Proteomic Analysis of Bovine Brain G Protein γ Subunit Processing Heterogeneity*

Lana A. Cook, Kevin L. Schey, Michael D. Wilcox, Jane Dingus, Rebecca Ettling, Troy Nelson, Daniel R. Knapp, and John D. Hildebrandt‡

We characterized the variable processing of the G protein γ subunit isoforms associated with bovine brain G proteins, a primary mediator of cellular communication. Gγ subunits were isolated from purified brain G proteins and characterized by Edman sequencing, by MALDI MS, by chemical and/or enzymatic fragmentation assayed by MALDI MS, and by MS/MS fragmentation and sequencing. Multiple forms of six different Gγ isoforms were detected. Significant variation in processing was found at both the amino termini and particularly the carboxyl termini of the proteins. All Gγ isoforms contain a carboxyl-terminal CAAX motif for prenylation, carboxyl-terminal proteolysis, and carboxymethylation. Characterization of these proteins indicates significant variability in the normal processing of all of these steps in the prenylation reaction, including a new variation of prenyl processing resulting from cysteinylatation of the carboxyl terminus. These results have multiple implications for intracellular signaling mechanisms by G proteins, for the role of prenyl processing variation in cell signaling, and for the site of action and consequences of drugs that target the prenylation modification. Molecular & Cellular Proteomics 5:671–685, 2006.

With access to the sequences of entire genomes, increasing attention is being focused on understanding the proteome, or protein complements of genomes (1–4). Substantial progress has been made on identifying proteins at a very large scale, on defining protein expression patterns in normal and disease states, and on defining protein-protein interactions on a near genome-size scale (5). The characterization of proteomes, however, is an even more demanding problem than sequencing entire genomes due to part in the greater chemical and combinatorial diversity of proteins. Approaches for carrying on such studies are still being developed as are both the information and ideas required to interpret the data generated. An important component of the complement of proteins in cells is their variability in processing after synthesis. This variability affects the volume of data likely to be acquired from the general analysis of cellular proteins as well as the potential functional significance of that data. One approach to understanding the significance of protein processing is to characterize cell-wide, or even genomewide, prevalence of specific processing events (1–4). A complementary approach, however, would be to focus on understanding the range and role of processing diversity in the function of specific proteins or protein families. In fact, the range and significance of such processing variability is incompletely understood.

G proteins are heterotrimeric GTP-binding proteins involved in signal transduction from receptors to intracellular effectors (6–10). They consist of a guanine nucleotide-binding α subunit that reversibly associates with a βγ dimer (11). Together these two components regulate numerous downstream effectors such as adenylyl cyclase, ion channels, and phospholipase C (6–8, 11–13). G proteins are a component of a ubiquitous cellular signaling system. More than 600 of the 25–30,000 genes found in the human genome code for G protein-coupled receptors (14, 15). These receptors are estimated to be the targets of 50% of clinically useful drugs (16) and an equally impressive array of endogenous regulatory compounds (17). The functional implication of these statistics is that the G proteins in cells process an immense amount of incoming information. Not surprisingly, the G proteins themselves are also structurally diverse. Over 20 α subunits (the products of 16 genes), five β subunits, and 12 γ subunits have been identified (9, 18). All of the known γ subunits of these proteins are substrates for prenylation, which appears to be essential for the normal function of the heterotrimeric G proteins.

About 2% of cellular proteins end in a CAAX motif that is a signal for prenylation (19–21). These include a broad range of proteins, many of which are involved in cell signaling and growth regulation, for example the Ras proto-oncogene products and the γ subunits of the heterotrimeric G proteins (20, 22–24). Prenylation is a complex modification that involves attachment of a geranylgeranyl or a farnesyl group to a Cys residue 4 amino acids from the carboxyl terminus of target proteins (19, 23). Which prenyl group is attached is determined by the carboxyl-terminal amino acid. Geranylgeranyl is added if this residue is a Leu or Phe, whereas Farnesyl is added if it is a Ser, Met, Gln, Cys, or Ala (19, 25). Following prenylation, the three carboxyl-terminal amino acids are re-
moved by a specific protease, and the protein is carboxymethylated. The fidelity and variability of these multiple enzymatic steps is not clear, particularly for native proteins, and may play a role in both the normal biochemistry of these proteins and the pathological consequences of their aberrant processing. These modifications and the enzymes involved are potential targets for drug development. For example, farnesyltransferase and geranylgeranyltransferase I inhibitors have potential as antitumor agents and are being used in clinical trials (26–28), but the exact mechanism of action of these inhibitors remains unclear. This is in part due to uncertainty about their real targets (29, 30) but also due to lack of information about the processes with which they interfere. In addition, the prenylation reaction turns out to be a heretofore unsuspected target of other antitumor agents, such as the statins that interfere with generation of the prenyl moiety (30) and methotrexate that interferes with the carboxymethylation reaction (31). What is lacking, in particular, is a clear understanding of the normal biology of this series of modifications and how variation in each step might affect function of the target proteins.

Brain cortex is a rich source of a diverse population of G proteins, which are regulated by many different signaling molecules. Past biochemical and molecular biology studies provide evidence for at least six G protein γ subunits in brain (32–41). When purified brain G proteins were analyzed by MALDI-TOF mass spectrometry, seven prominent signals in the Gγ subunit molecular weight range were found (32). Four of these molecular weights could be assigned to Gγ subunits known to be in bovine brain, Gγ2 (41), Gγ3 (38), Gγ5 (33), and Gγ7 (40), provided specific modifications of those Gγ subunits were proposed. Previously we described the characterization in these samples Gγ2 (42), Gγ10 (43), Gγ5 (44), a novel Gγ5 that is not proteolytically processed after prenyl terminus (44), and a Gγ2 subunit truncated and arginylated at the amino terminus as a signal for ubiquitylation (45). Throughout the study of all of these proteins, however, it was clear that the number of potential G protein γ subunits associated with brain Gγ/G proteins, due to variable processing, is much larger than originally appreciated.

Here we characterize the variability and processing of G protein γ subunits associated with G proteins purified from bovine brain. Significant variation in the carboxyl-terminal processing was found, including probable cysteinylation as a newly identified carboxyl-terminal modification of the prenylation site. In light of past studies characterizing the functional role for a number of these modifications, these results suggest that carboxyl-terminal variability could affect G protein signaling in a number of ways, including receptor recognition and fidelity, cellular localization, and even turnover of G protein functional components. These results also indicate the potential site of action of drugs that interfere with selective reactions in the prenylation process.

