as was [125I-Tyr<sup>3</sup>,Cys<sup>18</sup>]MP, except that the amount of iodoine was increased to 2 nmol. Purified peptides were spectroscopically identified, and the concentrations of the peptides were determined according to the absorbance of tyrosine (ε<sub>280</sub> = 14,000 M<sup>-1</sup> cm<sup>-1</sup> at 275 nm), monomoiodotyrosine (ε<sub>280</sub> = 2700 M<sup>-1</sup> cm<sup>-1</sup> at 283 nm), and diiodotyrosine (ε<sub>280</sub> = 2750 M<sup>-1</sup> cm<sup>-1</sup> at 287 nm) (14).

**Assays**—The regulatory activity of mastoparan analogs was routinely determined by enhancement of the steady-state GTPase activity of G proteins (15). The medium contained 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1.1 mM MgSO<sub>4</sub> plus [α-<sup>32</sup>PI]GTP and other additives at the concentrations shown. G proteins were assayed either in detergent solution or after reconstitution into vesicles of phosphatidylcholine, phosphatidylethanolamine, and phosphatidyethanolamine as described previously (5). The molar turnover number was calculated according to the total amount of G protein, which was determined by [35S]GTPαS binding as described previously (15, 16).

**Purification of G Proteins**—Bovine brain α<sub>o</sub> and β<sub>γ</sub> subunits were purified separately as reported previously (5). Recombinant α<sub>o</sub>, was expressed in Escherichia coli and purified according to the protocol of Linder et al. (17). For cross-linking experiments, 0.3 mol eq of β<sub>γ</sub> subunits from bovine brain were added to the α<sub>o</sub> subunit.

The amino terminus of α<sub>o</sub> (100 μg) was proteolyzed after activation by 20 μM AIC<sub>10</sub>, 6 mM MgCl<sub>2</sub>, 10 mM NaF (AMP) at 20 °C for 10 min in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.1% Lubrol 12A9. Then, 10 μg of L-1-tyosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was added and incubated at 30 °C for 1 h. The 39-kDa electrophoretic band of α<sub[o</sub>, disappeared during proteolysis, and a new 37-kDa band appeared. This band corresponds to α<sub[o</sub>, that lacks its amino-terminal fragment (18–20). The 37-kDa fragment was purified by chromatography on DEAE-Sephacel in the same buffer, but which contained 0.7% Lubrol (5, 21).

**Cross-linking**—Cross-linking of [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP to Go<sub>m</sub> was assayed in the presence of 5 mM DTT and the concentrations shown of either mastoparan (C, V) or [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP (O, V). Assays contained Lubrol PX at either 20 ppm (V, V) or 0.1% (O, O), effect of preincubation on the stimulatory activity of [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP. [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP (1 mM) was incubated in 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1.1 μM GTP, Go<sub>m</sub> was then diluted 5-fold into GTPase assay medium that contained 2 μM [γ-<sup>32</sup>PI]GTP with 0.1% Lubrol 12A9 (O, O, O) or without added Lubrol (O, O, O) (6 ppm of residual Lubrol) and the concentrations of DTT (0.1–10 mM) shown on the abscissa. GTP hydrolysis was measured at 20 °C for 30 min. GTPase activity is shown as a molar turnover number calculated according to the total G<sub>m</sub> determined by GTP·αS binding (4). C, effect of preincubation on the stimulatory activity of [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP. [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP (1 mM) was incubated in 50 mM NaHepes (pH 8.0) and 1 mM EDTA at 30 °C for 30 min under ambient atmosphere. The preincubated [Tyr<sup>3</sup>,Cys<sup>18</sup>]MP was then added to GTPase assay at the final concentrations shown. Assay mixtures contained 27 nM Go<sub>m</sub>, 1 μM [γ-<sup>32</sup>PI]GTP, and 0.1% Lubrol (O), 10 mM DTT (V), both Lubrol and DTT (O), or neither additive (V). Assays were carried out for 10 min at 20 °C.
removal of solvent. Based on autoradiography of the gel, two to three fractions that contained the [\textsuperscript{[35]S}-Tyr',Cys']MP-G\textsubscript{\alpha} adduct were pooled.

