We have purified to homogeneity a 23-kDa protein from bovine brain membranes using \( \text{[^{35}S]guanosine 5’-O-(3-thiotriphosphate)} \) (GTP\( \gamma \)S) binding as an assay. GTP\( \gamma \)S binding to the purified protein is inhibited by GDP, GTP, and GTP analogs but not by cGMP, GMP, or adenine nucleotides, consistent with the nucleotide-binding behavior of members of the family of GTP-binding regulatory proteins. On addition of the methyl donor S-adenosyl-L-methionine and a methyltransferase present in bovine brain membranes, the purified 23-kDa G-protein is carboxyl methylated. When subjected to limited tryptic proteinolysis, the 23-kDa protein is converted to a 22-kDa major fragment with concomitant release of a carboxyl methylated protein fragment of 1 kDa. Furthermore, when the cleaved protein is reconstituted with stripped bovine brain membranes, the small carboxyl-methylated fragment but not the 22-kDa major fragment is found to reassociate with the membranes. These results indicate that the site of carboxyl methylation and the region responsible for membrane anchoring, most likely, are localized to a small region at the carboxyl terminus. It is attractive to speculate that carboxyl methylation and membrane anchoring are interrelated processes and play key roles in the function of this small G-protein.

Recently, several laboratories have identified, by purification to homogeneity or molecular cloning approaches, small molecular weight GTP-binding proteins with homology to the \( \alpha \) subunits of the heterotrimeric regulatory G-proteins\(^1\) (Waldo et al., 1987; Touchot et al., 1987; Kikuchi et al., 1988; Matsui et al., 1988; Quilliam et al., 1988; Nagata and Nozawa, 1988; Bokoch et al., 1988). Although similar in molecular weight to the G-protein-related ras oncogene products, these small G-proteins comprise a homologous family distinct from that of the ras proteins. In addition to regions of homology at the GTP-binding site, the deduced cDNA sequences of a subgroup of these proteins predict a common cysteine residue at the carboxyl terminus (Touchot et al., 1987; Matsui et al., 1988). Although the functions of most members of this group of GTP-binding proteins remain unknown, two small molecular weight GTP-binding proteins from the yeast Saccharomyces cerevisiae, YPT1 and SEC4, have been implicated in the secretory process (Gallwitz et al., 1983; Salminen and Novick, 1987).

Clarke et al. (1988) have recently proposed that ras oncogene products, deduced to possess invariant -Cys-X-X-X carboxyl-terminal sequences (Barbacid, 1986), are post-translationally modified such that 3 residues from the carboxyl terminus are removed to yield a terminal cysteine, which they suggest may be carboxyl methylated. They have demonstrated that a fibroblast cell line transformed by the Ha-ras oncogene and grown in the presence of the methyl donor \( [\text{H}] \)AdoMet contains a carboxyl-methylated 21-kDa protein precipitable by the anti-ras monoclonal antibody Y13-259. Additional support for this hypothesis is provided by yeast (Anderegg et al., 1988), and fungal (Sakagami et al., 1981; Ishibashi et al., 1984) mating factor peptides, which have been shown to be carboxyl methylated at terminal cysteine residues. In the case of the yeast S. cerevisiae, methylation of the terminal cysteine residue of the mating a-factor peptide has been shown to be required for activity (Anderegg et al., 1988).

In addition to functioning as a probable site of carboxyl methylation, the invariant cysteine nearest the carboxyl terminus has been implicated as a site of lipidation in the ras proteins (Willumsen et al., 1984; Chen et al., 1985; Hancock et al., 1989). Substitution of this cysteine by site-directed mutagenesis is associated with the absence of acylation, failure to bind membranes, and a loss of transforming activity (Willumsen et al., 1984), whereas the restoration of membrane localization by acylation at the amino terminus of the protein has been shown to restore transforming activity (Buss et al., 1989). Moreover, the carboxyl-methylated terminal cysteine residues of the yeast (Anderegg et al., 1988) and fungal (Sakagami et al., 1981; Ishibashi et al., 1984) mating factor peptides have been demonstrated to possess sulphydryl-linked farnesyl moieties, and absence of this lipophilic modification is correlated with a decrease in activity (Anderegg et al., 1988).