EXPERIMENTAL PROCEDURES

**Purification of Bovine Brain G Protein Heterotrimers—**G proteins were purified from bovine brain cortex as described previously (46, 47). We generally obtained 30–60 mg of a mixture of Gα and Gβ isoforms. GTP·yS binding assays, protein quantitation, and Coomassie Blue staining polyacrylamide gels indicated that Gα and Gβ (and presumably Gγ2 subunits were purified in roughly stoichiometric amounts. Protein was stored at −80 °C until use in these studies in a buffer containing 25 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.1% Triton X-100. Gα was stored at −20 °C until further analysis. For separation of Asp-N-digested γ subunits, aliquots from the general HPLC separation (48) were dried under vacuum and resuspended in 5 μl of Solvent A (0.1% trifluoroacetic acid in 100% acetonitrile) over 140 min with a final hold at 75% B for 40 min. Eluted peptides were monitored at 210 nm. Peptides were either eluted onto PVDF membrane for Edman sequencing or hand-collected and stored at −20 °C for MS analysis.

**MALDI Mass Spectrometry—**MALDI-TOF analysis was performed on a PerSeptive Biosystems Voyager-DE mass spectrometer as described previously (48). Generally the matrix used was α-cyano-4-hydroxycinnamic acid, although sometimes results were verified using the alternative matrix sinapinic acid. Approximately 75–150 scans were averaged for a single representative MALDI spectrum. Spectra were analyzed under conditions that favored evaluation of the singly charged mass of each protein. Masses reported from MALDI MS were determined from spectra containing internal calibrants of known mass bracketing the signals being evaluated. Calibrants normally used and their associated [M + H]+ were horse cytochrome c (12,361), bovine insulin (5,735), and a synthetic Gγ2 peptide (1,701) with the sequence PASENPFREKKFFC. Average mass estimates come from multiple G protein preparations independently separated by HPLC and analyzed at different times with independent calibration of the mass spectrometer.

**Aspartate-Proline Bond Cleavage and MALDI Analysis—**HPLC fraction aliquots (50–100 μl) containing Gγ subunits were dried under vacuum, resuspended in 1:1 acetonitrile:water and 1–3% TFA. Samples were incubated at room temperature for at least 12 h and analyzed by MALDI mass spectrometry as described previously (48).

**Protein Sequencing—**Edman sequencing of 50–μl aliquots of HPLC fractions was performed in the Medical University of South Carolina Proteogenomics Facility on a Procise 494 Applied Biosystems instrument as described previously (45).

**Electrospray Ion Trap Mass Spectrometry—**ESI-MS/MS was performed either in line with HPLC on a Finnigan LCQ mass spectrometer or by direct infusion into a custom built nanospray source attached to the LCQ ion trap. Nanospray (flow, −30 nL/min) was

The abbreviations used are: GTP·yS, guanosine 5′-3-O-(thio)triphosphate; Rce, Ras-converting enzyme.
performed as described previously using a window of 1 or 2 m/z units for precursor ion selection (49).

**Statistical Analysis**—Masses detected in G protein preparations were quantified in multiple independent MALDI MS experiments using internal standards bracketing the measured mass. Measurements are reported as mean ± S.E. Typically S.E. was 1–2 Da for a 7–9,000-Da protein. A t test was used to evaluate consistency of observed masses with predicted masses compiled in a table of all possible combinations of plausible modifications of all known G protein subunits. This table contained about 2,500 total entries. Data are reported as the probability (p) that the theoretical mass is not different from the observed mass using the t distribution with n – 1 degrees of freedom and the observed S.E. Given the precision of mass spectrometry, this represents a fairly stringent test of an assignment given the large number of possible assignments. In effect, this test was used to support the assignment of a proposed structure to a mass only if the predicted and measured masses were within about 2–3 Da of 7–9,000 Da. Typically our assignments are consistent with only one possible structure of about 2,500 possible structures considered.

**RESULTS AND DISCUSSION**

**HPLC Separation of γ Subunits of Brain G Proteins**

Gγ subunits from bovine brain were separated from other components of the G protein heterotrimers by reverse-phase HPLC. The G protein preparations used in these studies were highly purified and contained protein with a specific activity of 12.4 ± 0.7 nmol of GTPγS binding activity/mg of protein (mean ± S.E. for 17 independent G protein preparations). Compared with a theoretical activity of 13.0 nmol/mg for a predicted 84,000-Da heterotrimer, this suggests greater than 95% purity of the proteins analyzed; this could be underestimated because of protein inactivity or to persistent binding of endogenous guanine nucleotides (50). These preparations are expected to contain stoichiometric amounts of Gγ subunits. HPLC fractions containing Gγ subunits were analyzed by SDS-PAGE and identified by silver staining and by immunoblotting with Gγ subunit-specific antisera (48). Fractions containing Gγ subunits were well separated in the elution profile from Gα and Gβ proteins. Fig. 1 shows MALDI analysis of the Gγ subunit mass range (6,500–9,000 Da) of fractions from a typical HPLC separation of bovine brain Gγ subunits. Fig. 1 shows spectra collected under constant ionization conditions where laser intensity was modulated to generate an ion current less than saturated for the completely processed Gγ2 (Fig. 1, Mass 25), which is consistently the Gγ isofrom with the strongest signal in these G protein preparations. Although there is not a simple relationship between MALDI signal intensity and the amount of sample because of the complex variables affecting ion generation, the abundance, distribution, and intensity of masses seen in Fig. 1 are typical of more than a dozen HPLC separations of bovine brain G proteins. Additional spectra were collected from the analysis of each fraction to provide high resolution mass estimates (data not shown but see supplemental data) based upon internal standards bracketing the signals being evaluated. Data on masses consistently seen in multiple G Protein preparations and numbered in Fig. 1 are summarized in Table I.

