Heterogeneous Processing of a G Protein γ Subunit at a Site Critical for Protein and Membrane Interactions†

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ABSTRACT: The G protein γ_5 subunit is selectively associated with specific G protein α subunits [Wilcox, M. D., et al. (1995) *J. Biol. Chem. 270*, 4189] and is localized preferentially in focal adhesion plaques [Hansen, C. A., et al. (1996) *J. Cell Biol. 126*, 811]. What determines the differential association of G proteins and their subunits with specific cellular structures or compartments is not clear, but one factor could be variation in the pattern of processing of the proteins. To study γ_5 subunit diversity and modifications, G protein subunits were fractionated on an HPLC phenyl column and analyzed with a γ_5-specific antiserum. The γ_5 eluted from the column as two peaks of immunoreactivity. Analysis by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and electrospray ionization tandem mass spectrometry revealed that the first immunoreactive peak corresponded to the predicted γ_5 isoform (N-terminally acetylated after removal of methionine, C-terminally geranylgeranylated and carboxymethylated with removal of the last three amino acids), while the second peak of immunoreactivity contained a γ_5 isoform isoprenylated at the C-terminus but retaining its three terminal amino acids. This alternatively processed protein is the predominant γ_5 subunit isoform associated with G_o and G_i proteins purified from bovine brain. These results describe a new C-terminal processing pattern for G protein γ subunits and establish the principle that G protein γ subunits can be heterogeneously modified at their C-termini. This is a site on the γ subunit critical for membrane and protein−protein interactions of G proteins. These results open the possibility that one determinant of the localization of G proteins in cells could be the pattern of processing of their γ subunit constituents.

G proteins are heterotrimeric GTP binding proteins composed of α, β, and γ subunits. They mediate the effects of a wide range of cell surface receptors on intracellular signaling pathways (*1*−*4*). There are multiple isoforms of each of the three kinds of subunits that make up G proteins, but the role of this subunit diversity in signaling processes is not entirely clear, particularly for the β and γ subunits (*5*−*7*). Studies in intact cells suggest that receptors communicate with intracellular effectors through heterotrimers composed of very specific α, β, and γ subunit combinations (*8*). Often, however, in vitro experiments do not suggest this same degree of specificity (*5*−*7*).

A likely important component of signaling specificity in intact cells, and one lost or disrupted in *in vitro* experiments, is the compartmentalization of the signaling components involved (*9*, *10*). Receptors (*11*, *12*), G protein subunits (*9*, *13*−*18*), and effectors (*10*, *16*) all show specific localizations in cells, although the mechanisms determining such compartmentalization are yet to be fully defined. The targeting of cellular proteins may be affected or regulated by their pattern of processing during and after their synthesis. For example, proteins that are modified by isoprenylation or by incorporation of other lipids may be preferentially associated with caveolae in cells (*19*, *20*).

All known G protein γ subunits are isoprenylated with either a geranylgeranyl (C-20) or a farnesyl (C-15) group on a cysteine four residues from the C-terminus. The isoprenyl group addition is directed by a CAAX motif at the C-terminus (where C = cysteine, A = aliphatic amino acid, X = any amino acid), and the type of group added is determined by the residue at position X (*21*). When X is a leucine, a geranylgeranyl group is added; but when X is a methionine, serine, glutamine, cysteine, or alanine, a farnesyl group is added (*22*). Isoprenylation is followed by removal of the terminal three amino acids and carboxymethylation (*21*, *23*). Similar processing affects a large number of proteins, many of which are involved in cell signaling (*21*, *24*).

The G protein γ_5 subunit is differentially associated with G protein α subunits (*25*) and is preferentially localized in focal adhesion plaques (*13*). Focal adhesions are specialized regions of the plasma membrane that anchor cells to their underlying extracellular matrix (*26*). Why γ_5 should be specifically associated with focal adhesions, when other G protein subunits such as γ_3 are not (*13*), is not known. Here we describe an alternative processing pattern for the γ_5 subunit, different from that found on other G protein γ subunits. Thus, this alternative pattern of processing provides one possible explanation for the unique association of γ_5 with focal adhesion plaques. Further, these results establish that Gγ subunits can be heterogeneously processed at the C-terminus. This could have important implications for

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receptor and effector interactions with G proteins, since this site of modification is involved in $\beta\gamma$ interactions with membranes and with other proteins.