The existence of a class of ras-like GTP-binding proteins with terminal cysteine residues suggests that these proteins may also be carboxyl methylated but without the proteolytic processing that would be required of ras proteins. Since those members of this class of proteins which have been purified have all been extracted from membranes, it is also likely that these proteins are lipidated on this terminal cysteine residue.

We report here the purification of a 23-kDa protein from bovine brain membranes using modifications of conventional methods for the purification of multisubunit regulatory G-proteins and demonstrate that the purified 23-kDa protein possesses guanine nucleotide-binding properties characteris-
tic of G-proteins. When reconstituted with [3H]AdoMet and a methyltransferase present in bovine brain membranes, the purified 23-kDa G-protein is methylated in a manner consistent with carboxyl-terminal methyl esterification. We demonstrate that limited tryptic digestion of the 23-kDa G-protein yields a 22-kDa major fragment and a small peptide that carries the carboxyl methyl ester and that the small peptide, and not the larger 22-kDa fragment, contains the domain responsible for membrane localization of this small G-protein.

EXPERIMENTAL PROCEDURES

Materials and Assays—Bovine brains were obtained from a local slaughterhouse. Protein concentrations were determined by the method of Bradford (1976) using γ-globulin from Bio-Rad as a standard. SDS-polyacrylamide gel electrophoresis (15%, 0.75-mm thickness gels) was performed according to Laemmli (1970). TPCP-trypsin was purchased from Worthington, and soybean trypsin inhibition was from Bio-Rad Chemicals. Cholic acid was purchased from Sigma and twice recrystallized prior to use. CHAPS was purchased from Pierce. [3H]AdoMet and [35S]GTPyS were purchased from Du Pont-New England Nuclear. Monoclonal antibodies Y13-259 against v-Ha-ras was a gift of Dr. Hsiang-Fu Kung (National Cancer Institute, NIH).

Buffered solutions utilized were: TED: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT; HA (hydroxyapatite) buffer: 20 mM Tri-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 3 mM MgCl₂, 0.6% CHAPS; Mono-Q buffer: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 0.6% CHAPS; GTP-γS-binding buffer: 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol PX, 25 mM MgCl₂, 200 mM NaCl; methylation buffer: 50 mM HEPES, pH 7.0, 100 mM NaCl, 5 mM MgCl₂, 0.1% Lubrol PX, 1 mM EDTA.

GTP-γS Binding and Competition—GTP-γS binding was determined by a modification of the method of Northup et al. (1982). Samples of column fractions or purified 23-kDa G-protein diluted to 20 μl were added to 2 μl of [35S]GTP-γS (~5000 cpm/pm mole in GTP-γS binding buffer) followed by incubation at 30°C for 2 h. Following incubation, samples were quenched by the addition of 3 ml of ice-cold binding buffer without Lubrol PX or DTT and rapidly filtered through Millipore type HA 0.45-μm pore membranes followed by washing with three additional 3-ml aliquots of the same buffer. Membranes were dried, placed in scintillation vials with liquisoil, and counted for 35S.

Competition of [35S]GTP-γS binding to the purified 23-kDa protein was performed as described above, with the inclusion of competing nucleotide at a final concentration of 100 μM.

Preparation of Stripped Brain Membranes—Bovine brain membranes were prepared exactly as described by Sternweis and Robishaw (1984). Washed membranes were extracted in 2 liters of TED, 1% sodium cholate also as described (Sternweis and Robishaw, 1984). Following centrifugation at 50,000 × g for 1 h, the pellets were washed with TED + 100 mM NaCl and resuspended in the same buffer. These stripped brain membranes were kept on ice and used as a source of methyltransferase in methylation the 23-kDa G-protein.