**Fig. 1.** Composite MALDI spectra of HPLC fractions containing G protein Gγ subunits. Twenty-five microliters of each fraction were analyzed by MALDI-TOF MS at a constant laser power and normalized to the relative signal intensity of the Gγ2 subunit (Mass 25). The numbered peaks indicate those with sufficient signal to be analyzed further that are shown in Table I.
Proteomic Analysis of Bovine Brain G Protein γ Subunit

A primary concern about the identity of the proteins corresponding to the masses found in Table I is that they could represent proteolytic fragments of either unrelated proteins or more likely the G protein subunits themselves. Several observations are inconsistent with this idea. When the distribution of masses with a signal/noise ratio greater than 2 was analyzed (48), approximately two-thirds of the signals were in the m/z range of 6,500–9,000, the mass range predicted from the DNA sequence of the known γ subunit isoforms. Significantly very few masses were smaller than m/z 6,500, arguing against the idea that many are proteolytic products of Gγ subunits. If most of these masses represent nonspecific proteolytic products of Gα or Gβ or of any protein for that matter, a fairly random distribution of masses might be expected, but most of the masses present are coincident with the mass range predicted for Gγ subunits. Finally Edman sequencing readily identified Gγ3 (Fig. 1, Mass 21), which has an unblocked amino-terminal Met residue, in appropriate HPLC fractions but, in general, failed to find appreciable amounts of protein that could be sequenced in most of our samples (see below for some significant exceptions). In a separation such as that shown in Fig. 1, samples of all fractions were sequenced, and multiple fractions yielded sequences of 15 cycles (maximum cycles processed) of Gγ2 protein (45) was detected in a single fraction at a level of about 70 pmol (Fig. 1, Mass 15). Altogether 1.4 nmol of sequenceable material was recovered from the separation; this, if all represents Gγ subunits of about 7.5 kDa, would account for about 10% of the initial Gγ protein preparation (if no correction is made for recovery). Recognizing that more than half of the Gγ3 appears to be N-acetylated on Met1 (Fig. 1, Mass 26) and allowing for recovery of protein, this would be a reasonable estimate of the abundance of Gγ3 in brain G proteins. Gγ2 and Gγ3 would be expected to be nearly equivalent in abundance in brain G protein prepara-

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.7</td>
<td>6,732.5</td>
<td>1.4</td>
<td>5</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>22.0</td>
<td>7,917.9</td>
<td>1.5</td>
<td>3</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>22.0</td>
<td>8,021.3</td>
<td>2.1</td>
<td>3</td>
<td>&lt;5,000</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>22.8</td>
<td>7,925.0</td>
<td>2.3</td>
<td>7</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>23.5</td>
<td>7,503.8</td>
<td>1.8</td>
<td>5</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>24.8</td>
<td>7,138.1</td>
<td>1.8</td>
<td>3</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>24.8</td>
<td>7,556.3</td>
<td>1.6</td>
<td>6</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>26.3</td>
<td>7,409.6</td>
<td>0.6</td>
<td>21</td>
<td>&gt;30,000</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>26.3</td>
<td>7,248.5</td>
<td>0.5</td>
<td>9</td>
<td>&lt;5,000</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
<td>26.5</td>
<td>7,736.2</td>
<td>0.7</td>
<td>7</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>27.0</td>
<td>7,169.8</td>
<td>1.6</td>
<td>5</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>27.3</td>
<td>7,684.5</td>
<td>0.9</td>
<td>5</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>28.0</td>
<td>7,840.1</td>
<td>0.8</td>
<td>7</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>29.3</td>
<td>7,501.5</td>
<td>0.5</td>
<td>5</td>
<td>15,000–30,000</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>29.3</td>
<td>7,593.7</td>
<td>1.0</td>
<td>4</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>29.3</td>
<td>7,737.0</td>
<td>0.5</td>
<td>5</td>
<td>&gt;30,000</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>29.4</td>
<td>7,880.9</td>
<td>0.9</td>
<td>5</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>30.0</td>
<td>8,280.7</td>
<td>1.3</td>
<td>6</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>30.0</td>
<td>8,425.4</td>
<td>1.4</td>
<td>7</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>30.3</td>
<td>7,324.2</td>
<td>1.3</td>
<td>6</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>33.0</td>
<td>8,295.6</td>
<td>0.4</td>
<td>8</td>
<td>5,000–15,000</td>
<td>100</td>
</tr>
<tr>
<td>22</td>
<td>33.6</td>
<td>7,481.1</td>
<td>1.4</td>
<td>22</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>23</td>
<td>33.8</td>
<td>7,903.8</td>
<td>2.2</td>
<td>13</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>34.1</td>
<td>7,938.2</td>
<td>0.5</td>
<td>16</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>34.3</td>
<td>7,751.3</td>
<td>0.3</td>
<td>26</td>
<td>&gt;30,000</td>
<td>100</td>
</tr>
<tr>
<td>26</td>
<td>35.8</td>
<td>8,337.6</td>
<td>0.7</td>
<td>18</td>
<td>15,000–30,000</td>
<td>100</td>
</tr>
<tr>
<td>27</td>
<td>35.8</td>
<td>8,068.5</td>
<td>3.0</td>
<td>3</td>
<td>&lt;5,000</td>
<td>100</td>
</tr>
<tr>
<td>28</td>
<td>40.8</td>
<td>7,194.9</td>
<td>0.7</td>
<td>11</td>
<td>15,000–30,000</td>
<td>75</td>
</tr>
<tr>
<td>29</td>
<td>41.5</td>
<td>8,339.8</td>
<td>2.0</td>
<td>14</td>
<td>&lt;5,000</td>
<td>75</td>
</tr>
<tr>
<td>30</td>
<td>42.0</td>
<td>7,752.2</td>
<td>0.5</td>
<td>17</td>
<td>15,000–30,000</td>
<td>75</td>
</tr>
</tbody>
</table>

a Mass number referring to index of supplemental information with mass spectra and other supporting evidence.

b Average HPLC retention time on a standardized HPLC separation (min).

c Average observed [M + H][+].

d S.E. of observed [M + H][+].

Number of observations (independent spectra) used to calculate the observed [M + H][+].

Approximate magnitude of the MALDI-TOF MS ion current in a typical HPLC separation calibrated against the γ2 peak (Mass 25), as in Fig. 1.

