Structural Analysis of Rod GTP-binding Protein, G<sub>α</sub>

LIMITED PROTEOLYTIC DIGESTION PATTERN OF G<sub>α</sub> WITH FOUR PROTEASES DEFINES MONOCLONAL ANTIBODY EPITOME*

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The epitope of monoclonal antibody (mAb 4A), which recognizes the α subunit of the rod G<sub>α</sub> protein, G<sub>α</sub>, has been suggested to be both at the carboxy terminus (Deretic, D., and Hamm, H. E. (1987) J. Biol. Chem. 262, 10839–10847) and the amino terminus (Navon, S. E., and Fung, B. K.-K. (1988) J. Biol. Chem. 263, 489–496) of the molecule. To characterize further the mAb 4A binding site on α<sub>α</sub> and to resolve the discrepancy between these results limited proteolytic digestion of G<sub>α</sub>, or α<sub>α</sub> using four proteases with different substrate specificities has been performed. Endoproteinase Arg-C, which cleaves the peptide bond at the carboxy terminal of arginine residues, cleaved the majority of α<sub>α</sub> into two fragments of 34 and 5 kDa. The α<sub>α</sub> 34-kDa fragment in the hokprotein, but not α<sub>α</sub>-guanosine 5′-O-(3-thiotriphosphate), was converted further to a 23-kDa fragment. A small fraction of α<sub>α</sub>-GDP was cleaved into 23- and 15-kDa fragments. Endoproteinase Lys-C, which selectively cleaves at lysine residues, progressively removed 17 and then 8 residues from the amino terminus, forming 38- and 36-kDa fragments. Staphylococcus aureus V8 protease is known to remove 21 amino acid residues from the amino-terminal region of α<sub>α</sub> with the formation of a 38-kDa fragment. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin cleaved α<sub>α</sub> progressively into fragments of known amino acid sequences (38, then 32 and 5, then 21 and 12 kDa) and a transient 34 kDa fragment. The binding of mAb 4A to proteolytic fragments was analyzed by Western blot and trypsin digestion. Under conditions that allowed sequencing of the 15- and 5-kDa fragments neither the 34- nor the 23-kDa fragments could be sequenced by Edman degradation, indicating that they contained a blocked amino terminus. The smallest fragment that retained mAb 4A binding was the 23-kDa fragment containing Met<sup>1</sup> to Arg<sup>204</sup>. Thus the main portion of the mAb 4A antigenic site was located within this fragment, indicating that the carboxy-terminal residues from Lys<sup>295</sup> to Phe<sup>350</sup> were not required for recognition by the antibody. Additionally, the antibody did not bind the 38- and 36-kDa or other fragments containing the carboxy terminus, showing that the amino-terminal residues from Met<sup>1</sup> to Lys<sup>12</sup> were essential for antibody binding to α<sub>α</sub>. 1

The light activation of rhodopsin, the rod outer segment light receptor, triggers the activation of an enzyme cascade which leads to cGMP hydrolysis and membrane hyperpolarization (Liebman et al. 1987). A guanine nucleotide-binding regulatory protein called transducin or G<sub>βγ</sub> communicates the signal from light-activated rhodopsin to a CTP phosphodiesterase (for review see Stryer and Bourne, 1986; Gilman, 1987; Hurley, 1987; and Liebman et al., 1987). Like all heterotrimeric G proteins, G<sub>α</sub> can be resolved into two functional subunits, α<sub>α</sub> (39 kDa) and β<sub>βγ</sub> (β<sub>β</sub>, 36 kDa; γ<sub>γ</sub>, 8 kDa). Photoisomerized rhodopsin catalyzes the exchange of GTP for GDP on α<sub>α</sub> (Fung and Stryer, 1980; Fung et al., 1981), which leads to the dissociation of subunits and activation of G<sub>α</sub>. In its GTP-bound form, the α<sub>α</sub> subunit activates the CTP phosphodiesterase, and this activation is terminated when the bound GTP is hydrolyzed to GDP by an intrinsic GTPase activity (Wheeler and Bitensky, 1977). Although the β<sub>βγ</sub> subunit does not participate directly in either GTP hydrolysis or phosphodiesterase activation its presence is important for effective binding of α<sub>α</sub> to light-activated rhodopsin and GDP exchange (Fung, 1983).

The key role of G proteins in signal transduction has evolved much in their structure and function and in the mechanism of their activation by receptors (for a review, see Gilman, (1987)). G<sub>α</sub> binds tightly to light-activated rhodopsin in intact rod outer segment membranes (Kühn, 1980). It also binds selectively to unbleached rhodopsin in rod outer segment membranes (Hamm et al., 1987) and in reconstituted phospholipid vesicles (Fung, 1983). The α<sub>α</sub> subunit can be ADP-ribosylated either by cholera toxin or pertussis toxin. Pertussis toxin, which catalyzes the transfer of ADP-ribose to Cys<sup>347</sup> (West et al., 1985), prefers the α<sub>α</sub>-GDP-bound form in the G<sub>α</sub> complex as substrate (Van Dop et al., 1984). The carboxy-terminal region of α<sub>α</sub> takes part in rhodopsin binding (Hamm et al., 1988) just as this region of α<sub>α</sub> is important for interaction with the β-adrenergic receptor (Sullivan et al., 1987). Pertussis toxin ADP-ribosylation can uncouple pertussis toxin substrates, G<sub>α</sub>, G<sub>β</sub>, and G<sub>γ</sub> from their cognate recep-