Methylation of 23-kDa G-protein—Methylation of the purified 23-kDa G-protein, as well as fractions from the various purification steps, was performed by a modified procedure of Swanson and Applebury (1983). Briefly, purified 23-kDa protein or column fractions in a total volume of 15 μl were mixed with 30 μl of [3H]AdoMet (0.5 μM, 79.6 Ci/mmol), 2% Triton X-100, and 10 μl of 1.5% CHAPS stripped brain membranes (25 μg of total protein). The mixture was allowed to incubate for 3 h at 37°C and the reaction terminated by the addition of 15 μl of 4× electrophoresis sample buffer (Laemmli, 1970). 25 μl of sample was run on an SDS-polyacrylamide gel followed by staining in Coomassie Blue. Radioactivity was determined either by excision of appropriate gel regions followed by digestion in 20% hydrogen peroxide at 65°C overnight and liquid scintillation counting, or by autoradiography using Kodak XAR-5 film following stabilization in the fluorographic reagent Amplify (Amersham Corp.).

Carboxyl methylation and membrane binding of 23-kDa G-protein, which readily hydrolyses the less stable d-aspartate β-methyl esters and L-isooaspartate α-methyl esters and leaves the more stable L-glutamate γ-methyl esters and carboxyl-terminal methyl esters intact (Clark, 1985). These stable methyl esters were distinguished from base-stable classes of stable methyl esters by the vapor-phase equilibration assay described by Clark et al. (1988).

Extraction of Methylation products—Methylated 23-kDa G-protein was extracted from the stripped bovine brain membranes for biochemical characterization. Routinely, 100 μg of purified 23-kDa G-protein was added to 500 μg (total protein) of stripped bovine brain membranes and [3H]AdoMet in a total volume of 1.0 ml. Following methylation as described above, membranes were pelleted by centrifugation at 25,000 × g for 15 min. Analysis of the supernatant routinely showed that greater than 90% of the 23-kDa G-protein remained with the membrane component. Membranes were washed twice in TED + 100 mM NaCl to remove free [3H]AdoMet. Following washing, pellets were reconstituted in TED + 100 mM NaCl, and an equal volume of 3% CHAPS in TED + 100 mM NaCl was added. After 15 min of incubation at room temperature, membranes were removed by centrifugation in a Beckman Airfuge for 3 min at 22 p.s.i. The supernatant, containing "H-labeled 23-kDa G-protein, was removed and used immediately.

Membrane Association of 23-kDa G-protein—The ability of the 23-kDa G-protein or its proteolytic fragments to reassociate with membranes was determined by reconstitution with stripped bovine brain membranes at a final detergent concentration no greater than 0.06% CHAPS, followed by incubation at 37°C for 15 min. In a typical experiment, 20 μg (total protein) of stripped bovine brain membranes was incubated with 2.5 μg of purified 23-kDa G-protein in TED + 100 mM NaCl in a total volume of 20 μl. Membranes were pelleted by centrifugation in a Beckman Airfuge at 22 p.s.i. for 3 min, and the supernatant was removed for analysis by SDS-PAGE. Pellets were dissolved directly in SDS-PAGE sample buffer and applied to SDS-polyacrylamide gels.

In assaying for binding of the proteolytic fragments to membranes, gels could not be stained as the small peptides were easily eluted from the gel. Consequently, samples were mixed with prestained molecular weight standards prior to SDS-PAGE to allow estimates of the locations of the bands of interest and to define the boundaries of each lane. Immediately following electrophoresis, gel lanes were cut into 1-mm slices and each slice was digested in 20% hydrogen peroxide at 65°C followed by liquid scintillation counting.

RESULTS

Purification of 23-kDa G-protein—Bovine brain membranes were prepared as described by Sternweis and Robishaw (1984). The washed membranes were extracted in a buffer of TED containing 1% sodium cholate, and the sodium cholate extract was chromatographed on DEAE-Sephacel as described previously for the purification of heterotrimeric G-proteins (Sternweis and Robishaw, 1984). A GTP-γS-binding profile of fractions eluted from the DEAE-Sephacel column and an SDS-polyacrylamide gel of selected fractions are shown in Fig. 1. The GTP-γS-binding profile reveals two partially resolved peaks of binding activity: the first correlating with the presence of the larger heterotrimeric G-proteins, and the second with a protein band of 23 kDa. Fractions containing the 23-kDa band were pooled, concentrated by ultrafiltration using an Amicon YM-30 membrane, and applied to a 1.8-liter column of Bio-Gel P-200 amidated with 20 mM TPCP-trypsin buffer containing 1% cholate + 100 mM NaCl. This purification step differed from the analogous step in the purification of the heterotrimeric G-proteins (Sternweis and Robishaw, 1984) and afforded higher resolution in the molecular mass range.
Carboxyl Methylation and Membrane Binding of 23-kDa G-protein

