Carboxyl Methylation and COOH-terminal Processing of the Brain G-protein γ-Subunit

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The enzymatic methylation of the guanine nucleotide-binding proteins (G-proteins) γ-subunit was investigated in brain membranes. Brain membranes were methylated in vitro using [3H-methyl]S-adenosylmethionine, and the G-protein γγ-complex was purified using an anti-γ antibody to assay for the protein during purification. The isolated G-protein γγ-complex was found to be carboxyl methylated on the γ-subunit. The methyl group was localized by tryptic digestion to the carboxyl-terminal of the protein. The methylated tryptic peptides contained a modified cysteine and were very hydrophobic, suggesting additional modification by lipidation. The evidence suggests that the COOH-terminal of G-γ is modified in a manner similar to the processing that occurs with the ras proteins.

The G-proteins are a family of membrane-bound proteins involved in the transduction of signals from cell surface receptors to a variety of effector systems (1, 2). All members of the G-protein family are heterotrimeric composed of a, β, and γ-subunits. The a-subunit contains the GTP-binding site and GTPase activity and can dissociate from the βγ-complex, while the βγ- and γ-subunits remain tightly associated under nondenaturing conditions. A cDNA encoding a γ-subunit of a G-protein from bovine brain has been recently cloned, and it appears to be a member of a multigene family (5, 6). The amino acid sequence of G-γ derived from the cDNA sequence has similarities with both transducin-γ and the ras proteins, particularly at the COOH terminus where the protein has the conserved sequence Cys=Aaa=Aaa=Xaa, where Aaa is an aliphatic amino acid and Xaa can be any amino acid. A multistep posttranslational modification of this COOH-terminal sequence has been shown to occur with the fungal mating factors (7, 8) and with the ras proteins (9-13). The modifications involve cleavage of the COOH-terminal tripeptide (9), the methylation of the α-carboxyl group of the resulting COOH-terminal cysteine to form a methyl-ester (9-11), and the addition of a farnesyl moiety as a thio-ether on the cysteine (12, 13). The processing of the COOH-terminal of the ras proteins is required for association with the membrane and oncogenic activity (14).

While the G-proteins are known to be membrane bound, the molecular basis for association with the membrane has not been determined. Based on the amino acid sequence of the a-subunits, there are no obvious regions of hydrophobicity to account for the membrane association. Some of the G-protein a-subunits from bovine brain, including a and α2, have been shown to be myristoylated (15). Recent evidence has shown that the addition of myristate at the NH2-terminal of α is required for association of the protein with the membrane (16). It has also been proposed that the βγ-complex serves to anchor the α-subunits in the membrane (17), however, the basis for βγ binding to the membrane has not been defined. Posttranslational modifications of the β- or γ-subunits of the G-proteins that may stabilize the βγ-complex in the membrane have not been previously reported. Due to the similarity between the COOH-terminal of G-γ and the ras proteins (5), it was of interest to determine if G-γ is modified in a similar manner. Here, we demonstrate that G-γ from brain membranes can be methylated in vitro and that the methylation appears to one of several posttranslational modifications at the COOH-terminal of G-γ.

EXPERIMENTAL PROCEDURES

Brain Membrane Preparation—Young rabbit brains (350 g, Pel-Freeze) were thawed into a homogenization buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, 0.1% 2-mercaptoethanol (TEM buffer), with the following additions: 0.1 mM MgSO4, 10% sucrose, 0.2 mM phenylmethylsulfonflu fluoride (PMSF), 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Except where indicated, all steps were carried out at 0-4 °C. The tissue was homogenized four times for 30 s in a blinder, and the homogenized sample was centrifuged at 500 × g for 10 min. The supernatant was collected and centrifuged again at 15,500 × g for 45 min. The pellet was resuspended in an equal volume of TEM buffer and stored at -70 °C. When the membranes were used for the methylation reaction, the membranes were thawed into an equal volume of TEM, 0.1 mM PMSF, and the suspension was centrifuged at 10,000 × g for 30 min in a SS-34 rotor (Sorval).