**SDS-Polyacrylamide Gel Electrophoresis**—For preparative purposes, a 1.5-mm gel was run at 15 mA overnight. Separated peptides were transferred to PVDF paper electrothermally at 50 V for 2 h in 10 mM Hepes (pH 8) and 20% methanol. Typically, ~50% of the labeled fragment was transferred to the first PVDF paper and 20% to a second paper, and 30% remained in the gel. The PVDF paper was stained with Amido Black, washed with 50% methanol several times, and dried. After the protein band at ~6 kDa was confirmed to have radioactivity by autoradiography, the band on the PVDF paper was cut for sequencing.

**Amino Acid Sequence**—Samples on PVDF paper were analyzed by automated amino acid sequencing with an Applied Biosystems Model 470A gas-phase sequenator equipped with a Model 120A on-line PTH-derivative analyzer, as reported previously (24).

**Miscellaneous**—Protein was assayed by the method of Schaffner and Weissmann (25) with bovine serum albumin as the standard. Chemicals were obtained from sources reported previously (5).

**RESULTS**

**Regulation of G Proteins by [Tyr',Cys']MP—[\textsuperscript{125]}I-Tyr',Cys']MP** was synthesized for use as a covalent probe of the mastoparan-binding site on G proteins. As expected from the structure-activity relationships of other mastoparan analogs (5), the replacement of Lys\textsuperscript{11} by Cys and of Leu\textsuperscript{1} by Tyr did not substantially reduce the ability of mastoparan to promote GDP/GTP exchange by G\textsubscript{\alpha} (or G\textsubscript{\beta} (data not shown)) when assays were performed in the presence of DTT (Fig. 1A). Stimulation of nucleotide exchange was measured according to enhancement of the steady-state GTPase activity of G\textsubscript{\alpha}, which is a good measure of GDP/GTP exchange at low exchange rates where hydrolysis is not rate-limiting (15, 26).

The assays shown in Fig. 1A used G\textsubscript{\alpha} that had not been reconstituted into phospholipid vesicles and were performed in medium that contained either 0.1% Lubrol 12A9 or 20 ppm of residual Lubrol. Consequently, maximum stimulation of GTPase activity was only ~7-fold, less than the 20-fold maximum that is obtained with reconstituted G proteins (see Ref. 5 for comparison).

In the absence of DTT, [Tyr',Cys']MP evoked complex nonlinear kinetic behavior that we did not attempt to analyze. However, if G\textsubscript{\alpha} was preincubated with [Tyr',Cys']MP in the absence of DTT, GTPase activity measured in a subsequent assay was linear with time. Preincubation with [Tyr',Cys']MP had no effect on GTPase activity when the assay was performed in the absence of both added thiol and excess Lubrol (Fig. 1B). The residual 6 \mu M [Tyr',Cys']MP did not cause any stimulation, as might have been expected from the data shown in Fig. 1A. However, assay in the presence of 10 mM DTT, but without Lubrol, revealed a 4-fold stimulation by the prior exposure to [Tyr',Cys']MP. When GTPase was assayed in the presence of 0.1% Lubrol, the effect of DTT on the activity of [Tyr',Cys']MP-pretreated G\textsubscript{\alpha} was reversed. Activity was stimulated 3-fold by pretreatment when assayed in the absence of DTT but with Lubrol, which is more than would be predicted from the data of Fig. 1A. There was no effect of pretreatment (or of carried over [Tyr',Cys']MP) when the assay medium contained both Lubrol and 10 mM DTT. For comparison, preincubation with 30 \mu M mastoparan caused slight stimulation that was consistent with the carry-over of 6 \mu M mastoparan into the assay volume. The GTPase activity of G\textsubscript{\alpha} after preincubation with mastoparan or without peptide was not altered by DTT.

The interacting effects of [Tyr',Cys']MP and DTT on G\textsubscript{\alpha} did not result from the covalent dimerization of [Tyr',Cys']MP during the assay. Incubation of [Tyr',Cys']MP for 30 min at 30 °C in mock assay medium did not alter its maximal effect or potency in a short (10 min) GTPase assay at 20 °C, and activity was not altered by the inclusion of DTT in the assay (Fig. 1C). Fig. 1C also shows that Lubrol decreased the potency of [Tyr',Cys']MP with or without DTT, as we reported previously for mastoparan (5).