**FIG. 1.** DEAE-Sephacel purification of the 23-kDa G-protein. Bovine brain membranes were prepared and extracted with a buffer of TED, 1% sodium cholate as described under "Results." The extract was applied to a 1-liter column of DEAE-Sephacel and eluted with a 2-liter gradient of 0-225 mM NaCl in TED containing 1% sodium cholate followed by further elution with 1 liter of 500 mM NaCl in TED containing 1% sodium cholate. *Upper panel,* GTPγS-binding activity profile, determined as described under "Experimental Procedures." *Lower panel,* SDS-polyacrylamide gel of fractions indicated. Fractions 210-260, containing the band at 23 kDa (solid arrow), were pooled and further purified. The open arrow indicates the position of a subunit of larger heterotrimeric G-proteins.

Fractions from the second Bio-Gel P-200 peak were diluted 1:4 in TED and loaded on a 2.6 × 10-cm Bio-Gel hydroxylapatite column equilibrated with HA buffer. After loading, the column was washed with HA buffer and the proteins eluted with a step of 10 mM NaH₂PO₄ in HA buffer. Fractions containing GTPγS-binding activity from the 10 mM NaH₂PO₄ elution step were pooled, concentrated, and loaded on a Mono-Q HR5/5 column equilibrated with Mono-Q buffer. Elution was performed with a linear gradient of 0-300 mM NaCl in Mono-Q buffer over 30 min at a flow rate of 0.5 ml/min. Fractions were assayed for both GTPγS-binding activity and methylation (Fig. 2, panel B). Two peaks of GTPγS binding were again observed, both correlating with the appearance of a major protein band of 23 kDa on a Coomassie Blue-stained SDS-polyacrylamide gel of 10 μl of each fraction methylated with [3H]AdoMet as described under "Experimental Procedures."

**FIG. 2.** Further steps in the purification of the 23-kDa G-protein. *A,* Bio-gel P-200 fraction profile showing [35S]GTPγS-binding activity, determined as described under "Experimental Procedures." *B,* Mono-Q HR5/5 fraction profile showing [35S]GTPγS-binding activity (○) and methylation (●) as determined by excision of the 21-31-kDa region of SDS-polyacrylamide gel of 10 μl of each fraction methylated with [3H]AdoMet as described under "Experimental Procedures."

**FIG. 3.** Methylation of the purified 23-kDa G-protein by stripped bovine brain membranes. Purified 23-kDa G-protein was methylated as described under "Experimental Procedures," using 5 μg of 23-kDa G-protein and 25 μg (total protein) of stripped bovine brain membranes where indicated, and substituted with the appropriate buffer where omitted. *A,* Coomassie Blue-stained SDS-polyacrylamide gel. *B, lane 1,* 23-kDa G-protein only; *lane 2,* brain membranes only; *lane 3,* 23-kDa G-protein + brain membranes; *lane 4,* 23-kDa G-protein + brain membranes denatured by incubation at 100 °C for 15 min; *lane 5,* 23-kDa G-protein + brain membranes denatured by incubation at 100 °C for 15 min + brain membranes. *B,* corresponding autoradiogram following incubation of gel in panel A in the fluorographic image enhancer Amplify.
SDS-polyacrylamide gel (Fig. 3A, lane 1). Both peaks of GTP\(\gamma\)S-binding activity were also coincident with methylation. The levels of GTP\(\gamma\)S binding and methylation were indistinguishable between the two peaks. Fractions comprising each peak were pooled, concentrated, frozen in liquid nitrogen, and stored at \(-20^\circ C\). Since the second peak appeared to contain minor impurities, whereas the first peak contained no other polypeptides detectable on a Coomassie Blue-stained SDS-polyacrylamide gel, all subsequent experiments described were performed using the pooled fractions from the first GTP\(\gamma\)S-binding peak.