Methylation and Isolation of G-protein βγ-Complex—The methylation reaction was carried out in 100 ml with a final concentration of 0.1 mM sodium phosphate, pH 6.8, 1 mM EDTA, 20 μM [3H-methyl]-AdoMet (500 Ci/mmol), 0.1 mM GDP, 8.1 mM PMSF, 20 μM leupeptin, 2 μg/ml aprotinin, and 2.5 μg of rabbit brain membranes. The sample was incubated for 2 h at 37 °C with gentle rocking. The reaction was stopped by addition of 200 ml of ice-cold TEM buffer, 0.1 mM PMSF, and the membranes were collected by centrifugation at 10,000 × g for 30 min. The membrane pellet was extracted with 1% sodium cholate in TEM buffer, 0.1 mM PMSF in a final volume of 100 ml for 1 h with stirring, followed by centrifugation at 10,000 × g for 45 min in an SS-34 rotor. The supernatant was applied to a column of DEAE-Sepharose (2.0 × 54 cm), Pharmacia LKB Biotechn
G-protein γ-Subunit Methylation

Isolation of G-γ and Proteinase Treatments—The γ-subunit was separated by HPLC using a C$_4$ reverse-phase column (250 × 4.6 mm, SphерoPak-RP4, Sphерo, Inc.). The column buffers consisted of buffer A (0.1% trifluoroacetic acid/H$_2$O) and buffer B (0.08% trifluoroacetic acid/CH$_3$CN). The column was equilibrated with 25% buffer B at a flow rate of 1 ml/min at 30 °C, and the samples eluted in a linear gradient to 60% buffer B in 40 min, followed by a linear gradient to 90% buffer B in 5 min. Column elution was monitored by absorbance at 215 nm, and 0.5 ml fractions were collected.

Trypsin digestion of the βγ-complex was performed in 0.4 ml containing 25 μg of the purified γ-subunit by HPLC, using a C$_4$ reverse-phase column (250 × 4.6 mm, SpheroPak-RP4, Sphero, Inc.). The column buffers consisted of buffer A (0.1% trifluoroacetic acid/H$_2$O) and buffer B (0.08% trifluoroacetic acid/CH$_3$CN). The column was equilibrated with 25% buffer B at a flow rate of 1 ml/min at 30 °C, and the samples eluted in a linear gradient to 60% buffer B in 40 min, followed by a linear gradient to 90% buffer B in 5 min. Column elution was monitored by absorbance at 215 nm, and 0.5 ml fractions were collected.

Carboxypeptidase-Y digestion was performed in 0.2 ml containing 25 μg of the purified βγ-complex by HPLC, using a C$_4$ reverse-phase column (250 × 4.6 mm, SpheroPak-RP4, Sphero, Inc.). The column buffers consisted of buffer A (0.1% trifluoroacetic acid/H$_2$O) and buffer B (0.08% trifluoroacetic acid/CH$_3$CN). The column was equilibrated with 25% buffer B at a flow rate of 1 ml/min at 30 °C, and the samples eluted in a linear gradient to 60% buffer B in 40 min, followed by a linear gradient to 90% buffer B in 5 min. Column elution was monitored by absorbance at 215 nm, and 0.5 ml fractions were collected.

Association of Methylated G-βγ with Transducin-α—Samples of the Mono-Q-purified methylated βγ-complex (102 μg of protein) were mixed with purified bovine transducin-α subunit (325 μg) (19), and diluted with 1% sodium cholate, 0.15 M Tris, pH 8.0, 1 mM dithiothreitol (CNDT buffer) in a final volume of 2 ml. In some experiments no transducin-α was added, and in other experiments the buffer was supplemented with 20 μM AlCl$_3$, 6 mM MgCl$_2$, 10 mM NaF (AMF). The samples were concentrated in a Centricon 30 centrifugal microconcentrator (Amicon) by centrifugation at 5,000 × g for 10 min (50 μl) with a 0.25-ml rinse of the microconcentrator with CNDT buffer (with or without AMF), and incubated at 30 °C for 30 min. After chilling on ice, the sample was applied to an ACA-44 Ultragel column (0.7 × 27.5 cm) equilibrated in CNDT buffer (with or without AMF), and the column was eluted with the same buffer at approximately 1 ml/h. The elution positions of the purified bovine γ-subunit and E. coli βγ-complex used were used to determine the presence of the βγ-complex.

Miscellaneous Methods—Protein determinations were performed by the method of Bradford, using bovine γ-globulin as a standard (20). Proteins were separated by SDS-PAGE, using gels of 10% acrylamide with a Tricine buffer system to resolve low molecular weight proteins (21). Amino acid composition was determined by hydrolyzing the protein samples for 20 h at 110 °C in 6 N HCl, followed by derivatization with phenylisothiocyanate. Mass spectrometric analysis was performed using a FINNIGAN MAT system (5890A) with separation conditions for physiological amino acids (22).