The effects of preincubation with [Tyr',Cys']MP on the GTPase activity of G\textsubscript{\alpha} can perhaps be rationalized by suggesting that [Tyr',Cys']MP and G\textsubscript{\alpha} form a covalent adduct that can be cleaved by DTT and that assumes an active conformation in the presence of excess Lubrol. Regardless of rationalization, the data of Fig. 1 indicate that [Tyr',Cys']MP stimulates nucleotide exchange by G\textsubscript{\alpha} and that at least some of its effects are prolonged in the absence of DTT.

**Cross-linking of [\textsuperscript{125]}I-Tyr',Cys']MP to G\textsubscript{\alpha}.—**The complex interactions between [Tyr',Cys']MP and DTT shown in Fig. 1 suggested that [Tyr',Cys']MP may form a disulfide cross-link with G\textsubscript{\alpha}. To detect covalent cross-linking, G\textsubscript{\alpha} and [\textsuperscript{125]}I-Tyr',Cys']MP were incubated under ambient atmosphere at 30 °C in GTPase assay medium without \textsuperscript{35}S and in the absence of DTT. Samples of the reaction mixture were analyzed by SDS gel electrophoresis after the addition of 5 mM NEM to inhibit subsequent thiol-disulfide exchange. Autoradiography of the gel showed that the \alpha subunit of G\textsubscript{\alpha} was selectively labeled by [\textsuperscript{125]}I-Tyr',Cys']MP (Fig. 2). Labeling of \alpha was time-dependent (Fig. 2A, lanes 2–6) and was largely complete by 30 min. If NEM was not added before denaturation, a faint band appeared that corresponded to minor labeling of the \beta subunit (data not shown). Preincubation of G\textsubscript{\alpha} with 5 mM NEM blocked the cross-linking of [I-Tyr',Cys']MP to the \alpha subunit (data not shown).

Covalent labeling of \alpha was inhibited competitively by 100...
Cross-linking of Mastoparan to Gα

Fig. 3. Cross-linking of [125I-Tyr3,Cys11]MP to Gα, A, effects of βγ subunits and guanine nucleotides. αα (25 pmol in 25 μl) was incubated with [125I-Tyr3,Cys11]MP for 15 min under the conditions described for Fig. 2. Each sample contained different amounts of βγ subunits (lane 1, none; lane 2, 8 pmol; lanes 3 and 5, 25 pmol; lane 4, 50 pmol). The sample shown in lane 5 contained 60 μM GDP instead of GTP. B, effect of GTPγS. Gα (38 pmol of αα, 80 pmol of βγ subunits in 50 μl) was incubated without [125I-Tyr3,Cys11]MP for 10 min under the same conditions as described for A, except that the medium contained 1.1 mM MgCl2 and either 10 μM GTP (lane 6) or 10 μM GTPγS (lane 7). [125I-Tyr3,Cys11]MP (30 pmol) was then added, and the incubation was continued for 30 min. Samples were treated into phosphatidylinositol vesicles. Go and ADP-ribosylated Go were ADP-ribosylated with pertussis toxin as shown in (Fig. 4). Although most cross-linking experiments were performed in 0.1% Lubrol, labeling of Gα with [125I-Tyr3,Cys11]MP appeared in few experiments to be slightly more efficient in 50 ppm Lubrol (data not shown). Gα was also efficiently labeled after reconstitution into phosphatidylinositol vesicles (Fig. 3C) or vesicles composed of mixed phospholipids (data not shown). ADP-ribosylation of αα by pertussis toxin inhibited labeling by [125I-Tyr3,Cys11]MP slightly, if at all (Fig. 3C).

Calculations based on the radiochemical specific activity of [125I-Tyr3,Cys11]MP indicated that 0.5–0.8 mol of peptide was cross-linked per mol of αα under optimum conditions. Because cross-linking decreases the electrophoretic mobility of αα stained gels could be used to estimate independently the fraction of αα that was cross-linked (Fig. 2B). Based on silver staining, ~50% of αα was cross-linked under these conditions. Only two silver-stained bands of αα were detected after cross-linking, the upper one of which corresponded to the band on the autoradiograph. These data suggest that [125I-Tyr3,Cys11]MP cross-links to only one site on αα.