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1 The abbreviations used are: G<sub>α</sub>, photoreceptor guanyl nucleotide-binding protein; α<sub>α</sub>, the α subunit of G<sub>α</sub>; β<sub>β</sub>γ, the β and γ subunit of G<sub>α</sub>; ROS, rod outer segment; mAb, monoclonal antibody; GTPyS, guanosine 5′-O-(3-thiotriphosphate); SOD, superoxide dismutase; MOPS, 3(N-morphollino)propanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride; TPCK, L-tosylamido-2-phenylthyl chloromethyl ketone; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography.
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itors (Kurose et al., 1983; Cote et al., 1984). Additionally the amino-terminal region of α has been shown to be important for interaction with the βγ subunit (Navon and Fung, 1987; Neer et al., 1988; Osawa et al., 1990b).

Specific antibodies against the α or β subunits of G proteins have been used widely to study the functional domains of these GTP-binding proteins (Cerione et al., 1988; McKenzie et al., 1988; Corrèze et al., 1987; Navon and Fung, 1988; Simonds et al., 1989). Monoclonal antibody 4A, which is directed against the α subunit of frog G, also recognizes the α subunit of bovine G (Witt et al., 1984) and cross-reacts with the α subunits of Gα and Gβ (α, > α, > α γ) (Yatani et al., 1988; Hamm et al., 1989). Binding of mAb 4A to G blocks light activation of photoreceptor cGMP phosphodiesterase by disrupting Gα-rodopsin interaction (Hammt et al., 1987), and the epitope is not available when G is bound to light-activated rhodopsin (Hamm et al., 1987; Navon and Fung, 1988). The binding of mAb 4A to α, also disrupts the G complex, with dissociation of subunits (Navon and Fung, 1988; Mazzoni and Hamm, 1989). Moreover, the antibody inhibits the pertussis toxin-mediated ADP-ribosylation of α, at Cysγ (Deretic and Hamm, 1987; Mazzoni and Hamm, 1989).

Localization of the mAb 4A antigenic site is important for understanding the basis of these multiple effects. However, conflicting experimental results have been reported (Deretic and Hamm, 1987; Navon and Fung, 1988), resulting in assignment of the mAb 4A epitope at either the carboxyl (Deretic and Hamm, 1987; Hamm et al., 1988) or the amino terminus (Navon and Fung, 1988). 2

The purpose of the present work was to reexamine and expand the proteolytic mapping of G, with mAb 4A binding. This was accomplished by cleaving either Gγ or α with four different proteases and analyzing antibody binding to the fragments on Western blots. The cleavage sites on αγ for two of these proteases, endoproteinase Arg-C and endoproteinase Lys-C, were also localized by determining the amino-terminal sequence of the proteolytic fragments. Monoclonal antibody 4A recognized proteolytic fragments of 34 and 23 kDa which contained the amino terminus of α and did not bind α after removal of 17–21 amino-terminal residues.

EXPERIMENTAL PROCEDURES

Materials and Miscellaneous Methods—TPCK-treated trypsin and soybean trypsin inhibitor were purchased from Worthington. GTP, GDP, GTP-S, endoproteinase Arg-C, endoproteinase Lys-C, and TlCK were products of Boehringer Mannheim. Staphylococcus aureus V8 protease, [32P]NAD and [35S]protein A were from ICN Biomedicals. Pertussis toxin and its catalytic subunit, the A protomer, were purchased from List Biological Laboratories. S. aureus cell suspension was from Sigma or Fisher. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976), using γ-globulin as standard (Bio-Rad). SDS-polyacrylamide gel (12.5 and 16%) electrophoresis was carried out according to the method of Laemmli (1970). Amino acid sequence analysis was performed using a pulsed liquid phase protein Sequencer (Applied Biosystems Inc. model 477A).

Preparation of Gα, αγ, and βγ Subunit—Bovine ROS membranes were prepared according to Papermaster and Dreyer (1974), with some modifications. Freshly collected retinas were suspended in a sucrose buffer solution containing 28% (w/w) sucrose, 80 mM KCl, 30 mM NaCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM PMSF, and 10 mM MOPS (pH 7.5). The bovine retina suspension was gently stirred on ice and then centrifuged at 4,000 rpm (SS-34 rotor) at 4°C for 4 min. The resulting pellet was resuspended in fresh sucrose buffer solution and centrifuged at 6,000 rpm (SS-34 rotor) at 4°C for 4 min. The supernatant was decanted, and the pellet was discarded. The combined supernatants were diluted with buffer (60 mM KCl, 30 mM NaCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM PMSF, and 10 mM MOPS, pH 7.5) and centrifuged at 19,000 rpm (SS-34 rotor) at 4°C for 20 min. The resulting pellet was resuspended in 24% (w/w) sucrose buffer solution and homogenized. The homogenized membranes were layered on the top of a discontinuous gradient containing 10 mM MOPS, pH 7.5, 60 mM KCl, 30 mM NaCl, 2 mM MgCl2, 1 mM DTT, and sucrose in incremental 8-ml density steps of 1.09, 1.1, and 1.13 g/ml. The gradient was centrifuged at 25,000 rpm (SW-28 rotor, Beckman Instruments) at 4°C for 30 min. The dense red band of ROS membranes which appeared at approximately the 1.09/1.11 g/ml interface was collected carefully. The ROS membranes were diluted with buffer and centrifuged at 19,000 rpm (SS-34 rotor) at 4°C for 20 min. The resulting pellet was resuspended in buffer, homogenized, and stored at -70°C. Bovine G was extracted from ROS membranes as described by Stryer et al. (1983). The α-GTP·P·S and βγ subunits were extracted from ROS membranes by using GTP·S and then purified by chromatography on Blue-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.), essentially as described by Kleuss et al. (1987). All the purified proteins were stored in 40% glycerol at -20°C.