RESULTS

The methylation of G-proteins by AdoMet was investigated by incubating rabbit brain membranes in vitro with [H-methyl]AdoMet. The βγ-complex was then isolated by sequential chromatography over DEAE-Sepharose, Sephacryl S-200, and Mono-Q columns. The anti-G-β antibody, MS, was used to follow the presence of the βγ-complex by immobiloblotting. The isolated G-protein βγ-complex contained radioactivity specifically associated with a protein migrating on SDS-PAGE in the region expected for the γ-subunit (Fig. 1, lanes A3 and B3). With the gel system used, a major Coomassie Blue-staining band migrated with an M, = 6600, and a minor band migrated with an M, = 6400. The βγ-complex could be dissociated into individual subunits and the γ-subunit isolated by HPLC. A single peak of radioactivity eluted in the gradient at about 55% acetonitrile (Fig. 2), and the radioactivity also comigrated with the Coomassie Blue-staining band on SDS-PAGE (Fig. 1, lanes A4 and B4).

In order to demonstrate that the isolated [H-methyl]labeled protein was part of a functional βγ-complex, the methylated protein was assayed for its capacity to associate with...
The included volume for this column was 11.2 ml.

The a-subunit of transducin in a fluoride reversible manner.

The association and fluoride-dependent dissociation of the methylated protein was part of a functional p-y-complex. The association of the proteins to form the heterotrimer was demonstrated by a shift in elution from the column of the MS circles, or with (open circles) AMF, as described under "Experimental Procedures." The arrow indicates the elution volume of the [3H] methylated G-protein y-complex in the absence of transducin-a.

Procedures." The Mono-Q-purified G-protein Py-complex was applied to a Synchropak RP-4 column and eluted with a gradient of the B buffer (CH,CN, 0.08% trifluoroacetic acid). The protein elution was monitored by absorbance at 215 nm. Fractions of 0.5 ml were collected and the radioactivity determined in 75~1 aliquots. The fractions containing the radioactive peak at 34 min were pooled and used for further analysis.

The association and fluoride-dependent dissociation of the methylated protein from transducin-a indicates that the methylated protein is part of a functional p-y-complex.

The [3H]methyl-labeled protein was further characterized by determination of the amino acid composition. The amino acid composition was very similar to that predicted by a cDNA clone encoding a y-subunit from bovine brain (Table I). A few minor discrepancies were observed between the measured composition and that predicted by the cDNA. There was 1 residue less than expected for Met and Ile, and 2–3 fewer residues for Ala, while an increase of 2 residues of Glu/Gln, and 1 residue each for Gly, Arg and Val was observed. These small differences could be explained by a number of factors. The loss of 1 methionine could be expected from cleavage of the initiation methionine, since G-y has been shown to have a blocked amino terminus. In addition, the C-terminal processing proposed below would also result in a decrease by 1 residue each for Ala, Ile, and Leu. Of these three COOH-terminal amino acids, Ala and Ile had lower than the expected amino acid residues. The material isolated here probably represents a mixture of different y-subunits in an unknown proportion, and at least two forms of y-subunits have been isolated. One unique peptide of an alternate G-y has substitutions of a Val for a Thr, and a Gly for a Pro (6). There could also be some minor species differences since the cDNA sequence was from a bovine source, while the methylated y-subunit was isolated from rabbit.

The type of covalent linkage of the radiolabeled methyl group to the protein was examined by determining the susceptibility of the methyl group to hydrolysis under basic conditions. The treatment of the purified βγ-complex at pH 11 for 16 h at room temperature converted more than 80% of the radiolabel to a volatile form, while at pH 8 less than 10% of the radiolabel was hydrolyzed. Hydrolysis of the methyl group under basic conditions is characteristic of a methyl-ester linkage, in contrast to the base stable N-methylations found on lysine, arginine and histidine. The G-y methylation is relatively stable at pH 8, making it more stable than typical aspartyl β-methyl-esters (23), and suggests that the methylation may be an ester of a COOH-terminal α-carboxyl group.

The HPLC-purified methylated G-protein γ-subunit (100 pmol) was hydrolyzed in 6 N HCl and the amino acids derivatized with phenylisothiocyanate (22). Quantitation of the amino acids represents the mean value of triplicate determinations.

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<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acids of purified methylated protein</th>
<th>Predicted from cDNA of G-γ (5, 6)</th>
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<tbody>
<tr>
<td>Asp</td>
<td>6.9</td>
<td>3</td>
</tr>
<tr>
<td>Asn</td>
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</tr>
<tr>
<td>Glu</td>
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<tr>
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</tr>
<tr>
<td>Lys</td>
<td>6.6</td>
<td>7</td>
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</table>

* ND, not determined.
In order to localize the methyl group on the γ-subunit, the purified methylated βγ-complex was digested with trypsin, and the resulting peptides were separated by HPLC. After treatment with trypsin for 4 h, the radioactive peak corresponding to intact Gγ disappeared, and a radioactive peak migrating slightly later in the gradient was formed. The amino acid sequence of this peptide was determined for 19 amino acid residues, and the sequence was identical to the sequence deduced from the cloned bovine brain γ-subunit cDNA (5, 6) starting from Ala\(^{a\beta}\). Since this peptide was radiolabeled, the methylation must be located somewhere in the protein to the carboxyl side of Ala\(^{a\beta}\).