Peptide Mapping of [125I-Tyr3,Cys11]MP Labeling Site on αα.—To estimate the number of [125I-Tyr3,Cys11]MP labeling sites on αα, in more detail, cross-linked αα was digested with CNBr, and the hydrolysate was analyzed by SDS gel electrophoresis. Autoradiography of the gel showed a single major labeled peptide of ~6 kDa above the smear of radioactivity from the [125I-Tyr3,Cys11]MP-NEM adduct and/or the [125I-Tyr3,Cys11]MP dimer (Fig. 4). Labeling of αα in the presence of 0.3 mol eq of βγ subunits increased the intensity of the 6-kDa band, in agreement with the enhanced labeling of intact αα. Two faintly labeled bands could also be seen above the major 6-kDa fragment in Fig. 4. When lower concentrations of CNBr were used, the band at 6 kDa was fainter, and these bands were darker, suggesting that the bands at higher molecular mass are due to incomplete hydrolysis of αα by CNBr. In several experiments, recovery of [125I-Tyr3,Cys11]MP cross-links ranged from 50 to 80% of that originally present in the intact [125I-Tyr3,Cys11]MP-αα adduct. Thus, whereas smaller, less

Fig. 4. CNBr hydrolysis of [125I-Tyr3,Cys11]MP cross-linked to αα, αα (100 pmol) either without (lanes 1 and 2) or with (lanes 3 and 4) 25 pmol of βγ subunits was incubated with 3 μM [125I-Tyr3,Cys11]MP (0.25 μCi) in a total volume of 50 μl for 15 min under the conditions described for Fig. 2 (except 1.1 mM MgCl2). The samples shown in lanes 1 and 3 (20 μl) were quenched with sample buffer. The samples in lanes 2 and 4 (20 μl) were hydrolyzed by CNBr as described under "Experimental Procedures." All samples were analyzed on gradient gels. The autoradiograph was developed for 1 day.

μM mastoparan, but was not significantly inhibited by 1 μM mastoparan (Fig. 2A). [Tyr3,Cys11]MP also inhibited labeling at similar concentrations. This concentration dependence is consistent with the relatively high EC50 displayed by mastoparan or [Tyr3,Cys11]MP in 0.1% Lubrol (Fig. 1A; see also Ref. 5). Labeling was also inhibited by inclusion of 5 mM DTT in the reaction mixture and was reversed by subsequent addition of 5 mM DTT or 1% 2-mercaptoethanol (data not shown). Although βγ subunits of Gα were not labeled by [125I-Tyr3,Cys11]MP, βγ subunits enhanced the labeling of the α subunit by 3–5-fold (Fig. 3A). Enhancement of labeling by βγ subunits saturated at ~0.3 mol of βγ subunits/mole of α subunit. These data suggest that βγ subunits act catalytically.

The efficiency of labeling was dependent on the identity of the nucleotide bound to Gα. Routinely, GTP was added to labeling reactions to stabilize Gα against denaturation. Similar labeling was observed with GTP or GDP (Fig. 3A), however, and labeling was also observed in the absence of added nucleotide (data not shown). (Purified Gα retains nearly stoichiometric bound GDP (27.) In contrast to GTP, the nonhydrolyzable GTP analog GTPγS inhibited labeling by 60–80% (Fig. 3B). Such inhibition is consistent with the observation that GTPγS-bound αα dissociates from βγ subunits (26, 28).

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Fig. 5. HPLC separation of CNBr fragments of adduct of [125I-Tyr3,Cys11]MP and bovine brain αα. [125I-Tyr3,Cys11]MP was cross-linked to bovine brain Gα. The reaction mixture was hydrolyzed with CNBr, and the hydrolysate was analyzed by reverse-phase HPLC (SynChrom C4 column, 5-mm diameter, 10 cm long, 1 ml/min flow rate). Solvents were 0.1% trifluoroacetic acid (A) and 10% acetonitrile plus 0.1% trifluoroacetic acid (B). The elution profile was: 5 min, 20% solvent B; 40 min, linear gradient to 60% solvent B; and 10 min, linear gradient to 100% solvent B. Ten μl of each fraction was lyophilized, resuspended in sample buffer, and electrophoresed on a polyacrylamide gradient gel. Protein was stained with silver (A), and 125I was visualized by autoradiography (16 h at ~80 °C (B)).
efficiently labeled fragments of α, may be hidden in the smear at the bottom of the gel, it is likely that the 6-kDa fragment is the only labeled fragment of Gs and that, consequently, only one molecule of [125I-Tyrγ,Cys11]MP was cross-linked per molecule of αs.