Proteolysis of G and αγ Subunit—Trypsinization of G and α-GTP·P·S was performed essentially as described by Fung and Nash (1983). Either Gγ (2 mg/ml) or α-GTP·P·S (1 mg/ml) in buffer A (200 mM NaCl, 2 mM MgCl2, 1 mM DTT, 10 mM MOPS, pH 7.5) containing 5% glycerol was incubated at 37°C. The reaction was terminated by addition of TLCK (1.0 mg/ml) and the samples were boiled for 5 min.

Proteolysis of α-GTP·P·S by S. aureus V8 protease was carried out as described by Navon and Fung (1987). Either Gγ (1.2 mg/ml) or α-GTP·P·S (0.6 mg/ml) was digested in buffer A by S. aureus V8 protease for 2 h at room temperature. The protease to α-GTP·P·S ratio was 1:50 (w/w). The reactions were stopped by the addition of TLCK and were then boiled for 5 min. The digests were denatured by boiling for 5 min.

Proteolysis of G and α-GTP·P·S by either endoproteinase Arg-C or endoproteinase Lys-C was carried out in buffer A at room temperature. For endoproteinase Arg-C, either Gγ (1.6 mg/ml) or α-GTP·P·S (0.8 mg/ml) was digested at a protease/Gγ ratio of 1:3 (w/w). At each time point, aliquots of the reaction mixture were removed, and the reactions were stopped by the addition of TLCK to a final concentration of 2.4 mg/ml. The samples, after addition of the electrophoresis sample buffer (Laemmli, 1970), were then boiled for 5 min. Endoproteinase Lys-C was used to cleave either Gγ (1 mg/ml) or α-GTP·P·S (0.5 mg/ml) at a protease/Gγ ratio of 1:100 (w/w). Aliquots of the reaction mixture were removed at different times, and proteolysis was terminated by the addition of TLCK to a final concentration of 54 μg/ml. The electrophoresis sample buffer (Laemmli, 1970) was added, and the samples were boiled for 5 min. The proteolytic fragments were purified by reversed phase HPLC (Brownlee, Aquapore RP-300, C8) the reactions were stopped by the addition of 1% trifluoroacetic acid and 6 μM guanidine HCl (final concentrations), applied to the column, and eluted with a linear acetonitrile gradient in 0.1% trifluoroacetic acid.

ADP-ribosylation of G and α-GTP·P·S—Pertussis toxin-catalyzed ADP-ribosylation of either Gγ or α-GTP·P·S was carried out as described previously (Mazzoni and Hamm, 1989). The [32P]ADP-ribosylated proteins were substrates for proteolysis by trypsin, endoproteinase Arg-C, and endoproteinase Lys-C.

Proteolysis of G, prior to sequencing was catalyzed by the active subunit alone, the A protomer, at a ratio of 50:1 (G/A protomer, w/w) for 90 min at 30°C. Labeled G, was isolated from the A protomer and untreated [32P]G by gel filtration on Sephacryl S-300 equilibrated in buffer A and eluted with buffer A followed by repeated washing with buffer A of the pooled protein in a Centricon 30 filtration unit (Amicon).

Immunological Procedures—Monoclonal antibody 4A was generated by immunization of mice with the soluble fraction of ROS proteins, purified, and characterized as described by Hamm and Elkind (1981) and Witkin et al. (1984). The antibody was isolated from the SDS-polyacrylamide gel to nitrocellulose (0.1 μm, Schleicher

While this paper was under review a similar finding was reported (Hingorani, V. N., and Ho, Y.-K. (1990) J. Biol. Chem. 265, 19923–19927).
& Schuell) essentially as described by Townin et al. (1979). The immunoblots were incubated in 150 mM NaCl, 50 mM Tris/HCl, pH 8.5 (TBS), containing 3% ovalbumin (OTBS) for 3 h at room temperature to block nonspecific binding and then transferred to OTBS containing mAb 4A (50 μg/ml) and incubated overnight. After two washes in TBS, one wash in TBS at room temperature, and two more washes, the immunoblots were incubated in TBS for 3 h and then transferred to OTBS containing 125I-protein A (0.5 μCi/ml, specific activity, 30 μCi/μg) and incubated at room temperature. After 4 h of incubation the immunoblots were rinsed as described above and dried. Autoradiography was performed by exposing the immunoblot to a Kodak XAR-2 x-ray film with an intensifying screen. Immunoprecipitation of αt and αb, after cleavage by S. aureus V8 protease was carried out as described previously (Mazzoni and Hamm, 1989). After digestion of αt by the protease, the sample was treated with TPCK, TLCK, and FMSP at final concentrations of 50, 50, and 200 μg/ml, respectively. The mixture was incubated at 4°C for 4 h. The proteolysis was terminated by adding 2× Laemmli buffer. The mixture was heated to 75°C for 5 min. The addition of mAb 4A. The antibody-antigen immunocomplexes were precipitated using S. aureus cell suspension as described (Mazzoni and Hamm, 1989).