Frequency of observation in four internally calibrated runs.
### Table II

Summary of structural analysis of masses in bovine brain G protein preparations

<table>
<thead>
<tr>
<th>Mass number</th>
<th>Observed [M + H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>S.E.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Predicted structure of amino terminus&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Predicted structure of carboxyl terminus&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Predicted [M + H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>t&lt;sup&gt;h&lt;/sup&gt;</th>
<th>p&lt;sup&gt;i&lt;/sup&gt;</th>
<th>Descriptive designation&lt;sup&gt;j&lt;/sup&gt;</th>
<th>Estimated abundance (percentage of isoform)&lt;sup&gt;k&lt;/sup&gt;</th>
<th>Evidence&lt;sup&gt;l&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7,925.0</td>
<td>2.3</td>
<td>Ac-(M)S&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,928.2</td>
<td>1.397</td>
<td>0.212</td>
<td>Predicted γ&lt;sub&gt;12&lt;/sub&gt;</td>
<td>50–100&lt;sup&gt;m&lt;/sup&gt;</td>
<td>A, B, D, E</td>
</tr>
<tr>
<td>6</td>
<td>7,138.1</td>
<td>1.8</td>
<td>Ac-(M)S&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,138.2</td>
<td>0.054</td>
<td>0.962</td>
<td>Unprenylated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>1–5</td>
<td>A, B, D, G</td>
</tr>
<tr>
<td>10</td>
<td>7,736.2</td>
<td>0.7</td>
<td>Ac-(M)S&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;IL&lt;sub&gt;2&lt;/sub&gt;</td>
<td>7,736.1</td>
<td>0.109</td>
<td>0.917</td>
<td>Unproteolyzed γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>5</td>
<td>A, B, D, E</td>
</tr>
<tr>
<td>12</td>
<td>7,684.5</td>
<td>0.9</td>
<td>Ac-(M)A&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;far&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,683.0</td>
<td>1.671</td>
<td>0.170</td>
<td>Farnesylated γ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1–3</td>
<td>A, B, D, E, F</td>
</tr>
<tr>
<td>13</td>
<td>7,840.1</td>
<td>0.8</td>
<td>Ac-(M)A&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;Cys&lt;sup&gt;s&lt;/sup&gt;</td>
<td>7,840.3</td>
<td>0.166</td>
<td>0.874</td>
<td>Cysteinylated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>3–5</td>
<td>A, B, D, E, F</td>
</tr>
<tr>
<td>16</td>
<td>7,737.0</td>
<td>0.5</td>
<td>Ac-(M)A&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,737.0</td>
<td>0.176</td>
<td>0.869</td>
<td>Unmethylated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>30–50</td>
<td>A, B, D, E, F</td>
</tr>
<tr>
<td>20</td>
<td>7,324.2</td>
<td>1.3</td>
<td>(MASNNITASIA) γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,322.7</td>
<td>1.144</td>
<td>0.304</td>
<td>Truncated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>1–3</td>
<td>A, B, C, D, E, F</td>
</tr>
<tr>
<td>21</td>
<td>8,295.6</td>
<td>0.4</td>
<td>M&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>8,294.9</td>
<td>1.727</td>
<td>0.128</td>
<td>Predicted γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>10–20</td>
<td>A, B, C, D, E, F</td>
</tr>
<tr>
<td>22</td>
<td>7,481.1</td>
<td>1.4</td>
<td>Ac-(M)A&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>7,478.6</td>
<td>1.757</td>
<td>0.094</td>
<td>Unprenylated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>&lt;1</td>
<td>A, B, D, G</td>
</tr>
<tr>
<td>26</td>
<td>8,337.6</td>
<td>0.7</td>
<td>Ac-M&lt;sup&gt;s&lt;/sup&gt; γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>Cys&lt;sup&gt;s&lt;/sup&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>8,337.0</td>
<td>0.934</td>
<td>0.364</td>
<td>Acetylated γ&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>70–80</td>
<td>A, B, D, E, F</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mass number referring to peaks labeled in Fig. 1 and Table I and index of supplemental information with mass spectra and other supporting evidence.

<sup>b</sup> Average observed [M + H]<sup>+</sup>.

<sup>c</sup> S.E. of observed [M + H]<sup>+</sup>.

<sup>d</sup> Predicted structure of the amino terminus of the γ subunit. Residues in parentheses would be removed by proteolysis.

<sup>e</sup> Predicted γ subunit isoform.

<sup>f</sup> Predicted structure of the carboxyl terminus, starting with the predicted prenylated Cys<sup>s</sup>, carboxymethylation; +gg, geranylgeranylation; +far, farnesylation; +Cys, an additional Cys residue added to the carboxyl terminus in the absence of carboxymethylation.

<sup>g</sup> Mass of +1 charge state of predicted γ subunit structure.

<sup>h</sup> t statistic for the difference between the observed and predicted mass.

<sup>i</sup> Probability that the predicted mass is within the S.E. of the observed average mass based upon a t test with n – 1 degrees of freedom.

<sup>j</sup> Descriptive designation of the predicted processed γ subunit isoform.

<sup>k</sup> Abundance of the different structural forms have been estimated for discussion purposes only and represent the percentage of the total isoform expressed in brain that is thought to be modified as indicated by the proposed structure. These estimates are based upon the relative MALDI signal intensity in multiple separations as in Fig. 1 but pertain only to comparing differently modified forms of the same Gγ subunit isoform. This assumes that all of the differently modified forms of a single Gγ isoform have the same ionization characterization, which has not been experimentally verified.

<sup>l</sup> The support for each assignment is based upon the following data and/or evidence. A, replicate MALDI mass. B, predicted mass is within the error range of the observed mass. C, Edman sequencing. D, MS/MS or MS/MS/MS sequencing on an ion trap LCQ mass spectrometer. E, MS/MS evidence of prenylation (loss of 272 Da for geranylgeranyl or 204 Da for farnesy1 from the parent ion). F, Asp-Pro (acid hydrolysis) fragmentation with predicted amino-terminal and carboxyl-terminal fragments. G, MS/MS fragmentation of these proteins did not yield mass losses consistent with loss of a farnesyl or a geranylgeranyl group. Although not proof that these lack prenyl groups, this is consistent with all of the other data on these proteins and differentiates them from a large number of γ subunits that do lose such a mass and are predicted to be prenylated.

<sup>m</sup> Although this is the only γ<sub>12</sub> variant fully characterized, Mass 2 has the predicted mass of the unmethylated form of γ<sub>12</sub>, and Mass 3 has the predicted mass of the carboxyl-terminally cysteinylated form. However, these structures have not been characterized rigorously.
estimates of precision can be obtained from errors determined by repeated independent measurements of our masses, which are generally in the range of 1–2 Da (Table I). These measurements have 95% confidence intervals of about 6 Da (i.e. ±3 Da on either side of the observed mass).

Several approaches were used to specifically identify G\_\gamma subunit isoforms in HPLC fractions or modifications of those G\_\gamma subunits (Table II and Fig. 2). The proteins summarized in Table II have multiple lines of support for their structural assignments. One way that we generated an initial hypothesis for many of these structures was to compare the observed mass with a library of about 2,500 masses generated from all known G\_\gamma sequences modified by all variations of known modifications (such as presence or absence of N-acetylation or geranylerganylation versus farnesylation) or plausible modification such as phosphorylation. Generally there is only one plausible structure within the 95% confidence intervals for each observed mass. If an observed mass corresponded to one of the possible masses, we used the techniques below to seek supporting evidence for this assignment. In some cases, observed masses were not compatible with the predicted mass of any known G\_\gamma isofrom modified by any known combination of posttranslational modifications. In such cases, we used the strategies below to elucidate the structure but still imposed the requirement that the final proposed structure had to have a mass within the 95% confidence intervals of observations in Table II.