Identification of [125I-Tyrγ,Cys11]MP Labeling Site—To identify the site of [1-Tyrγ,Cys11]MP labeling on αs, the [125I-Tyrγ,Cys11]MP-labeled CNBr fragment was purified in larger quantity. Bovine brain αs (8 nmol) and bovine brain βγ subunits (2.5 nmol) were incubated with 5 μM [125I-Tyrγ,Cys11]MP; the mixture was hydrolyzed by CNBr as described above; and the hydrolysate was fractionated by reverse-phase HPLC. HPLC fractions were analyzed by SDS gel electrophoresis. Fig. 5 shows both protein staining and autoradiography of the HPLC fractions that contained peptides (fractions 27–45). The 6-kDa fragment labeled with [125I-Tyrγ,Cys11]MP was eluted at fractions 39–41 (54–56% solvent B). Because these three fractions contained several peptides of different molecular masses, they were pooled, and the peptides were separated by electrophoresis on a 1.5-mm-thick gradient gel. After electrotransfer to PVDF paper, the 6-kDa labeled band was cut out and subjected to automated amino acid analysis. The amino acid sequence corresponding to [1-Tyrγ,Cys11]MP was detected with reasonable yield according to protein staining or radioactivity. However, no other amino acid residues were detected. Three separate experiments gave the same result. This suggested that the amino-terminal residue of the fragment of αs to which [125I-Tyrγ,Cys11]MP was cross-linked may be blocked.

The amino terminus of mature bovine brain αs (Gly3) is blocked by N-myristoylation (29). Because recombinant βγ subunits produced in E. coli have free amino termini (17), the same procedures of [125I-Tyrγ,Cys11]MP cross-linking, hydrolysis, and purification of the cross-linked fragment were performed using recombinant αs mixed with bovine brain βγ subunits. Fig. 6 shows protein staining and autoradiography of the SDS-polyacrylamide gels of the relevant C4 HPLC fractions, 27–45, from one such experiment. The 6-kDa labeled fragment of recombinant αs eluted at a lower concentration of acetonitrile (46–48% solvent B, fractions 31–33) than did the fragment of αs from bovine brain, which indicates that it is less hydrophobic. Fractions 31–33 were pooled, fractionated on a preparative gradient gel, and transferred to PVDF paper. The 6-kDa labeled fragment was subjected to amino acid sequencing. The residues detected in the sequenator, shown in Table I, corresponded reasonably well to those predicted for an equimolar mixture of [1-Tyrγ,Cys11]MP and the amino-terminal CNBr fragment of αs. Two independent preparations and analyses gave essentially identical results. The amino-terminal CNBr fragment is expected to have a molecular mass of 5.46 kDa, which is consistent with the observed mobility of the fragment.