**Nucleotide-binding Specificity of the 23-kDa Protein**—Table I illustrates the specificity of binding guanosine di- and tri-nucleotides and GTP analogs to the purified 23-kDa protein. As with the class of heterotrimeric G-proteins and other small molecular weight G-proteins, the binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S} is competitively inhibited by GDP, GTP, Gpp(NH)p, and GTP\(\gamma\)S, whereas binding is not inhibited by cGMP, GMP, and adenosine nucleotides. Photoaffinity labeling using \[^{\alpha-32}\text{P}]\text{GTP}\gamma\text{S} showed that a band at 23 kDa is labeled and that labeling can be specifically inhibited by co-incubation with GDP but not ATP (data not shown). These results suggest that the purified protein is a member of a class of small molecular weight GTP-binding proteins with homology to the \(\alpha\) subunit of larger G-proteins. Immunocytochemical studies using a panel of polyclonal and monoclonal antibodies showed that the 23-kDa G-protein does not cross-react with the anti-ras antibodies Y13-293, 142-24E5, or 147-67C6.

**Carboxyl Methylation of 23-kDa G-protein**—When the purified 23-kDa G-protein was reconstituted with stripped bovine brain membranes and \[^{3}\text{H}]\text{AdoMet}, incubated at 37 °C, and subjected to SDS-PAGE and autoradiography, radioactivity was observed to incorporate in a band at 23 kDa (Fig. 3, lanes 3). Determination of \(^{\text{3}}\text{H}\)-methyl incorporation into the 23-kDa G-protein by excision of the appropriate gel band, solubilization in 20% \(\text{H}_2\text{O}_2\) overnight at 65 °C, and liquid scintillation counting revealed 0.1 mol of methyl group incorporated/mol of 23-kDa G-protein. When radioactivity incorporated in the 23-kDa G-protein was subjected to the vapor-phase equilibration assay described under "Experimental Procedures," greater than 85% of the total counts were observed to be base labile, demonstrating that the 23-kDa G-protein is carboxyl methylated. Incorporation of radioactivity in the 23-kDa band was not observed in the absence of brain membranes (lanes 1) or in stripped bovine brain membranes alone (lanes 2). Methylation was greater than 90% inhibited in the presence of the AdoMet metabolite, S-adenosylhomocysteine,

**TABLE I**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>% Maximum binding</th>
</tr>
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<tbody>
<tr>
<td>cGMP</td>
<td>100</td>
</tr>
<tr>
<td>GMP</td>
<td>100</td>
</tr>
<tr>
<td>GDP</td>
<td>10.4</td>
</tr>
<tr>
<td>GTP</td>
<td>7.1</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>29.4</td>
</tr>
<tr>
<td>GTP(\gamma)S</td>
<td>4.7</td>
</tr>
<tr>
<td>cAMP</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
</tbody>
</table>

suggesting that incorporation of radioactivity in the 23-kDa band is indeed a competitive enzymatic process (lanes 4). Similarly, when brain membranes or purified 23-kDa protein was denatured by heating at 100 °C, incorporation of \[^{3}\text{H}]\text{AdoMet} was essentially blocked (lanes 5 and 6). These results demonstrate that a methyltransferase present in stripped bovine brain membranes is capable of methylating the purified 23-kDa G-protein. Conversely, brain cytosol obtained during preparation of the bovine brain membranes showed no methyltransferase activity, indicating that the 23-kDa G-protein is not carboxyl methylated by soluble methyltransferases. Further experiments showed that bovine retinal rod outer segment membranes, but not cytosol, contain a methyltransferase capable of carboxyl methylating the purified 23-kDa G-protein (data not shown).

Attempts to extract the 23-kDa G-protein from the stripped brain membranes both before and after methylation by washing with low or high ionic strength buffers failed to remove the reconstituted protein, suggesting that the small G-protein binds to the membranes through a highly lipophilic domain. Consequently, extraction with the detergents sodium cholate or CHAPS was successful. The use of brain membranes previously stripped by 1% sodium cholate as a source of methyltransferase allowed the extraction of methylated 23-kDa G-protein with few contaminating proteins.