EXPERIMENTAL PROCEDURES

G Protein Heterotrimer Purification. G protein heterotrimers ($\alpha\beta\gamma$) were prepared from bovine brain cortex membranes by the method of Sternweis and Robishaw (*27*), with modifications as described previously (*28, 29*). The resulting G protein preparation is a mixture of G_o and G_i isoforms (*25*), 85−90% of which is a G_o variant representing the most abundant brain G protein and accounting for as much as 1% of total brain particulate protein (*27, 30*). This G protein preparation contains very little G_s, G_z, or G_q which are also known to be contained in bovine brain but at lower levels than those of G_o.

HPLC[1] *Separation of γ Isoforms*. Purified G protein heterotrimer (2−4 mg) was passed over a 220 × 4.6 mm Aquapore 7 μm phenyl column for the reverse-phase HPLC separation of the γ isoforms and their resolution from α and β subunits. Solvent A contained 10% acetonitrile, 90% water, and 0.1%TFA, and solvent B, 75% acetonitrile, 25% 2-propanol, and 0.095% TFA. The elution conditions (1 mL/min) were 0−40% B over 10 min, 40−46% B over 65 min, and 46−100% B over 5 min. Fractions of 1 mL were collected and stored at −20 °C.

MALDI Mass Spectrometry. Aliquots of 25−100 μL of HPLC fractions were dried under vacuum and resuspended at up to a 50-fold concentration in 47.6% 1-propanol, 47.6% water, 4.76% acetonitrile, and 0.095% TFA. A 0.5 μL sample was usually mixed at a 1:3 ratio of sample with the matrix α-cyano-4-hydroxycinnamic acid (50 mM in 70% acetonitrile, 0.1% TFA) for MALDI analysis. Samples were air-dried and analyzed on a PerSeptive Biosystems Voyager-DE mass spectrometer. For internal calibration, standards were spotted first and air-dried, followed by spotting of 0.5−1 μL of sample diluted in matrix. Mass estimates were based on two-point internal calibration using horse cytochrome *c* ($[M+H]^+$, *m*/*z* 12361) and bovine insulin ($[M+H]^+$, *m*/*z* 5735) as standards for intact γ subunits, and a γ_2 peptide (sequence: P-A-S-E-N-P-F-R-E-K-K-F-F-C, $[M+H]^+$, *m*/*z* 1701) and horse cytochrome *c* for acid hydrolysis fragments. External calibration was performed using the same standards. Approximately 75−150 scans were averaged for a representative MALDI spectrum.

Generation of Antisera. The γ_5 anti-peptide rabbit polyclonal antiserum, G5NA, was generated to the acetylated amino-terminal peptide acetyl-S-G-S-S-S-V-A-A-M-K-K-C (*31, 32*) using the method of Green et al. (*33*).

Immunoblotting. HPLC fractions containing isolated γ isoforms were diluted 1:40 with buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 mM DTT, and 1% acetonitrile) and spotted on nitrocellulose in a Schleicher & Schuell mini-fold II slot-blotter apparatus under vacuum. For Western blotting, 20 μL aliquots of the HPLC fractions were dried under vacuum and resuspended in Laemmli sample buffer (*34*), followed by SDS−PAGE on a 10−20% gradient gel and transferred to nitrocellulose with a Hoeffer Semi-Phor transfer apparatus as described by Towbin et al. (*35*).

Aspartate−Proline Bond Hydrolysis. Acid hydrolysis of the isoforms was performed by drying 50−100 μL of HPLC fractions under vacuum and resuspending them at up to 50-fold concentration in water and acetonitrile (1:1) with 1−3% TFA. Samples were incubated for at least 12 h at room temperature followed by MALDI analysis.

Electrospray Ionization Tandem Mass Spectrometry. Aliquots of HPLC fractions were dried under vacuum and resuspended in either 47.6% 1-propanol/47.6% water/4.76% acetonitrile/0.095% TFA or 50% methanol/26% water/20% 1-propanol/4% acetic acid. Samples were introduced into a Finnigan LCQ ion trap mass spectrometer by direct injections of 5 μL into a flowing stream (50 μL/min) of 47% water/47% methanol/6% acetic acid (v/v/v) directed to the standard ESI source. Alternatively, samples were introduced by nanospray (continuous flow of nanoliters per minute) using a custom-built nanospray source consisting of a pulled glass capillary sputter coated with gold and filled with ~1 μL of sample. A voltage of 1500 V was applied to the capillary positioned ~1 mm from the heated metal capillary of the Finnigan electrospray source. Tandem mass spectrometry was accomplished by selecting the *m*/*z* ratio of the precursor ion of interest which was subsequently fragmented. Fragment ions were then mass-analyzed. MS/MS/MS experiments were accomplished by two sequential stages of *m*/*z* selection followed by dissociation and mass analysis. A window of 1 or 2 *m*/*z* units was employed in the precursor ion selection. MacBioSpec software v. 1.0.1 (PE SCIEX Instruments, 1992, Thornhill Ontario, Canada) was used to generate predicted ion *m*/*z* values.