RESULTS

To determine the localization of mAb 4A epitope on the αt subunit, antibody binding to proteolytic fragments of αt was analyzed. Four different proteases were used to cleave either Gt or the αt subunit. The fragments were separated by SDS-gel electrophoresis (Laemmli, 1970), transferred to nitrocellulose, and mAb 4A binding to these fragments was determined using 125I-protein A.

Trypsin was the first protease used to cleave either Gt or αt, since the proteolytic fragments are well characterized (Fung and Nash, 1989; Hurley et al., 1984). The time course of limited trypsin digestion of Gt, on a 16% SDS-polyacrylamide gel is shown in Fig. 1A (left panel). Transient proteolytic fragments (α38, α34, and α23) appeared; however, the major final fragments were α38, α34, α23, β32, and β12, in agreement with Fung and Nash (1983) and Hurley et al. (1984). A duplicate gel was blotted onto nitrocellulose, and immunoblot analysis showed that mAb 4A bound to the αt subunit and two transient fragments of 34 and 23 kDa (Fig. 1A, right panel). The 23-kDa fragment could not be localized in the gel since it disappeared and was obscured by the more abundant β32 fragment. The cleavage sites on αt with the sequential formation of the 38-, 34-, 32-, 23-, 21-, and 12-kDa fragments are known (Hurley et al., 1984). However, the origin of the 34- and 23-kDa fragments has not been elucidated clearly (Fung and Nash, 1983).

To determine if the 34- and 23-kDa fragments still contained the carboxyl-terminal region of αt, trypsinization was carried out using Gt, previously 32P-labeled at Cys47 by pertussis toxin-mediated ADP-ribosylation (Fig. 2). The quantity of the 34-kDa fragment was reduced for both ADP-ribosylated protein Gt and digested with trypsin under conditions used for ADP-ribosylation which included increased reducing agent. However, the overall pattern of trypsin peptides was the same for both unmixed and ADP-ribosylated Gt, i.e., the same major final fragments (α38, α34, α23, β32, and β12). Additionally, the overall conformation of the ADP-ribosylated Gt, used in the experiments was very similar to the native conformation of the protein since studies of various pertussis toxin substrates have shown that the effect of ADP-ribosylation is at the level of receptor-G-protein interaction. ADP-ribosylated Gt and Gt show normal nucleotide binding and GTPase activity (UI, 1986; Snyuer et al., 1989) as well as unchanged interaction with βγ (Neer and Clapham, 1990). Similarly, for Gt, Ramdas and Wensel (1990) have shown that although pertussis toxin labeling blocks rhodopsin-catalyzed nucleotide exchange there is no effect on the rate of nucleotide exchange in the absence of light-activated rhodopin and that modified Gt, also activates photoreceptor cGMP phosphodiesterase in the presence of AlF4-. The autoradiogram (Fig. 2, right panel) shows that αt, α38, and α23 are 32P-labeled as well as an early minor fragment (15 kDa) seen in the Coomassie Blue-stained gel at 1, 2, and 5 min. This transient fragment, the product of cleavage at Arg340 rather than at Arg310 has also been reported by Fung and Nash (1985). The 34- and 23-kDa fragments were not 32P-labeled and therefore had lost the carboxyl-terminal region containing Cys47.

When αt-GTPγS was used as a substrate for trypsinization the cleavage pattern was different from that of Gt (Fig. 1, compare A and B, left panels). The amount of the 34-kDa fragment was decreased dramatically, and the 32-kDa fragment was not cleaved further to 21- and 12-kDa fragments. Accumulation of the 34-kDa fragment appeared to be dependent on the conformation of the αt subunit, the presence of reducing agents (compare Fig. 1A and Fig. 2) and the βγ subunit (compare Fig. 1A and B). For αt-GTPγS the absence of βγ, and the presence of GTPγS enhanced cleavage at Lys18 and protected at Arg204. The two protein bands of 23 and 14 kDa represent trypsin and a trypsin fragment resulting from enzyme self-digestion, respectively. Western blot analysis of mAb 4A binding to the fragments (Fig. 1B, right panel) showed that the antibody bound only the αt subunit and the 34-kDa fragment.