When the βγ-complex was incubated with trypsin for longer times (19 h), the radioactive trypsin fragment formed at 4 h of incubation disappeared and three peaks of radioactivity were observed eluting later in the gradient (Fig. 4). The two most hydrophobic radiolabeled peptides (Tryp II and III) accounted for most of the radioactivity. Acid hydrolysis of the peptide Tryp II produced phenylalanine and lysine (2:1), and acid hydrolysis of Tryp III produced only phenylalanine. In order to determine if a cysteine was present, the peptides were oxidized with performic acid before hydrolysis, and no cysteic acid was observed for either Tryp II and III. In order to test for the presence of a cysteine modified to a thioether, such as a thioether linkage.

The COOH-terminal amino acid of the methylated γ-subunit was examined by carboxypeptidase-Y digestion of the HPLC-purified γ-subunit. When the γ-subunit was incubated with carboxypeptidase-Y, the release of phenylalanine increased with time to 0.6 mol of phenylalanine/mol Gγ after 2 h of incubation. At the same time, carboxypeptidase-Y also hydrolyzed the labeled methyl group, as detected by conversion of the radiolabel to a volatile form. After 1 h of incubation, 74% of the \(^3\)H label was hydrolyzed, while 0.4 mol of phenylalanine/mol of Gγ was released, indicating that the methyl group was hydrolyzed more rapidly than the phenylalanine.

The stoichiometry of methyl group incorporation indicates that the majority of Gγ had the same COOH-terminal amino acid composition. No additional amino acids appeared to be released by carboxypeptidase-Y digestion under these conditions.

The addition of a cytosolic fraction back to the membranes during the incubation did not significantly increase the release of Gγ. The stoichiometry of methyl group incorporation did not increase with the addition of GTPγS, but the total yield of methyl group was increased.

The incorporation of the labeled methyl groups under these in vitro conditions was calculated to equal about 0.5 mol% methyl groups/γ-subunit. Since brain membranes were used as the substrate for Gγ methylation, it seemed possible that most of the Gγ protein was methylated before the in vitro reaction, so that the stoichiometry for methylation of Gγ was much higher than indicated by the incorporation of radiolabeled methyl groups. The amount of methylated tryptic peptides recovered from the HPLC (Fig. 4) indicates a high stoichiometry of methylation for Gγ. When 6.7 nmol of the G-protein βγ-complex was digested with trypsin, 0.38 nmol of the methylated Tryp II and 0.35 nmol of the methylated Tryp III peptides were recovered, based on the amino acids recovered after acid hydrolysis. This represents a recovery of 5.7 mol% for Tryp II and 5.3 mol% for Tryp III, for a combined total of 11 mol%. When Gγ was purified by HPLC from the intact βγ-complex, the recovery of Gγ from the column was about 16–20%. Therefore, recoveries of methylated peptides were similar to the recovery of intact Gγ, indicating that most of Gγ was methylated. If the bulk of Gγ was not methylated, the quantity of amino acids recovered from the...
The hydrophobicity of the methylated Tryp II and III peptides also indicates the presence of a further hydrophobic modification, possibly a lipid moiety, at the COOH-terminal. The conserved COOH-terminal Cys-Aaa-Aaa-Xaa sequence has been identified in a number of proteins, and appears to be a signal for further processing. For the fungal mating factors (7, 8), and the ras proteins (9–13), the COOH-terminal tripeptide is cleaved, the α-carboxyl group of the cysteine is methylated, and a lipid is added to the cysteine. The methylation and cleavage of the C terminus of G-γ demonstrated here is the first direct evidence that G-γ is also modified in a manner similar to ras proteins. Modifications of the ras proteins at the COOH terminus are required for membrane attachment and function (14). The similarities in the modifications of G-γ with those of the ras proteins suggest that these reactions may also anchor G-γ to the plasma membrane, and further characterization of the G-γ modifications will be required in order to define their function.

Acknowledgments—We would like to thank Mary P. Padgett for determination of the peptide amino acid sequences and Dr. Cecille Unson for the peptide synthesis.

Note Added in Proof—Subsequent to submission of this paper, Fung et al. (Fung, B. K.-K., Yamane, H. K., Ota, I. M., and Clarke, S. (1990) FEBS Lett. 260, 313–317) have reported the carboxy methylylation of a cysteine residue of the G-protein γ subunit when the purified αγ complex was reconstituted with detergent-stripped brain membranes.

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