RESULTS

Bovine brain Gγ subunits were separated from α and β subunits of purified heterotrimeric G proteins by reverse-phase HPLC (Figure 1A). Aliquots of HPLC fractions were analyzed on slotblots with a γ_5 subunit-specific antiserum, G5NA (Figure 1B). Included on the slotblot to demonstrate the specificity of the antisera was the γ_5 peptide to which the antiserum was made, the analogous peptides to N-terminal sequences of γ_2 (G2NA), γ_3 (G3N) and γ_7 (G7NA) and a peptide homologous to the C-terminus of γ_2 (γ common) and shared by many γ subunit sequences. Interestingly, immunoreactivity for γ_5 was seen in two noncontiguous fractions in the separation. One of these reacted strongly with the antiserum (fraction 32, late γ_5), the other weakly (fraction 30, early γ_5). Both fractions contained G5NA-reactive bands in Western blots, with the stronger reactivity again present in fraction 32 (Figure 1C). Overall recovery of γ_5 immunoreactivity from the HPLC separation was close to 50%. Quantitative comparison of the starting G protein preparation and the γ_5 pools on immunoblots with the G5NA antiserum indicated that the late γ_5 accounted for 40%, and the early γ_5 fraction 5%, of the γ_5 immunoreactivity in the starting material (Figure 2).

Analysis by matrix-assisted laser desorption ionization (MALDI) mass spectrometry showed that the early γ_5 fraction (Figure 3A) contained multiple signals in the γ subunit mass range (mass/charge = *m*/*z* 7167, 7410, 7680, 7735, 7838). One of those signals (*m*/*z* 7167) corresponds

[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; *m*/*z*, mass-to-charge ratio; MALDI, matrix-assisted laser desorption ionization mass spectrometry.

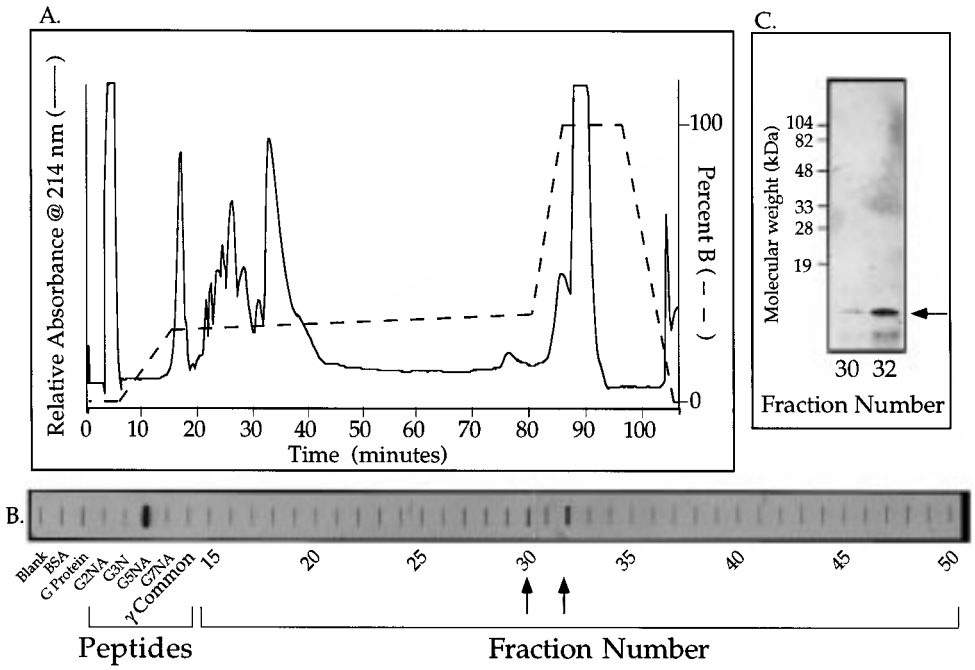


FIGURE 1: (A) HPLC absorbance profile of the separation of G protein γ subunits measured at 214 nm. (B) Slotblot for γ_5 of HPLC fractions using a γ_5-specific antiserum, G5NA. The arrows indicate the fractions containing γ_5 immunoreactivity. (C) Western blot of fractions 30 and 32 separated on a 10−20% SDS−polyacrylamide gel using a γ_5-specific antiserum, G5NA. The arrow indicates the location of γ_5 immunoreactivity in both fractions.