S. aureus V8 protease removes 21 residues from the amino terminus of αt (Navon and Fung, 1987), with the formation of a stable 38-kDa fragment. Using Western blot analysis, Navon and Fung (1988) have shown that mAb 4A does not recognize the 38-kDa fragment. In a similar experiment (Fig. 3), αt-GTPγS was cleaved with S. aureus V8 protease. After blocking the reaction, aliquots containing 20 μg of protein were applied to two gels for protein stain and immunoblot, respectively. Immunoblot analysis (Fig. 3, right panel) showed that mAb 4A did not bind to the 38-kDa fragment, which lacked the amino-terminal 21 amino acids. In addition, mAb 4A did not immunoprecipitate the 38-kDa fragment (Fig. 4, lane 4) but was capable of immunoprecipitating uncleaved αt (Fig. 4, lane 2). Control experiments showed that the presence of the enzyme and inhibitors did not affect the immunoprecipitation of uncleaved αt (data not shown).

As a further step in the analysis of mAb 4A binding to proteolytic fragments of αt, the cleavage pattern of either Gt or αt, after digestion with endoproteinase Arg-C was determined. Ho et al. (1989) reported that endoproteinase Arg-C makes a single cut at Arg310 of αt, generating a 34- and a 5-kDa fragment containing the amino and carboxyl termini of αt, respectively. Fig. 5A shows the time course of ADP-ribosylated Gt obtained by digestion with endoproteinase Arg-C. The Coomassie Blue-stained gel (Fig. 5A, left panel) shows that although αt was not a particularly good substrate for this enzyme, digestion resulted in 34- and 23-kDa fragments, and the proteolytic pattern was the same whether Gt or ADP-ribosylated Gt, was used as a substrate (Fig. 5, compare A and B left panels). The βγ subunits were uncleaved. In the autoradiogram (Fig. 5A, right panel), the 32P label was associated mainly with the αt subunit, a 5-kDa fragment, and a minor ~15-kDa fragment. The 34- and 23-kDa fragments were not 32P-labeled, indicating that the fragments did not contain Cys47. Immunoblot analysis of these proteolytic fragments showed that the antibody bound αt, the 34- and 23-kDa fragments, and a ~12-kDa fragment which appeared very late in the proteolysis (Fig. 5B, right panel). The total intensity of the 125I-labeled bands in each lane appeared to be
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Fig. 1. Time course of proteolysis of Gα and α, GTPγS by TPCK-treated trypsin and Western blot analysis of mAb 4A binding to the fragments. Purified bovine Gα (1 mg) or α, GTPγS (0.5 mg) was digested with 40 μg of TPCK-treated trypsin in 1 ml of buffer A (see "Experimental Procedures") on ice. At the indicated time points, 80 μl of the mixture was removed, and the reaction was stopped by adding 4 μl of trypsin inhibitor (8 mg/ml). The proteolytic fragments (A, 35 pg of Gα; B, 17 pg of α, GTPγS) were separated by electrophoresis on duplicate SDS-polyacrylamide gels (16%). One gel was stained with Coomassie Blue (A and B, left panels), and the other (A and B, right panels) was blotted onto nitrocellulose. Antibody binding to fragments was detected as described under "Experimental Procedures." Molecular weight standards are indicated, as are the sizes and origin of the tryptic fragments. C, Gα; N, α, GTPγS; T, trypsin; I, trypsin inhibitor.

equal, suggesting that the αi subunit and its fragments bound a similar amount of antibody.

When compared with Gα, the cleavage pattern of α, GTPγS showed that the 23-kDa fragment was not produced (Fig. 6). This suggests that the presence of GTPγS prevented endo-proteinase Arg-C cleavage at Arg304, similar to digestion with trypsin, in which bound GTPγS eliminates Arg304 as a cleavage site (Fig. 1B) (Fung and Nash, 1983). A fraction of the 34-kDa fragment was converted to two fragments of ~33 and 32 kDa after many hours of digestion (Fig. 6).

To define both cleavage sites on αi, the fragments obtained after digestion of [32P]ADP-ribosylated Gα were purified by reversed phase HPLC and sequenced. The analysis revealed that the 5-kDa fragment had an amino-terminal sequence of Asp-Val-Lys-Ile-Tyr-Ser-His-Met-Thr-Cys-Ala-Thr-Asp, and the 15-kDa fragment had an amino-terminal sequence of Lys-Lys-Trp-Ile-His-Cys-Phe-Glu-Gly-Val-Thr-Cys-Ile-Ile, which corresponded to αi residues 311-324 and 205-218, respectively (Yatsunami and Khorana, 1985; Tanabe et al., 1985; Lochrie et al., 1985; Medynski et al., 1985). We were unable to sequence the 34- and 23-kDa fragments. This suggested that the 34-kDa fragment contained the amino terminus which is known to be blocked to sequencing (Hurley et al., 1984). Therefore, in agreement with a previous report.
**Fig. 2.** Time course of proteolysis of pertussis toxin ADP-ribosylated G, by TPCK-treated trypsin.
Purified bovine G, (325 μg) was ADP-ribosylated with pertussis toxin (PT) as described (Mazzoni and Hamm, 1989) and digested immediately with 13 μg of TPCK-treated trypsin in 380 μl of reaction mixture. The reaction was stopped by adding 2 μl of trypsin inhibitor (8 mg/ml) to 47 μl of the reaction mixture at the time points indicated. The fragments (35 μg/lane) were separated by electrophoresis on an SDS-polyacrylamide gel (16%). The gel was stained with Coomassie Blue (left panel), dried, and autoradiographed (right panel) using a Kodak XAR-2 film with an intensifying screen. Molecular weight standards and tryptic fragments are labeled as described in Fig. 1.