**Table I**
Amino acid sequence analysis of the [125I-Tyrγ,Cys11]MP-labeled CNBr fragment of αs

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Detected amino acid</th>
<th>Predicted amino acid</th>
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<tr>
<td></td>
<td>[125I-Tyrγ,Cys11]MP</td>
<td>αs</td>
</tr>
<tr>
<td>1</td>
<td>Gly (53), Ile (16), Ser (9)</td>
<td>Ile Gly</td>
</tr>
<tr>
<td>2</td>
<td>Gly (12), Asn (6)</td>
<td>Asn Cys</td>
</tr>
<tr>
<td>3</td>
<td>Thr (6), Trp/I-Tyr</td>
<td>I-Tyr Thr</td>
</tr>
<tr>
<td>4</td>
<td>Leu (18), Lys (24)</td>
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<tr>
<td>5</td>
<td>Ala (29), Ser (5)</td>
<td>Ala Ser</td>
</tr>
<tr>
<td>6</td>
<td>Ala (23), Leu (19)</td>
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<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>Ala (21), Glu (14)</td>
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</tr>
<tr>
<td>9</td>
<td>Leu (8), Arg (trace)</td>
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</tr>
<tr>
<td>10</td>
<td>Ala (21)</td>
<td>Ala</td>
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<tr>
<td>11</td>
<td>Ala (23)</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>Arg (5), Leu (4)</td>
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<tr>
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</tr>
<tr>
<td>16</td>
<td>Lys (2)</td>
<td>Lys</td>
</tr>
<tr>
<td>17</td>
<td>Ala (3)</td>
<td>Ala</td>
</tr>
<tr>
<td>18</td>
<td>Ile (3)</td>
<td>Ile</td>
</tr>
</tbody>
</table>

*Values in parentheses represent picomoles.

**Table II**
Effect of mastoparan and the βγ subunits on the GTPase activity of intact and amino-terminally prenylated αs

<table>
<thead>
<tr>
<th>GT-Pase Activity</th>
<th>Control</th>
<th>Proteolized</th>
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<tr>
<td></td>
<td>−βγ</td>
<td>+βγ</td>
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<tr>
<td>No peptide</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>0.42</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Fig. 7. Cross-linking of [125I-Tyrγ,Cys11]MP to G protein α subunits. [125I-Tyrγ,Cys11]MP was reacted with G protein α subunits in 20 μl of the medium described for Fig. 2 that also contained 0.5 μM of bovine brain βγ subunits. Reaction was for 20 min at 30°C. The α subunits were 2 μg of bovine brain αs (lane 1), 1 μg of recombinant αs, (lane 2), 1 μg of recombinant αs, (lane 3), 0.5 μg of recombinant αs, (lane 4), 0.5 μg of recombinant αs, long form (lane 5), 0.3 μg of recombinant αs, short form (lane 6), 1 μg of the PT mutant of αs (lane 7), and 2 μg of recombinant αs (lane 8). The reaction was stopped by the addition of 5 mM NEM, and the mixture was hydrolyzed with 100 mg/ml CNBr for 1 day at room temperature. After lyophilization, samples were analyzed by electrophoresis on gradient gels. The autoradiograph was exposed for 1 day at −80°C.
sistent with the electrophoretic mobility of the cross-linked fragment. It contains only 1 Cys residue, the second residue from the amino-terminal Gly.

**MP Does Not Regulate Go, That Has Been Proteolyzed at Amino Terminus of a Subunit—**Trypsain selectively proteolyzes the amino termini of activated α subunits (18, 30). To determine the importance of the amino terminus in responding to mastoparan, α was activated with AMF, treated with trypsin, and then deactivated. Formation of the 37-kDa product was monitored by gel electrophoresis (data not shown). As shown in Table II, the GTPase activity of amino-terminally proteolyzed α was insensitive to both stimulation by mastoparan and inhibition by βγ subunits. No response to mastoparan was observed at concentrations up to 100 µM in either the presence or absence of βγ subunits. In parallel experiments, intact α, displayed both these expected responses, although stimulation by mastoparan was only ~2-fold because the assay was performed in Lubrol solution. The insensitivity of proteolyzed α subunits to regulation by βγ subunits has been reported previously (20, 31). The unresponsiveness of proteolyzed α to mastoparan is consistent with the importance of the amino terminus for regulation by mastoparan.

**Cross-linking of [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP to Other G Proteins—**To test the generality of the results obtained with Go, we incubated [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP with the three forms of Go, the long and short forms of Go, and an α mutant that contains a pertussis toxin substrate site near its carboxyl terminus (32). Go was included as a positive control. Each G protein was selectively labeled in its α subunit (data not shown). CNBr hydrolysis of the adducts followed by electrophoresis of the hydrolys products on a gradient gel showed that the band with covalently bound [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP appeared at about the same position for each G protein: ~6 kDa for α, and the α proteins and ~8 kDa for both forms of α (Fig. 7). Some larger fragments were also observed. These were probably products of incomplete hydrolysis (see legend to Fig. 7). These data are consistent with the covalent cross-linking of [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP to Cys<sup>11</sup> in each of the G protein α subunits. The Cys<sup>11</sup> residue is conserved among all the α subunits tested, as is the Met residue (Met<sup>210</sup> in αs) that is cleaved to produce the ~6-kDa CNBr fragment (see Ref. 1 for sequences). There is a γ-amino acid insertion near the amino terminus of α, which presumably accounts for the greater apparent size of its labeled fragment. The three α proteins also contain a Met at position 18, which should have produced a fragment too small to be resolved from the smear of [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP in the electrophoresis system used here. We assume that the ~6-kDa fragment of the [3H-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP-α adduct in Fig. 7 is the result of incomplete hydrolysis.