**Localization of the Membrane-binding Domain to a Carboxyl-methylated Tryptic Peptide**—The purified 23-kDa G-protein was readily cleaved by trypsin to yield a major fragment of 22 kDa (Fig. 4, panel A) and a smaller fragment (or fragments) of less than 1 kDa which were too small to be observed by SDS-PAGE. When the 23-kDa G-protein was methylated, extracted by 1.5% CHAPS, and subjected to limited tryptic digestion, radioactivity in the 23-kDa protein...
was observed to decrease, whereas radioactivity appearing at the dye front concomitantly increased (Fig. 4, panel B). This result strongly suggests that a small fragment, most likely derived from the carboxyl terminus, is removed by tryptic digestion.

Upon reconstitution with stripped bovine brain membranes, the purified 23-kDa G-protein was observed to reassociate quantitatively with the lipid matrix (Fig. 5, panel C). When the purified small G-protein was subjected to limited trypsin proteolysis to yield 22- and 1-kDa fragments prior to reconstitution, the 22-kDa major fragment was observed in the supernatant and not in the membranes (panel D). Protein bands in the 22-23-kDa range were not observed in the supernatants of control preparations of stripped bovine brain membranes alone (panel A) or in the presence of trypsin quenched by soybean trypsin inhibitor prior to reconstitution (panel B), indicating that the 22-kDa band is not derived from the cleavage of membrane proteins.

The observation that the 22-kDa major tryptic fragment does not reassociate with membranes suggested that the 1-kDa carboxyl-methylated fragment contains the membrane-binding domain. To test this hypothesis, purified 23-kDa G-protein was methylated by stripped bovine brain membranes, extracted, and converted to its 22-kDa and 1-kDa fragments by tryptic digestion. The digested mixture and a control undigested preparation were reconstituted with stripped brain membranes and the membranes pelleted by centrifugation. Radioactivity profiles of the membrane pellets separated by SDS-PAGE are shown in Fig. 6. The undigested preparation shows membrane-bound radioactivity in the position corresponding to ~23 kDa, whereas the trypsin-treated sample shows membrane-bound radioactivity appearing at the dye front. These results show that the site of carboxyl methylation and the domain responsible for membrane anchoring reside within approximately 10 amino acid residues of each other and possibly on a single residue.

![Figure 5](image)

**FIG. 5.** Membrane binding of native and cleaved 23-kDa G-protein. Stripped bovine membranes (20 µg of total protein) were incubated with buffer (panel A), trypsin prequenched by soybean trypsin inhibitor (panel B), purified 23-kDa G-protein (2.5 µg) + soybean trypsin inhibitor (panel C), and purified 23-kDa G-protein (2.5 µg) subjected to limited trypsin digestion as described under "Experimental Procedures" (panel D). Membranes were pelleted by centrifugation in a Beckman Airfuge for 3 min at 22 p.s.i., and the membrane pellets (M) and supernatants (S) were run on separate lanes of an SDS-polyacrylamide gel. The positions of the native (23 kDa) and major trypptic fragment (22 kDa) are indicated by arrows. TI indicates the position of soybean trypsin inhibitor used in quenching the proteolytic activity of trypsin.

![Figure 6](image)

**FIG. 6.** Mapping of membrane-binding domain to carboxyl-methylated terminal region. Purified 23-kDa G-protein was methylated and extracted with 1.5% CHAPS in TED + 100 mM NaCl as described under "Experimental Procedures." 10 µl of extract was digested with TPK-trypsin for 5 min at room temperature, and the reaction was terminated by the addition of soybean trypsin inhibitor. A control mixture without TPK-trypsin was also prepared. The digested and control extracts were added to 25 µg of stripped brain membranes and incubated for 15 min at 37 °C. Following incubation, both samples were centrifuged in a Beckman Airfuge for 3 min. Pellets were then resuspended and subjected to SDS-PAGE. Values on the abscissa represent radioactivity measured by cutting each lane into 1-mm wide slices, digestion of each slice in 20% hydrogen peroxide, and liquid scintillation counting for control (C) and trypsin-treated (T) samples.