One of the first strategies used to identify masses in Table I was Edman sequencing. As noted above, however, this was often not a useful approach because most masses contained blocked amino termini. In addition, in general this approach cannot be used for identifying protein modifications. Nevertheless prominent signals were obtained for G\_\gamma (Fig. 1 and Table I, Mass 21), for an N-arginylated G\_\gamma variant previously characterized (45) (Fig. 1 and Table I, Mass 15), and for a G\_\gamma fragment related to the arginylated protein but starting with Thr\(\_6\) (Mass 20). We also found a sequence co-eluting with the major G\_\gamma sequence that could be G\_\gamma, another G\_\gamma subunit predicted to contain an unprotected amino terminus. This signal was very weak, however, and we are unable to identify a specific mass corresponding to a modified bovine G\_\gamma in our preparation, although this could be a minor signal not seen consistently.

A second approach to confirm structures for masses of predicted G\_\gamma subunits and to obtain information for identifying unknown masses, took advantage of the characteristic single aspartate-proline bond found in all known G\_\gamma isoforms except G\_\gamma_{10} (43). This Asp-Pro bond is susceptible to hydrolysis at low pH, generating amino- and carboxyl-terminal products (≈4,500–6,000 and 2,000–3,000 Da, respectively) that can be compared with known G\_\gamma subunit sequences with different possible modifications (48). This technique was used previously to characterize G\_\gamma_{2} (42). Because all of the modifications described here were found at the amino or carboxyl
terminus of the proteins, this technique was particularly useful for confirming the site of variable modification, in the case of proteins that corresponded to predicted masses, or for providing clues to the Gγ isoform corresponding to a mass for proteins with unknown or novel modifications. Fig. 3 shows a MALDI spectrum of an HPLC fraction containing a complex set of Gγ subunit species and the analysis of the acid digest of this fraction to identify amino-terminal and carboxyl-terminal fragments of Gγ subunits with newly described modifications, a farnesylated species of Gγ5, and a cysteinylated species of Gγ2.

A third approach to confirming or identifying structural assignments was sequencing by mass spectrometry using an LCQ ion trap instrument. This was either based upon MS/MS of intact Gγ subunits (49) or MS/MS and MS/MS/MS sequencing of proteolytic fragments generated with trypsin or Asp-N and separated by in-line HPLC (48). In many cases, this approach was used to confirm hypothesized structures of modified Gγ isoforms based upon application of the earlier described approaches and generated from consistency of the observed MALDI mass with reasonable modifications of known Gγ isoforms. In particular, this approach was useful to
show the presence of an isoprenyl group on many of the subunits listed in Table II. This thioether bond is readily fragmented by CID, and the existence of an isoprenyl group can be inferred from the loss of the mass (272 Da for geranylgeranyl and 204 Da for farnesyl) from the intact protein (49). An example of this is shown in Fig. 4 where a farnesylated G\textsubscript{AH9253}\textsubscript{2} subunit was characterized. One of the advantages of the ion trap mass spectrometer is that ions can be sequentially selected for multiple rounds of fragmentation. In general the molecular ions of the intact G\textsubscript{AH9253}\textsubscript{subunits appear to have preferred charge states in the MS of 5\textsuperscript{+} to 8\textsuperscript{+}. Although the LCQ has insufficient resolution for unambiguous assignment of the charge state from isotopic distribution of fragment ions, assignments were facilitated by identification of signals compatible with multiple charge states of fragment ions and by the ability to associate nearly all major signals with predicted b and y ions of structures proposed by multiple independent lines of evidence as summarized above.

Modification Patterns Observed

Some of the masses observed corresponded to those that would be expected for the known G\textsubscript{Y} isoforms with their predicted modifications (Tables I and II and Fig. 2). For example, signals indicating the presence of G\textsubscript{Y2} (Mass 25, m/z 7,751.3), G\textsubscript{Y5} (Mass 11, m/z 7,169.8), and G\textsubscript{Y7} (Mass 8, m/z 7,409.6) were routinely found and agree with previous predictions (32). In addition, a signal consistent with G\textsubscript{Y12} (Mass 4, m/z 7,925.0) was found in a fraction that was immunoreactive with the antiserum to G\textsubscript{Y7} (data not shown). (The antiserum was generated against an amino-terminal G\textsubscript{AH9253}\textsubscript{7 peptide, and cross-reactivity is due to their similar amino-terminal sequences.) There was also a G\textsubscript{Y10} isoform (Mass 28, m/z 7,134.4) found (43). All of these masses were compatible with proteins that are fully processed, i.e. acetylated at the amino terminus after Met\textsuperscript{1} cleavage and prenylated at the carboxyl terminus followed by proteolytic removal of the last three residues and carboxymethylation of the new carboxyl terminus. In addition, we previously found a broad MALDI signal that could not differentiate between two differently modified forms of G\textsubscript{Y3} (32). In the analysis here, both of these forms were elucidated, one with an unblocked amino terminus (Mass 21, m/z 8,295.6) and the other N-acetylated on Met\textsuperscript{1} (Mass 26, m/z 8,337.6). Aside from these seven proteins, however, many of the masses could be accounted for by versions of these proteins with alternative processing patterns. All but two of these alternatively processed proteins (Masses 29 and 30 appear to be exceptions but were not satisfactorily resolved in these studies, but see below) were modified at either the amino terminus or the carboxyl terminus, which appear to be regions of hypervariability.