**DISCUSSION**

The activation of G proteins by mastoparan is mechanistically similar to activation by receptors (4, 5). The structure of membrane-bound mastoparan, an α helix with four positive charges directed toward the aqueous medium (6), is also reminiscent of cationic regions on the cytoplasmic surface of G protein-coupled receptors that are thought to interact with G proteins (33). Mastoparan may therefore be useful as a low molecular mass model of receptors for studying receptor-(= G protein) interactions. However, cationic amphipathic peptides exert many nonspecific effects, some of which may reflect merely disordering of cell membranes. The utility of mastoparan as a model for receptors would therefore be supported by the definition of a specific mastoparan-binding site on G proteins.

The data shown here indicate that [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP spontaneously cross-links to α through a disulfide bridge between Cys<sup>11</sup> of [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP and Cys of α. Mastoparan itself, which has no Cys residue, blocked formation of the cross-linked product when present at a concentration adequate to catalyze activation of Go. G protein βγ subunits catalytically enhanced the cross-linking of [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP to α, without themselves undergoing cross-linking. Activation of Go with GTPyS inhibited cross-linking, which is consistent with the tendency of activated α subunits to dissociate from βγ subunits. Cross-linking was selective for α, even in preparations that were <10% pure (data not shown), which indicates that [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP does not act nonspecifically as a sulfhydryl reagent. These observations all support the specificity of cross-linking.

We could identify the [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP labeling site on α as Cys<sup>11</sup> because this is the only Cys residue in the amino-terminal CNBr fragment of α. The identity of the fragment was determined by amino acid sequence analysis of the CNBr fragment of the adduct of [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP and recombinant α. The two PTH-derivatives released at each sequenator cycle were those predicted from the sequences of [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP and α, and their yields were similar, suggesting a 1:1 stoichiometry. Other residues were not detected. Identification was supported by the fact that no sequence other than that of [I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP was obtained from the same-sized CNBr fragment of the adduct of [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP and α from bovine brain, whose amino terminus is blocked by myristoylation of Gly<sup>2</sup> (29). The labeled CNBr fragment from cross-linked bovine brain α was also eluted from the C<sub>4</sub> HPLC column at a higher concentration of acetonitrile than was the fragment from the recombinant α consistent with greater hydrophobicity from the myristoyl blocking group. Other silver-stained fragments of recombinant and natural α were eluted at about the same positions.

These data suggest, but do not prove, that [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP cross-linked to a single site on α. First, calculations based on the radiochemical specific activity of [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP indicated that 0.5–0.8 mol of [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP could be covalently bound per molecule of α. In addition, cross-linking altered the electrophoretic mobility of α in SDS to produce a single new band of increased size that corresponded to the single band on the autoradiographs. Last, we identified only a single labeled peptide after CNBr hydrolysis of the [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP-α adduct, and this fragment accounted for 50–80% of the radioactivity that was incorporated into α.

In our experience, such recovery after CNBr digestion is quite good. Other sites of labeling, if they exist, are therefore minor. Of the four other predicted cysteine-containing CNBr fragments, two are larger than the major labeled fragment and, if labeled, would have been noticed on the gradient gels. The other two, of M<sub>r</sub> 2390 and 4690, may have been missed at the bottom of the gels if they were only weakly labeled and were not separated from the mass of [3H-I-Tyr<sup>3</sup>,Cys<sup>11</sup>]MP by HPLC.