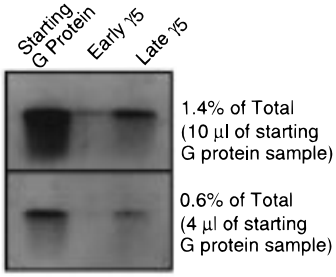


FIGURE 2: Recovery of G protein γ_5 during HPLC separation of G protein γ subunits on a phenyl column. Immunoblot with anti-Gγ_5 antiserum detected with ^{125}I-labeled second antibody and visualized on a Molecular Dynamics PhosphorImager. Equal percentages of the starting G protein sample injected on the column and the two γ_5 isoforms recovered from the column were each run at two different dilutions (corresponding to 4 and 10 μL of the starting G protein sample) on a 10−20% SDS−polyacrylamide gradient gel and analyzed for γ_5 immunoreactivity. At either dilution alone, 100% recovery would require that the immunoreactivity of the two γ_5 samples added together be equal to that of the starting G protein sample. It can be seen in the figure that the late γ_5 recovered equivalent to 10 μL of starting material has immunoreactivity corresponding to 4 μL of starting G protein, indicating that the late γ_5 recovered represents 40% of the γ_5 injected on the column.

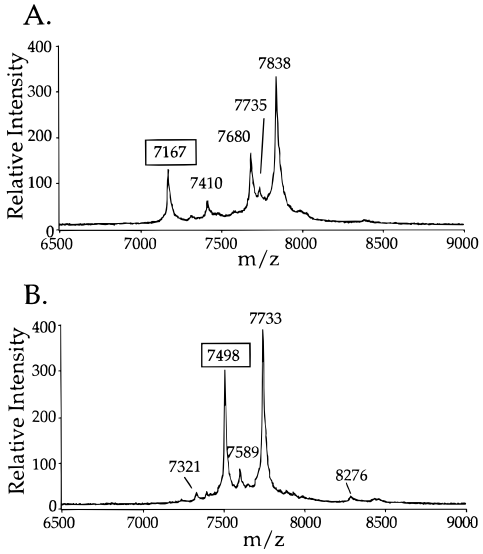


FIGURE 3: (A) MALDI mass spectrum of an HPLC fraction containing early γ_5 with predicted processing pattern (*m*/*z* 7167), average of 106 scans. (B) MALDI mass spectrum of an HPLC fraction containing late γ_5 subunit (*m*/*z* 7498), average of 152 scans.

to the predicted fully processed γ_5 isoform (predicted singly charged ion or $[M+H]^+$, *m*/*z* 7168) (*31*). This ion was observed previously (*32*) and corresponds to the mass of a γ_5 subunit N-terminally acetylated after removal of methionine, and geranylgeranylated on the cysteine residue four amino acids from the C-terminus, followed by proteolysis and carboxymethylation. The other signals in the spectrum also correspond to γ subunits (L. A. Cook, M. D. Wilcox, J. Dingus, K. L. Schey, and J. D. Hildebrandt, in preparation). Figure 3B shows the MALDI mass spectrum of the late γ_5 fraction. Two prominent signals were observed, one at *m*/*z* 7498 and the other at *m*/*z* 7733. The ion at *m*/*z* 7733 is a γ_2 isoform (data not shown), but the ion at *m*/*z* 7498 does not correspond to any predicted γ isoform.