Amino-terminal Variants of G\textsubscript{Y} Isoforms and Related Modifications

Although most of the variation in G protein modifications described here occurs in association with the carboxyl-terminal prenylation site, there was also variation in amino-terminal processing detected, particularly for the most abundant isoform, G\textsubscript{Y2}. Most brain G protein \textit{y} isoforms are predicted to be acetylated after removal of Met\textsuperscript{1}. This was found to be the case for G\textsubscript{Y2}, G\textsubscript{Y5}, G\textsubscript{Y7}, G\textsubscript{Y10}, and G\textsubscript{Y12}; for G\textsubscript{Y3}, more than one-half was acetylated on Met\textsuperscript{1} according to MALDI signal intensity, and the rest was unmodified (Fig. 1 and Table II). In addition to G\textsubscript{Y3}, Edman sequencing identified a low level of a peptide homologous to the unblocked amino terminus of G\textsubscript{Y4} (MKEGMSNNST). The G\textsubscript{Y4} sequence, like that of G\textsubscript{Y3}, could remain unacetylated at the amino terminus in the cell because its amino-terminal

![Fig. 4. Electrospray ionization MS/MS analysis of farnesylated G\textsubscript{Y2} isoform. A, proposed structure of farnesylated G\textsubscript{Y2} indicating the b- and y-ions observed in the MS experiment. B, MS/MS spectrum of farnesylated G\textsubscript{Y2} isoform, 7+ charge state selected, m/z 1,098.5, average of 41 scans. C, table of masses indicating expected and observed ions.](image-url)
sequences are similar. However, neither MALDI nor ESI analysis identified the expected Gγ4 mass in that fraction.

Recently we described (45) an N-arginylated variant of the Gγ2 protein that seems to be generated by a complex processing pathway leading to a Gγ2 species with an N-end rule ubiquitylation signal (51). The N-arginylated Gγ2 protein (45) is missing its first five residues (MASNN), which have been replaced by the sequence “RD.” Portions of an enzymatic pathway that could generate this protein have been described previously (51). These include an amino-terminal deamidase that could use as a substrate the Asn at position 5 in Gγ2 protein (after proteolysis of the MASNN sequence) and a protein arginylltransferase that could arginylate the resulting amino-terminal Asp. The RD-Gγ2 protein that we previously described is the first protein identified that would be a product of the complete pathway, and so this pathway has not yet been established definitively. Proteins characterized here that are likely to be part of this pathway include a truncated form of Gγ2 identified by Edman sequencing with the following amino-terminal sequence: TASIAQARKLVEQLK. This protein is identical in sequence to processed Gγ2 (45), which has a 1-Da increase in mass from the correctly processed Gγ2 protein (after proteolysis of the MASNN sequence) and a pro-

Carboxyl-terminal Variants of Gγ Isoforms

Unmethylated Gγ2—Carboxymethylation is the final reaction in the progressive steps involved in prenylation of the CAAX motif proteins. Although expectations were that blocking the carboxymethylase would have relatively modest effects on the function of Ras (53), this turned out not to be the case. Deletion of the carboxymethylase gene is early embryonic lethal (53) and appears to block the transforming activity of activated K-Ras and N-Ras (54). Surprisingly inhibition of carboxymethylation appears to be a secondary effect of antitumor agents such as methotrexate and may be a primary mode of action in its suppression of tumor cell growth (31). For the G proteins, carboxymethylation is a reversible reaction (55), possibly regulated by receptors (56), that may influence turnover (57) and membrane association of the Gβγ dimer (58, 59). Thus, it is of considerable interest whether or not our studies provide evidence of variation in carboxymethylation of native G protein γ subunits. In one case, a signal at m/z 7,737.0 (Tables I and II, Mass 16) had an Asp-Pro fragment ([M + H]+, 5,159.8 ± 3.8) compatible with the amino terminus predicted for Gγ2 ([M + H]+, 5,161.9), but the corresponding carboxyl-terminal fragment ([M + H]+, 2,590.2 ± 2.0) was compatible with a Gγ2 lacking a carboxymethyl group at the carboxyl terminus ([M + H]+, 2,594.2) and not the predicted Gγ2 isoform ([M + H]+, 2,608.2). ESI-MS/MS experiments supported this assignment (supplemental data). The generality of these putative structures is suggested by identification of intact masses compatible with unmethylated forms of Gγ3 (Mass 18) and Gγ12 (Mass 2) (Table I and Fig. 1), although we do not now have additional supporting data on these for assignment of a structure. The presence of unmethylated Gγ2 was not a nonspecific phenomenon either because the apparent amounts of unmethylated and methylated forms of the proteins varied substantially from being a large and prominent signal for Gγ2 (Mass 16, unmethylated versus Mass 25, methylated) to not being observable as a significant signal for nearly equally prominent Gγ7 (Mass 8).

Farnesylated Gγ2—The carboxyl-terminal residue of CAAX motif (X) is the primary determinant of the type of lipid group added to the cysteine (19, 25). A 20-carbon geranylgeranyl group is added if the final residue is a Leu (or Phe), and a 15-carbon farnesyl group is added if the final residue is a Ser, Met, Gin, Cys, or Ala (21). The Gγ2 isoform contains CAAX as its carboxyl-terminal signal for geranylgeranylation, which is the major γ isoform observed (Fig. 1, Mass 29). Surprisingly, however, a MALDI signal at m/z 7,684.5 (Fig. 1 and Table II, Mass 12) was compatible with a farnesylated Gγ2 isoform. Acid hydrolysis to cleave the predicted Asp-Pro bond in Gγ2 generated confirming fragments at m/z 5,160.7 and 2,539.1 (compared with predicted m/z 5,161.9 and 2,540.1) (Fig. 3). MS/MS sequencing on an LCQ ion trap mass spectrometer confirmed this structural assignment, generating peaks compatible with b and y ions for the sequence of Gγ2 as well as evidence supporting the presence of a farnesyl prenylation modification (Fig. 4). The 7+ charge state of farnesylated Gγ2, m/z 1,098.5, was selected for MS/MS fragmentation. The MS/MS spectrum in Fig. 4B shows signals of m/z 1,247.2 and 1,069.3, compatible with the 6+ and 7+ charge states of Gγ2 subunit after loss of 204 mass units, the mass of the farnesyl group. In contrast, the fragmentation spectrum did not generate identifiable ions consistent with the loss of geranylgeranyl group from this protein; loss of geranylgeranyl group was readily identifiable in most proteins predicted to contain this prenyl group (supplemental data).