**DISCUSSION**

We demonstrate here that a small molecular weight G-protein purified from bovine brain membranes and reconstituted with a methyltransferase from the same tissue is carboxyl methylated. The availability of a purified substrate enabled the identification of the protein as a member of the family of GTP-binding proteins. Although our calculations indicate a typical incorporation of 0.1 mol of methyl group/mol of 23-kDa G-protein, this relatively low value most likely reflects a slow turnover of the methyl ester and possible inefficiencies associated with the reconstituted system. Our demonstration of carboxyl methylation in a small molecular weight G-protein is consistent with the hypothesis of Clarke et al. (1988), which postulates that carboxyl methylation of ras proteins occurs at a terminal cysteine residue following proteolytic processing of three carboxyl-terminal residues, and the recent discovery of a class of small molecular weight membrane-bound GTP-binding proteins with common carboxyl-terminal cysteine residues. Although the stability of the methyl ester at the alkaline pH of our SDS-PAGE system does not exclude the possibility of an L-glutamate γ-methyl ester, the fact that no L-glutamate γ-methyl esters have ever been reported in eukaryotic cells supports our conclusion of a carboxyl-terminal α-methyl ester. In further support of this hypothesis, Ota and Clarke (1989) have recently demonstrated that a 23-kDa protein in bovine retinal rod outer segment membranes is carboxyl methylated at a terminal cysteine residue.

The availability of a purified small G-protein substrate and a methyltransferase source devoid of other substrates also enabled us to demonstrate that the membrane-binding domain of the 23-kDa G-protein can be localized to a 1-kDa tryptic fragment containing the carboxyl methyl esters. This result is consistent with similar modifications of ras proteins, which have been demonstrated to be lipidated at an invariant cysteine residue at the carboxyl terminus (Willumsen et al., 1984; Chen et al., 1985; Hancock et al., 1989). Moreover, yeast (Anderegg et al., 1988) and fungal (Sakagami et al., 1981;
Ishibashi et al., 1984) peptidyl mating factors have been shown to possess both a methyl ester and a sulfhydryl-linked farnesyl moiety at a carboxyl-terminal cysteine. By analogy, it is likely that the 23-kDa G-protein also carries a lipid functionality at a carboxyl-terminal cysteine, which allows the small G-protein to bind to membranes. It is attractive to speculate that the close association of the site of methylation and a site of possible lipidation defines a role for carboxyl methylation in the regulation of membrane binding.

Backlund and Aksamit (1988) have recently reported the GTP-dependent methylation of a 20–23-kDa membrane protein in RAW264 cells. Although we do not observe a GTP effect on methylation in our system, this may be the result of having removed receptors and/or effectors during the purification of the 23-kDa protein and consequently lacking the total machinery required to observe a GTP dependence. Additionally, there is evidence that the methylation observed by Backlund and Aksamit and the methylation of our 23-kDa G-protein may represent distinct processes. In particular, the 23-kDa G-protein is a substrate only for membrane-bound methyltransferases from bovine brain and retina. This possibility that each member of this class of small molecular weight G-proteins may be methylated by a distinct methyltransferase according to its specific function is an attractive one and remains to be seen.

The evidence that carboxyl methylation and lipidation may be closely linked to the binding of guanine nucleotides suggests that this class of proteins may fulfill a regulatory role involving membrane compartments. As in the case of the heterotrimeric regulatory G-proteins, the binding of GTP would initiate the activation of such a process, whereas the intrinsic GTPase activity would signal the completion of the task and reset the system. Such a theme is already well characterized in the elongation factor-mediated translocation of the appropriate aminoacyl tRNA to an mRNA-ribosome complex in the process of protein synthesis. In this process, a molecule of elongation factor Tu "charged" with a GTP molecule binds an aminoacyl tRNA in the cytosol. Upon successful delivery of the appropriate aminoacyl tRNA to the ribosome, elongation factor Tu hydrolyzes its bound GTP to GDP and inorganic phosphate, signaling its release from the protein synthesis machinery. By analogy and based on our observation that the substrate and methyltransferase are localized in membranes, GTP might serve as a signal to deliver the small G-proteins to their destined membrane targets at which carboxyl methylation would subsequently act as a signal for lipidation or alternatively for triggering other processes.

Whether carboxyl methylation in this class of small molecular weight GTP-binding proteins serves as a regulatory signal for membrane localization and whether the intrinsic GTPase activity plays a role in this process similar to that in the case of elongation factor Tu remains to be seen; however, the evidence presented herein for carboxyl methylation and for its relationship to membrane binding in these proteins provides the strongest insight thus far as to their possible function.

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