Mastoparan mimics the function of G protein-coupled receptors and would be expected to bind to an α subunit at its receptor-binding site. However, previous studies suggested that the amino-terminal region of G protein α subunits is primarily important for interaction with βγ subunits. Proteolytic cleavage of the amino terminus of the α subunit inhibits interaction with βγ subunits (Refs. 20 and 31; also Table II), and amino-terminal myristoylation of α and enhances interaction with βγ subunits (34).

Receptors have been assumed to bind to a site on G proteins near the carboxy terminus of the α subunit (1, 2). This is the
site of pertussis toxin-catalyzed ADP-ribosylation of the $\alpha$ subunits of $G\alpha_1$ proteins and $\alpha_2$ (35, 36) and the site of the urea mutation in $\alpha_2$ (37), both of which block regulation by receptors. In addition, peptides based on the carboxyl-terminal sequence of $\alpha_2$ stabilize the meta-I1 conformation of rhodopsin (38), as does G1, itself; and antibodies to carboxyl-terminal epitopes of $\alpha_2$ inhibit rhodopsin-G interaction (39). Mastoparan action is also linked to carboxyl-terminal structures. ADP-ribosylation by pertussis toxin blocks regulation by mastoparan (4, 40); adenyllylcyclase in membranes of the unc mutant of S49 lymphoma cells (37, 41) is insensitive to G$_2$-activating peptides; and an antibody against the carboxyl terminus of $\alpha_2$ blocked regulation of G$_{12}$ by mastoparan (40) (confirmed for $G$, in this laboratory).

Although our data argue for the importance of the amino terminus of the $\alpha$ subunit for mastoparan, they do not exclude the possibility that mastoparan also binds to the carboxyl-terminal region of $\alpha$ subunits. With the present approach, $[^{125}I]$$\text{Tyr}^3$$\text{Cys}^{15}$MP bound near the carboxyl terminus would not have been cross-linked to $\alpha_2$, unless there was a Cys residue that was correctly oriented relative to Cys$^{15}$ of the bound peptide. Taken together, the data presented here and the data cited above suggest that both amino- and carboxyl-terminal regions of $G\alpha$ protein $\alpha$ subunits are essential for regulation by mastoparan.

There are at least two ways to interpret the present data. First, amino and carboxyl termini of an $\alpha$ subunit may be juxtaposed within the three-dimensional structure of the protein. Recent chemical cross-linking studies of $G$, suggest that the amino- and carboxyl-terminal domains may lie close together (42). If so, one molecule of mastoparan may interact directly with both the amino- and carboxyl-terminal regions, with cross-linking occurring only at one cysteine residue. Alternatively, more than one molecule of mastoparan may bind to a single $\alpha$ subunit at sites near the amino and carboxyl termini, but with only the amino-terminal site providing a cysteine capable of cross-linking to $[^{125}I]$$\text{Tyr}^3$$\text{Cys}^{15}$MP. Indeed, the high Hill coefficient ($n = 2$–4) of the mastoparan dose-response curve (5) suggests that multiple molecules of mastoparan must bind to promote guanine nucleotide exchange. In addition, recent site-directed mutagenesis studies of G protein-coupled receptors also suggest that multiple cationic domains on the receptors' second and third extracellular loops are required for specific interaction with G proteins (7). The observation that ADP-ribosylation of $\alpha_2$ by pertussis toxin inhibited activation by mastoparan (4, 40) but did not significantly inhibit the cross-linking of $[^{125}I]$$\text{Tyr}^3$$\text{Cys}^{15}$MP to $\alpha_2$ is also consistent with mastoparan's interaction with both regions, although ADP-ribosylation may inhibit coupling without physically blocking the receptor-binding site.

Our data indicate that the mastoparan-binding region of G protein $\alpha$ subunits consists minimally of an amino-terminal sequence, but do not exclude the involvement of a carboxyl-terminal region as well. The observation that $\beta$ subunits enhanced the cross-linking of $[^{125}I]$$\text{Tyr}^3$$\text{Cys}^{15}$MP to $\alpha_2$, suggests that binding sites for mastoparan and $\beta$ subunits are closely linked, at least for function. It will now be interesting to use new reactive mastoparan analogs to test directly whether carboxyl-terminal portions of $\alpha$ subunits actually contribute to the binding site for mastoparan or receptor.

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REFERENCES