As far as we know, the variability in the prenylation pattern of native proteins has not previously been systematically investigated. Previous studies have shown that recombinant G
Proteomic Analysis of Bovine Brain G Protein γ Subunit

protein γ subunits (60) and expressed RhoB (61–63) can be prenylated with either geranylgeranyl, their predicted prenyl group, or farnesyl, albeit at substantially different levels under comparable conditions. Further Ras, predicted to be farnesylated, can also be geranylgeranylated in cells treated with a farnesyl protein transferase inhibitor (64–67). This could be due in part to acceptance of alternative substrates by geranylgeranyltransferase I and farnesyltransferase (68), but the sequence determinants of this appear to be complex and may differ between proteins (69). Given the controversy over the site of action of farnesyl protein transferase inhibitors and the increasingly apparent complexity of the enzymology associated with protein prenylation (as described here), it is important to know which pathways and processes are biologically relevant to normal cells and tissues, which are pharmacologically induced, which are due to pathologic processes, and which are possibly related only to the processing of overexpressed recombinant proteins. An early protein identified as variably prenylated as a recombinant protein was RhoB (61), which is predicted to be geranylgeranylated but was also found to be farnesylated. Although different mechanisms of farnesylation of this protein have been proposed (62, 63), immunologic evidence suggests that the farnesylated form is expressed both as a recombinant and as an endogenous protein in cells (70). Those studies also suggested, however, that this dual prenylation is sequence-specific and coded not specifically cleave all Gγ targets, something that we did not observe (see above). Thus, although we cannot rule out absolutely generation of unprenylated proteins during isolation, there is also the real possibility that this is a physiological process. Given what is generally thought to be the role of the prenyl group in membrane localization, such a reaction would produce cytosolic Gβγ dimers and would provide a potential intracellular signaling pattern in G protein responses.

Unprenylated Carboxyl Terminus of Gγ—Generally it is believed that prenylation of CAAX motif proteins involves a sequence of reactions including prenylation of Cys at the –4 position, proteolysis of the carboxyterminal three amino acids, and carboxymethylation of the new carboxy terminus. The proteolysis step is mediated by one of two enzymes in yeast (77) and by one known enzyme in mammalian cells, referred to as Ras-converting enzyme 1 (Rce1). Absence (knock-out) of this enzyme is embryonic lethal in mice and disrupts Ras localization (78) and leads to abnormal cardiac function and eventual death if disrupted cardioselectively (79). Rce1 has been targeted for development of antitumor drugs (29). Previously (44) we showed that the major Gγγ protein expressed in bovine brain is an exception to this rule; it retains its three carboxyterminal amino acids after prenylation without carboxymethylation (Fig. 1 and Table II, Mass 14), and a minor fraction of the protein is processed as is predicted by current understanding of this reaction (Mass 11). One thing that we looked for in our data was the generality of this observation. The only other Gγ subunit for which we could find evidence of lack of Rce1 processing was Gγ7, which was found to be prenylated (60) and expressed by ESI-MS, unprenylated proteins can be generated by CID (60), similar to our recently described characterization of intact Gγ subunits (49). This does not explain the results reported here because the prenylated and unprenylated proteins travel with different average retention times (Table I) on HPLC. It is possible to generate an unprenylated protein by acid hydrolysis as during the HPLC separation in the presence of TFA. However, masses corresponding to unprenylated variants were not generated in samples containing intact Gγ subunits and stored in HPLC buffer before analysis, and acid hydrolysis at higher TFA concentrations to produce Asp-Pro fragments (Fig. 3) did not generate unprenylated proteins even though these conditions readily cleaved the acid-sensitive Asp-Pro bond. Finally although prenylcysteine lyase has not been shown to metabolize prenyl peptides (75), perhaps it could do so at a low level if released from lysosomes during tissue preparation. Presumably such release would be nonspecific, however, and other enzymes, such as carboxymethylases, would nonspecifically cleave all Gγ targets, something that we did not observe (see above). Thus, although we cannot rule out absolutely generation of unprenylated proteins during isolation, there is also the real possibility that this is a physiological process. Given what is generally thought to be the role of the prenyl group in membrane localization, such a reaction would produce cytosolic Gβγ dimers and would provide a potential intracellular signaling pattern in G protein responses.

Unprenylated Carboxyl Terminus of Gγ7—Protein prenylation through a thioether linkage is a relatively stable bond. Studies of the complexity of metabolizing prenylcytide degradation products of these proteins resulted in the description of a lysosomal prenylcysteine lyase that is an FAD-dependent thioether oxidase (75, 76). This enzyme, however, will not process prenylated peptides (75), only the free prenylcysteine, and as far as we know, no enzyme activity has been described that specifically removes a prenyl group from a functional prenylated protein. Nevertheless we characterized in purified bovine brain Gγ (Fig. 1 and Table II, Mass 22) and Gγ7 (Fig. 1 and Table II, Mass 6) proteins that were otherwise processed normally but lacked a prenyl group. When fragments of expressed Gγ subunits are assayed by ESI-MS, unprenylated proteins can be generated by CID (60), similar to our recently described characterization of intact Gγ subunits (49). This does not explain the results reported here because the prenylated and unprenylated proteins travel with different average retention times (Table I) on HPLC. It is possible to generate an unprenylated protein by acid hydrolysis as during the HPLC separation in the presence of TFA. However, masses corresponding to unprenylated variants were not generated in samples containing intact Gγ subunits and stored in HPLC buffer before analysis, and acid hydrolysis at higher TFA concentrations to produce Asp-Pro fragments (Fig. 3) did not generate unprenylated proteins even though these conditions readily cleaved the acid-sensitive Asp-Pro bond. Finally although prenylcysteine lyase has not been shown to metabolize prenyl peptides (75), perhaps it could do so at a low level if released from lysosomes during tissue preparation. Presumably such release would be nonspecific, however, and other enzymes, such as carboxymethylases, would nonspecifically cleave all Gγ targets, something that we did not observe (see above). Thus, although we cannot rule out absolutely generation of unprenylated proteins during isolation, there is also the real possibility that this is a physiological process. Given what is generally thought to be the role of the prenyl group in membrane localization, such a reaction would produce cytosolic Gβγ dimers and would provide a potential intracellular signaling pattern in G protein responses.
general explanation for the lack of processing of $G_\gamma_5$ and $G_\gamma_7$.

In addition, the $\alpha$ and $\beta$ subunits of farnesylated rabbit skeletal muscle glycogen phosphorylase kinase have been shown to have a similar processing pattern (80). There is no obvious carboxyl-terminal motif common to $G_\gamma_5$ and $G_\gamma_7$ that might direct this alternative processing pattern, and it is not clear that previous surveys of the specificity of, for example, the yeast Rce-related enzymes would adequately predict these observations in mammals (77). Nevertheless given the results with Rce1 knock-out mice and the mislocalization of Ras in cells from these animals (78), it is a reasonable hypothesis that these alternatively processed forms of $G_\gamma$ subunits would provide for alternative targeting sites for their associated G proteins.