**Fig. 3.** Digestion of α,-GTPyS by S. aureus V8 protease and Western blot analysis of mAb 4A binding to the fragment.
Purified α,-GTPyS (40 μg) was digested with 0.8 μg of S. aureus V8 protease in 58 μl of buffer A for 2 h at room temperature. The reaction was stopped by adding 5 μl of PMSF (35 mg/ml) and 10 μl of TLCK (18 mg/ml). The sample (20 μg of protein/lane) was loaded on duplicate 12.5% SDS-polyacrylamide gels and electrophoresed. One gel was stained with Coomassie Blue (left panel), and the other (right panel) was blotted onto nitrocellulose. Antibody binding to the 38-kDa fragment was detected as described under “Experimental Procedures.” lane 1, α; lane 2, α38.

**Fig. 4.** Immunoprecipitation of α, and α, after digestion with S. aureus V8 protease (α38) by mAb 4A. Purified α,-GTPyS (10 μg) was digested with 0.2 μg of S. aureus V8 protease in 21 μl of buffer A containing 6.5 mM DTT for 2 h at room temperature. The reaction was stopped by the addition of 2 μl of TPCK (50 mM), 2 μl of TLCK (50 mM), and 1 μl of PMSF (100 mM). The sample volume was brought up to 100 μl with buffer A containing 0.4% Lubrol PX and 0.1 mM DTT, and the pH was adjusted to 7.5. Monoclonal antibody 4A (76 μg) was added to the mixture and incubated for 1 h at room temperature. The immune complex was precipitated as described previously (Mazzoni and Hamm, 1989). The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel (12.5%) electrophoresis and stained with Coomassie Blue. Control, α (lane 1); α + mAb 4A (lane 2); control, α38 (lane 3); α38 + mAb 4A (lane 4). HC and LC are antibody heavy and light chain, respectively.
Fig. 5. Time course of proteolysis of G, and pertussis toxin-catalyzed ADP-ribosylated G, by endoproteinase Arg-C and Western blot analysis of mAb 4A binding to fragments. A, purified G, (410 μg) was ADP-ribosylated with pertussis toxin (PT) as described previously (Mazzoni and Hamm, 1989) and then digested with 150 μg of endoproteinase Arg-C in 340 μl of reaction mixture at room temperature. The reaction was stopped by adding 7.4 μl of TLCK (18 mg/ml) to 58 μl of the reaction mixture at the time points indicated. The proteolytic fragments (30 μg/lane) were separated on a 16% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue/ and the other panel, B, purified G, (520 μg) was digested with 160 μg of endoproteinase Arg-C in 315 μl of buffer A at room temperature. At the indicated time points, 49 μl of the mixture was removed, and the reaction was stopped by the addition of 7.4 μl of TLCK (18 mg/ml). The proteolytic fragments (40 μg/lane) were separated on duplicate 16% SDS-polyacrylamide gels. One gel was stained with Coomassie Blue/ (left panel), dried, and autoradiographed (right panel). B, purified G, (520 μg) was digested with 160 μg of endoproteinase Arg-C in 315 μl of buffer A at room temperature. At the indicated time points, 49 μl of the mixture was removed, and the reaction was stopped by the addition of 7.4 μl of TLCK (18 mg/ml). The proteolytic fragments (40 μg/lane) were separated on duplicate 16% SDS-polyacrylamide gels. One gel was stained with Coomassie Blue/ (left panel), and the other (right panel) was blotted onto nitrocellulose, and mAb 4A binding to the fragments was detected as described under "Experimental Procedures." Molecular weight standards and proteolytic fragments are labeled as described in Fig. 1. G, G,; I, TLCK; E, endoproteinase Arg-C.

(Ho et al., 1989), the first cleavage occurred at Arg301. Additionally, we observed a second cleavage at Arg431. The fourth protease used to cleave either G, or the α,, subunit was endoproteinase Lys-C. Fig. 7 shows the time course of proteolysis of pertussis toxin-catalyzed ADP-ribosylated G, (A, left panel) and α,-GTPγS (B, left panel). A similar cleavage pattern was obtained when either unmodified G, or α,-GTPγS was used as substrate for digestion with endoproteinase Lys-C (data not shown). The Coomassie Blue-stained gel of digested α,-GTPγS (Fig. 7B, left panel) shows that α, was cleaved to a transient 38-kDa fragment, which was further converted to a stable 36-kDa fragment. At a protease/α,, subunit ratio of 1:50 (w/w) the proteolysis was almost complete after 2 h. When G, was used as substrate (Fig. 7A, left panel), the 36-kDa fragment was not clearly visible since it was obscured by the β, band. The β, and γ, subunits were not cleaved (data not shown). Autoradiography of the gels (Fig. 7, A and B, right panels) shows that the 32P label remained associated with the 36-kDa fragment. When ADP-ribosylated G, was digested, a minor labeled fragment (~15 kDa) appeared late in the time course. These results suggested that the two major cleavage sites were located near the α, amino terminus. To determine the exact location of these cleavage sites on α,-GTPγS the fragments were purified by reversed phase HPLC and sequenced. This resulted in assignment of amino-terminal sequences Lys-Leu-Lys-Glu-Val-Leu-Leu-Leu to the 38- and the 36-kDa fragments, respectively (α, residues 18-27 and 26-34) (Yatsunami and Khorana, 1985; Tanabe et al., 1985; Lochrie et al., 1985; Medynski et al., 1985). Therefore, endoproteinase Lys-C cleaves α, first at Lys3 and then at Lys25.