One strategy for identifying unknown γ subunits and their modifications is to cleave the protein into smaller fragments before analysis by mass spectrometry. To examine fragments of γ subunits, we took advantage of the single acid-labile aspartate−proline (D−P) bond present in all γ isoforms cloned to date, except γ_{10}. Acid treatment of γ subunits will generate two fragments, an N-terminal fragment in the 4500−5800 mass range, and a C-terminal fragment in the 2000−3000 mass range. The exact masses of these fragments can be predicted for each γ subunit from their DNA

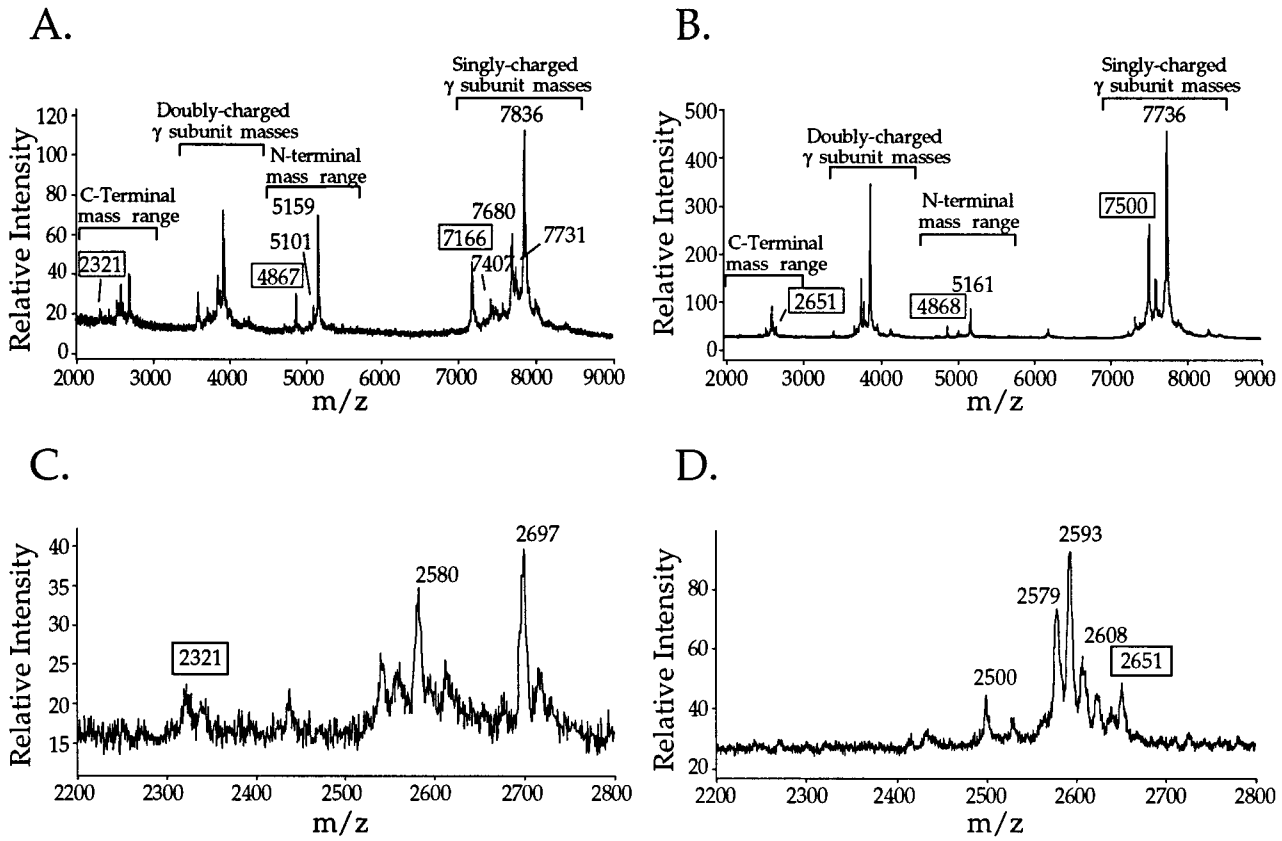


FIGURE 4: (A) MALDI mass spectrum of a fraction containing early γ_5 isoform after acid hydrolysis, average of 109 scans. (B) MALDI mass spectrum of a fraction containing late γ_5 isoform after acid hydrolysis, average of 152 scans. (C) Expanded region from (A) showing the C-terminal fragment of the early γ_5 (*m*/*z* 2321). (D) Expanded region from (B) showing the C-terminal fragment of the late γ_5 (*m*/*z* 2651). Other peaks in (C) and (D) represent C-terminal fragments of the other γ subunits in the fractions, triply charged ions of the intact γ subunits, or doubly charged ions of the N-terminal fragments.