**Carboxyl-terminal Cysteinylation of G Protein $\gamma$ Subunits—**

The array of $G_\gamma$ subunits described above can be explained as variations of previously described prenylation-related modifications. In addition to these, however, a $G_\gamma_2$ species was found that is ~89 mass units higher than completely processed $G_\gamma_2$ (Tables I and II and Fig. 1, Mass 13, m/z 7,840.1). That this is related to $G_\gamma_2$ is based upon analysis of Asp-Pro cleavage patterns (Fig. 3) and ESI-MS analysis (Fig. 5 and supplemental data). The Asp-Pro analysis indicated that the 89-Da addition was associated with the carboxyl-terminal fragment of the protein. We isolated the peptide fragment analogous to this by HPLC after Asp-N digestion of the protein. Edman sequencing of this peptide showed that its amino-terminal sequence was that predicted for $G_\gamma_2$ (data not shown). MS/MS followed by MS/MS/MS fragmentation of this peptide disclosed a fragmentation pattern characteristic of a Cys residue at the carboxyl terminus of the protein (Fig. 5). The data were compatible with the Cys linked either through a peptide bond or as a thioester. Attempts to alkylate the protein with several different reagents (N-ethylmaleimide and

---

**Fig. 5.** MS/MS and MS/MS/MS analysis of a carboxyl-terminal Asp-N fragment of cysteinylated $G_\gamma_2$. An HPLC fraction containing an $m/z$ of 7,840.1 (Mass 13 in Fig. 1 and Table I) was digested with Asp-N as described previously (48). Peptides were separated by HPLC and analyzed by nanospray on an LCQ ion trap mass spectrometer. A, MS/MS spectra of the 3+ charge state of a parent Asp-N carboxyl-terminal fragment of the intact $G_\gamma_2$ with $m/z$ 938.3. Fragmentation generated a mass corresponding to the parent -gg and multiple y ions corresponding with loss of a 323-Da fragment. B, MS/MS/MS spectrum of the y16 ion from the assay in A that has lost a 323-Da fragment ($m/z$ 974.5). The spectrum shows consecutive losses of 103, 28, and 41 mass units, compatible with the loss of a cysteine residue, assuming the loss of 323 includes the geranylgeranyl group attached to another cysteine residue. C, a structure compatible with the sequence of $G_\gamma_2$ and fragmentation patterns in A and B showing sequential loss of geranylgeranylcysteine with water (mass = 323), cysteine from the carboxyl terminus (mass = 103), a carbonyl group (mass = 28), and the remaining peptide residue of the geranylgeranylcysteine. D, alternate structure for the carboxyl-terminal cysteine attached to the protein through a thioester linkage also compatible with the fragmentation data.
Diversity and Function of G Protein γ Subunit Isoforms

The most abundant Gγ subunit in bovine brain is Gγ2. Seven of the masses described here or previously represent differently processed forms of Gγ2 that vary at either the amino or carboxyl termini (Tables I and II and Fig. 1, Mass 12, farnesylated m/z 7,684.5; Mass 13, cysteinylation m/z 7,840.1; Mass 16, unmethylated m/z 7,737.0; Mass 20, truncated TASS-Gγ2 m/z 7,324.2; Mass 15, RDTASIA-Gγ2 m/z 7,593.7; Mass 6, unprenylated m/z 7,481.1; Mass 25, fully processed Gγ2 m/z 7,751.3). Most of the processed forms of this protein represent variations at the site of prenylation. It is possible that the variable processing of Gγ2 is indicative of what would also be found for the other G protein γ subunits if expressed at a comparable level, but some forms seem to be more prominent for some Gγ isoforms than for others.

Although some alternatively processed forms, such as the unproteolized Gγ2 and unmethylated Gγ2, are major forms of their respective proteins, others of these signals suggest that the modified forms are minor components of the total γ subunit present. Although this might minimize the biological role of these variants, this would also be expected if these represent transitional variants responding to incoming signals of one kind or another. For example, this might apply to the potentially unmethylated isoforms found at low levels (i.e., Gγ3, Mass 18, and Gγ12, Mass 2), which are in contrast to the unmethylated γ2 (Mass 16) that appears to represent a sizable fraction of the protein. This would be compatible with previous suggestions that carboxymethylation of these proteins is regulated (55, 56), and our present data would suggest that Gγ2 is preferentially affected by this at least under the specific conditions with which these proteins were isolated. Similar considerations would apply to proteins that have lost their prenyl group or are amino-terminally modified in transit to the ubiquitylation pathway. Although found at low levels, these proteins may be indicative of important regulatory processes altering the distribution and level of G proteins in cells.

In contrast to low levels of modified forms that may represent transient states of the protein, low levels of farnesylated Gγ subunits that are usually geranyleranlated may result from the nonspecificity of the transferases involved (68), although even this seems to be sequence-specific even if complex (69) and suggests specificity to this variation. Regardless of the mechanism of generation, however, a population of such Gγ subunits (farnesylated when predicted to be geranyleranlated) do exist in cells and should have altered signaling responses to receptors (60). Similarly the unprenylated variants, if generated in cells, would be predicted to lead to altered membrane association of the Gβγ dimer and almost assuredly altered signaling properties. Thus, the processing variants described here, when viewed in light of past studies of the role of prenylation in cell, could have a dynamic interplay existing among them that will contribute to the responses in cells following regulation of these proteins. It is important also to recognize that level of expression is a relative value. Whereas many of the modified forms of Gγ2, in particular, appear to be low relative to the predicted form of this protein, G proteins in brain are expressed at truly remarkable levels, approaching 1% of particulate protein. In most cells G protein subunits would constitute on the order of 0.01% of even membrane protein. Thus, although many of the modified forms described here are low compared with the high levels normally found in brain, they are comparable to the protein levels found for G proteins in other cells, and so they may be predominant forms of the protein in restricted compartments even within brain.

Evaluation of the proteome of different cells and disease states is a challenging endeavor. To begin with there is the enormous variation in protein sequence and chemistry magnified further by posttranslational processing of the proteins that can be, as demonstrated here, quite variable for what has been believed to be a well defined processing reaction. Ultimately characterization of the complete complement of proteins in cells will have to take into account all of these factors. A major step in the process will be defining the fidelity and variability associated with posttranslational processing events.
as is reported here for the prenylation reaction associated with the γ subunit of the heterotrimeric G proteins. A primary conclusion of our work is that signaling proteins, such as G proteins, that are posttranslationally modified at potentially important functional sites may vary greatly at these sites, and their role in normal or pathological function will not be apparent without taking these posttranslational variations into account. These results indicate too that the characterization of the proteome expressed in cells will have to ultimately incorporate a strategy to account for functionally significant variation in protein processing patterns.


Proteomic Analysis of Bovine Brain G Protein γ Subunit

Molecular & Cellular Proteomics 5.4