Western blot analysis of mAb 4A binding to the proteolytic fragments shows (Fig. 8, right panel) that the antibody recognized only α, and did not bind the major fragments, 38 and 36 kDa, which were missing the first 17 or 25 amino-terminal residues.

The major cleavage sites on α, for the four proteases used in this work are summarized in Fig. 9. The figure clearly shows that α, has three regions that are readily available for proteolytic digestion: Arg204, Arg210, and Lys17-Lys25. Trypsin and endoproteinase Arg-C each had a common cleavage point at Arg204 and Arg210. Recent work with chymotrypsin indicated...
and Hamm (1987) reported the binding of mAb 4A to α, tryptic and chymotryptic fragments using epitope mapping on Western blots. The results showed that none of the major tryptic fragments bound the antibody, but a 34-kDa fragment and several minor transient fragments of 26, 23, 18, 16 and 12 kDa which comigrated with 32P-ADP ribosylated fragments did bind the antibody. Size analysis of these antigenic fragments suggested that the main portion of the mAb 4A epitope could be localized to the carboxyl-terminal region between Arg196 and Lys202. However, these fragments were not sequenced so their origins could not be proven.

In the present work the antibody bound the 34- and 23-kDa tryptic fragments. The transient fragments of 26, 18, 16, and 12 kDa were not normally observed when trypsin activity was completely blocked by trypsin inhibitor before the addition of electrophoresis sample buffer. However, when trypsin inhibitor and electrophoresis sample buffer were added together before the addition of G,, trypsin activity was not completely blocked, and the minor fragments appeared (data not shown).

The results of Deretic and Hamm (1987) were supported by ELISA competition studies between synthetic α, peptides and G, for mAb 4A binding (Hamm et al., 1988). The α, peptides Glu139-Val209 and Ile208-Phe210 inhibited antibody binding whereas peptide Asp220-Ala225 did not compete (Hamm et al., 1988). Another amino-terminal peptide, Met2-Ala25, also had no effect (Hamm et al., 1990). Since the amino terminus of α, is blocked, acetyl-1-23-NH2 was recently synthesized, and this peptide also did not compete with α,-GTPYs for mAb 4A binding (data not shown). Thus, competition ELISA suggested that the carboxyl terminus may also be recognized by the antibody. Navon and Fung (1988) have reported that a synthetic α, peptide derived from the amino-terminal sequence, Cys7-Tyr-Asp2-Ala252 competes with G, for antibody binding in a solid phase immunoassay. However, the addition of two amino acids not present in the native sequence and the requirement for 10-fold higher concentrations of this peptide as compared with carboxyl-terminal peptides (Hamm et al., 1988) make this result difficult to interpret. Another difficulty in the interpretation of these contrasting results arises from the indirect nature of the measurement. Peptides that compete for antibody binding to G, may do so directly, by binding to the antibody itself or to a site on G, which is recognized by the antibody, or allosterically, by binding α, at a site away from, but affecting, the epitope. It is not possible to distinguish among these alternatives with peptide competition ELISA.

Complete studies on the antigenicity of other proteins such as lysozyme have shown that neither Western blot nor peptide competition ELISA is adequate to specify a monoclonal antibody epitope completely, as finally determined by x-ray crystallography of the antigen-antibody complex. In fact, for the known protein/Fab crystal structures, the epitope is usually formed by several regions of the protein, with approximately 15 amino acids taking part in binding to the antibody (Amit et al., 1986; Sheriff et al., 1987; for review see Davies et al., 1988; Laver et al., 1990; Janin and Chothia, 1990).

The work presented here shows that on Western blots the presence of the amino-terminal region is the main criterion for antibody-α, binding. However, the epitope on the native protein could be linear, continuous over a single length of amino acids, or discontinuous, composed of amino acids from disparate parts of the primary sequence which are brought together on the surface when the protein assumes its native conformation. If the mAb 4A epitope is linear it could be contained completely within the amino terminus, as proposed.

**DISCUSSION**

The major finding of this study is that the main portion of the mAb 4A epitope on α, is located in the amino-terminal region of α,. This conclusion is based on real-time analysis and expansion of previous peptide mapping studies and clearly emphasizes the importance of the amino-terminal region. Specifically, after digestion with endoproteinase Arg-C and trypsin digestion of α, mAb 4A bound the 34- and 23-kDa fragments. Since the endoproteinase Arg-C fragments were not sequenceable it was assumed that these fragments contained the amino terminus, which is reported to be blocked to sequencing (Hurley et al., 1984). The 34- and 23-kDa fragments lack 41 and 106 carboxyl-terminal residues, respectively. Furthermore, mAb 4A did not recognize the tryptic, endoproteinase Lys-C, or S. aureus V8 protease 38- or 36-kDa fragments lacking 17-25 amino-terminal residues. Since the 23-kDa fragment was recognized by the antibody, residues from Lys210 to Phe210 were not required for antibody binding. In contrast, the amino-terminal residues from Met1 to Lys17 appear to be required for antibody-α, binding.