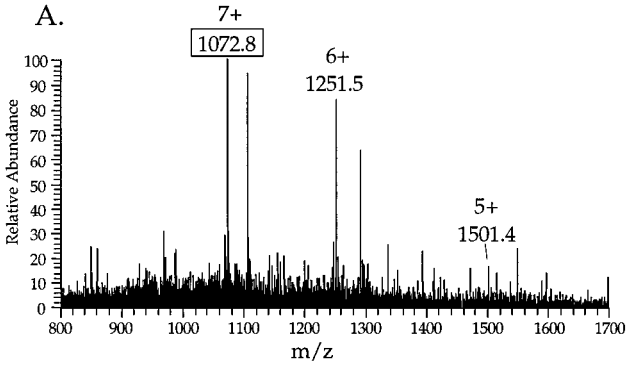
sequence. Acid hydrolysis of early (Figure 4A,C) and late (Figure 4B,D) γ_5 fractions produced new MALDI signals in the predicted mass ranges. Acid hydrolysis of early γ_5 generated only two ions, *m*/*z* 4867 and *m*/*z* 2321 (Figure 4A), whose combined mass minus that of water (*m*/*z* 7170) was close to that of the parent mass (*m*/*z* 7167). These ions correspond to the predicted γ_5 N-terminal and C-terminal fragment ions of *m*/*z* 4870 and *m*/*z* 2318, respectively. This supports the conclusion that the ion at *m*/*z* 7167 is a "fully processed" γ_5 isoform with the predicted modifications.

Acid hydrolysis of a the late γ_5-containing fraction (Figure 4B,D) also generated signals in the predicted range. Amino- and carboxy-terminal fragment ions compatible with a 7500 parent mass were seen at *m*/*z* 4868 and *m*/*z* 2651. The fragment at *m*/*z* 4868 is essentially identical to the N-terminal fragment of the fully processed γ_5 isoform (predicted $[M+H]^+$, *m*/*z* 4870). These data suggest that the 7498 mass originally seen in Figure 3B (*m*/*z* 7500 in Figure 4B) was responsible for the γ_5 immunoreactivity seen in this fraction (Figure 1). However, the corresponding C-terminal fragment ion at *m*/*z* 2651 is 333 mass units greater than the predicted γ_5 C-terminal fragment (*m*/*z* 2318). These results suggested that the 7498 ion is either a γ_5 subunit with a unique modification near the C-terminus or, alternatively, a C-terminal γ_5 splice variant.

To confirm that the 7498 mass protein was a γ_5 isoform and to determine its structure, the 7498 mass was analyzed further by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in a Finnigan LCQ ion trap mass spectrometer. In a tandem mass spectrometry experiment, proteins are fragmented predominantly at peptide bonds, yielding amino acid sequence and posttranslational modification information. MS/MS fragment ions are usually of a type referred to as b or y ions (*36*) and have an index number indicating the number of residues from the amino or carboxy terminus of the precursor ion, respectively.[2] Figure 5 shows the electrospray mass spectrum of a fraction containing the late γ_5 isoform ($[M+H]^+$, *m*/*z* 7498), along with the γ_2 isoform noted earlier. As is typically observed in ESI mass spectra, each protein was observed as multiple ions in different charge states. In this case, the late γ_5 isoform ($[M+H]^+$, *m*/*z* 7498) was observed in its 7+, 6+, and 5+ charge states at *m*/*z* 1072.8, 1251.5, and 1501.4, respectively (Figure 5). The precision of the LCQ instrument is greater than that of the MALDI instrument, and the average molecular weight for the three charge states of the late γ_5 was 7502.5 ± 0.5.

The ion corresponding to the 7+ charge state (*m*/*z* 1072.5) was mass-selected and fragmented to obtain the tandem mass spectrum in Figure 6. A signal at *m*/*z* 1205.8 is compatible with the 6+ charge state of the precursor ion after loss of a geranylgeranyl group, as predicted for γ_5. Possible N-terminal fragments of γ_5, labeled b_{34}, b_{46}, and b_{49}, as well as possible C-terminal fragments, labeled y_{16}, y_{22}, y_{42}, and y_{64}, were readily observed in the spectrum (Figure 6). As can be the case with fragmentation of larger peptides or proteins,