Previous studies from this laboratory have suggested that mAb 4A binds to the carboxyl-terminal region of α,. Deretic

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**Fig. 6. Time course of proteolysis of α,-GTPγS by endoproteinase Arg-C.** Purified α,-GTPγS (260 µg) was digested with 160 µg of endoproteinase Arg-C in 315 µl of buffer A at room temperature. At the indicated time points, 49 µl of the mixture was removed, and the reaction was blocked by adding 7.4 µl of TLCK (18 mg/ml). The proteolytic fragments (19 µg/ml) were separated with 16% SDS-polyacrylamide gel electrophoresis. Molecular weight standards and proteolytic fragments are labeled as described in Fig. 1. α,-GTPγS; I, TLCK; E, endoproteinase Arg-C.

Coomassie blue stain

![Coomassie blue stain](image)

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**G**
FIG. 7. Time course of proteolysis of pertussis toxin-ADP-ribosylated G, and α,·GTPγS by endoproteinase Lys-C. Either G, (A, 150 μg) or α,·GTPγS (B, 150 μg) was ADP-ribosylated with pertussis toxin (PT) as described (Mazzoni and Hamm, 1989) and then digested with endoproteinase Lys-C (1.5 and 3 μg, respectively) in 157 and 314 μl of the reaction mixtures, respectively. The reactions were blocked by adding 5 and 10 μl of TLCK (0.28 mg/ml) to 21 and 42 μl of the reaction mixtures, respectively, at the time points indicated. The proteolytic fragments (20 μg/lane) were separated on 12.5% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue (A and B, left panels), dried, and autoradiographed (A and B, right panels). E, endoproteinase Lys-C.

by Navon and Fung (1988). However, Western blot analysis and peptide competition assays cannot determine if additional amino acids form a discontinuous epitope.

G, structure function studies have revealed that the rhodopsin binding domain is complex. The carboxyl terminus of α, interacts directly with rhodopsin (Hamm et al., 1988, 1991), but other regions of α, may also be involved in rhodopsin binding. Hingorani et al. (1988) suggested on the basis of cross-linking experiments that the rhodopsin binding domain of α, contains both the amino and carboxyl termini of the protein. We have found that amino-terminal peptides as well as carboxyl-terminal peptides can disrupt rhodopsin-G, interaction (Hamm et al., 1988), supporting this suggestion. However, association of α, and βγ, subunits is required for G, interaction with rhodopsin, and the amino-terminal region of α, has also been suggested as the site of interaction with βγ,. Proteolytic removal of the amino-terminal 23 amino acids from α, causes dissociation from βγ, (Navon and Fung, 1987), and G,·G, chimera studies demonstrate that disruption of internal regions near the amino terminus (α, residues 15–41, 62–71, and 87–144) also inhibits interaction with βγ (Osawa et al., 1990b). If rhodopsin binds both the amino and carboxyl termini then these regions may be physically close, as has been suggested by several recent studies (Dhanasekaran et al.,
**Coomassie blue stain**

**mAb 4A Western-Blot**

**time (min)**

**fig. 8.** Time course of proteolysis of \( \alpha \)-GTP\(_{yS} \) by endoproteinase Lys-C and Western blot analysis of mAb 4A binding to fragments. Purified \( \alpha \)-GTP\(_{yS} \) (200 \( \mu \)g) was digested with 4 \( \mu \)g of endoproteinase Lys-C in 440 \( \mu l \) of buffer A at room temperature. At the indicated time points, 50 \( \mu l \) of the mixture was removed, and the reaction was stopped by adding 5 \( \mu l \) of TLCK (0.52 mg/ml). The proteolytic fragments (10 \( \mu g/lane \)) were separated on duplicate 12.5% SDS-polyacrylamide gels. One gel was stained with Coomassie Blue (left panel) and the other was blotted onto nitrocellulose. mAb 4A binding to fragments was detected as described under "Experimental Procedures."

**fig. 9.** Summary of the major proteolytic cleavage sites on \( \alpha \), T, trypsin; K, endoproteinase Lys-C; V, Staphylococcus aureus V8 protease; R, endoproteinase Arg-C; C, chymotrypsin; *, Cys\(^{17} \), site of ADP ribosylation by pertussis toxin.

1988; Hingorani and Ho, 1988; Osawa et al., 1990a, 1990b).

Monoclonal antibody 4A binding to \( \alpha \) has several important functional consequences. It disrupts \( \alpha \) interaction with rhodopsin (Hamm et al., 1987) and causes \( \alpha \) subunit dissociation (Mazzoni and Hamm, 1988). \( \alpha \) binding to light-activated rhodopsin interferes with mAb 4A binding to its epitope (Hamm et al., 1987; Navon and Fung, 1988). The functional effects of mAb 4A are most likely related to the close functional relationship between the rhodopsin and \( \beta_{1} \) binding sites on \( \alpha \), and may be suggestive of interaction between the amino and carboxyl termini of \( \alpha \).

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**REFERENCES**


mAb 4A Epitope Mapping of \(G_i\)