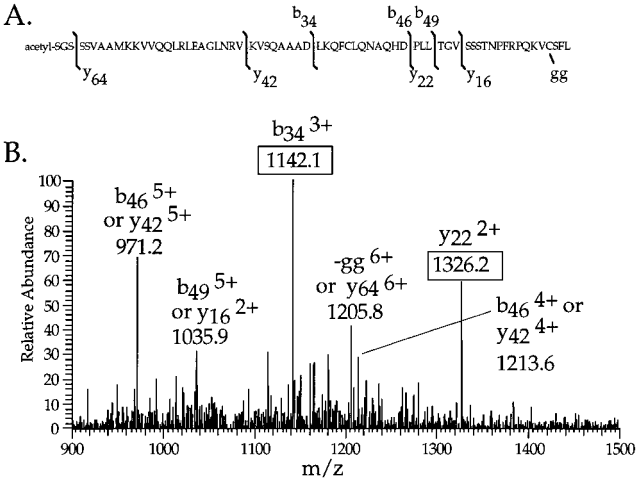
[2] Here we have chosen to index the ion by the amino acid position in the cloned sequence, not taking into account possible modifications.



B.

Residues	Predicted mass	z^a	Predicted Ion	Observed Signal
(Ac)2-68+gg	7501.8	7+	1072.5	1072.8
(Ac)2-68+gg		6+	1251.1	1251.5
(Ac)2-68+gg		5+	1501.2	1501.4

FIGURE 5: (A) LCQ electrospray mass spectrum of late γ_5 isoform, 65 scans averaged. (B) Table of predicted and observed ion signals: $^a z$ = charge state of protein.



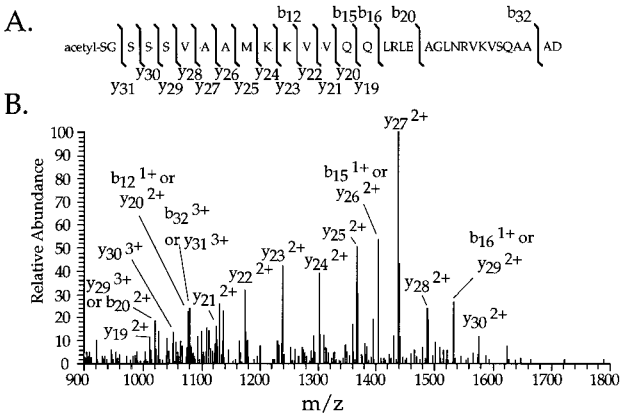
C.

Fragment	Residues	Predicted mass	z^a	Predicted Ion	Observed Signal
loss of gg	(Ac)2-68	7229.4	6+	1205.7	1205.8[b]
b_{49}	(Ac)2-49	5175.0	5+	1035.8	1035.9[b]
b_{46}	(Ac)2-46	4851.6	5+	971.1	971.2[b]
b_{46}	(Ac)2-46	4851.6	4+	1213.6	1213.6[b]
b_{34}	(Ac)2-34	3424.9	3+	1142.3	1142.1
y_{64}	5-68+gg	7228.6	6+	1205.6	1205.8[b]
y_{42}	27-68+gg	4848.7	5+	970.5	971.2[b]
y_{42}	27-68+gg	4848.7	4+	1212.9	1213.6[b]
y_{22}	47-68+gg	2651.3	2+	1326.1	1326.2
y_{16}	53-68+gg	2070.5	2+	1035.8	1035.9[b]

FIGURE 6: (A) Sequence of γ_5 identifying the possible b and y ions observed. (B) MS/MS spectrum of 7502.5 dalton protein after selection of the $[M+7H]^{7+}$ ion (m/z 1072.5), average of 30 scans. (C) Table of predicted and observed ion signals: $^a z$ = charge state of protein or fragment; [b]multiple possibilities for ions.

some ions were compatible with assignment to more than one fragment.

To identify key b and y ions generated in the experiment in Figure 6, we took advantage of the ability to perform an MS/MS/MS experiment using the Finnigan ion trap mass spectrometer. In this experiment, fragment ions are selected from an MS/MS experiment (as in Figure 6) and further fragmented, and then the generated fragments are analyzed to obtain sequence information. Figure 7 shows an MS/MS/MS spectrum for the b_{34} ion (predicted N-terminal fragment ion of γ_5) seen in Figure 6. Most major masses in this spectrum (Figure 7) could be accounted for as ions from the



C.

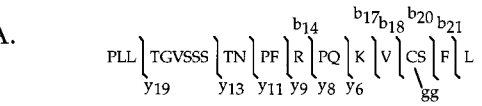
Fragment	Residues	Predicted mass	z^a	Predicted Ion	Observed Signal
b_{32}	(Ac)2-32	3238.8	3+	1080.3	1079.7[b]
b_{20}	(Ac)2-20	2043.4	2+	1022.2	1021.8[b]
b_{16}	(Ac)2-16	1531.8	1+	1531.8	1532.1[b]
b_{15}	(Ac)2-15	1403.6	1+	1403.6	1403.7[b]
b_{12}	(Ac)2-12	1077.2	1+	1077.2	1076.4[b]
y_{31}[c]	4-34	3238.8	3+	1080.3	1079.7[b]
y_{30}[c]	5-34	3151.7	3+	1051.2	1051.5
y_{30}[c]	5-34	3151.7	2+	1576.4	1576.5
y_{29}[c]	6-34	3064.7	3+	1022.2	1021.8[b]
y_{29}[c]	6-34	3064.7	2+	1532.8	1532.1[b]
y_{28}[c]	7-34	2977.6	2+	1489.3	1488.7
y_{27}[c]	8-34	2878.4	2+	1439.7	1439.3
y_{26}[c]	9-34	2807.4	2+	1404.2	1403.7[b]
y_{25}[c]	10-34	2736.3	2+	1368.6	1368.0
y_{24}[c]	11-34	2605.1	2+	1303.0	1302.7
y_{23}[c]	12-34	2476.9	2+	1239.0	1238.7
y_{22}[c]	13-34	2348.7	2+	1174.9	1174.7
y_{21}[c]	14-34	2249.6	2+	1125.3	1124.6
y_{20}[c]	15-34	2150.5	2+	1075.7	1076.4[b]
y_{19}[c]	16-34	2022.3	2+	1011.7	1011.2

FIGURE 7: (A) Sequence of γ_5 amino acids 2−34 identifying the possible b and y ions observed. (B) MS/MS/MS spectrum of N-terminal b_{34} fragment ion of the 7502.5 dalton protein, m/z 1142.0 from Figure 5 selected, average of 93 scans. (C) Table of predicted and observed ion signals: $^a z$ = charge state of fragment; [b]multiple possibilities for ions; [c]this ion is a C-terminal fragment of a b ion and not that of an intact peptide. Consequently, the actual C-terminus of this ion is not the same as that of traditional y ions, but is that of a traditional b ion (*36*).

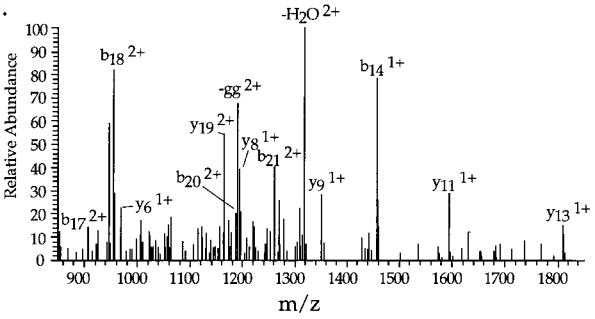
N-terminus of γ_5. Notably, we observed ions compatible with the loss of 12 consecutive residues (residues 4−15) at the N-terminus of γ_5, which is the most variable region of γ subunits. These data strongly support the conclusion that the 7500 dalton protein is in fact a γ_5 subunit isoform.

Figure 8 shows the MS/MS/MS analysis of a possible C-terminal fragment ion (y_{22}) selected from the MS/MS spectrum, m/z 1326.2 (Figure 6). A series of y ions in this spectrum (y_6, y_8, y_9, y_{11}, y_{13}, and y_{19}) were compatible with C-terminal fragments of a γ_5 isoform 333 mass units greater than that of the predicted protein. Further analysis of the MS/MS/MS spectrum revealed sequence information about the C-terminus of the protein. The signal at m/z 1261.2 (labeled b_{21} in Figure 8) is compatible with the loss of a leucine from the y_{22} fragment, implying that leucine (or isoleucine) is the C-terminal residue. This is significant because the last four residues of γ_5 are CSFL, suggesting that the 7502.5 dalton protein is a γ_5 isoform that is not proteolytically processed even though it is geranylgeranylated. The b_{20} and b_{18} ions in the MS/MS/MS spectrum (Figure 8) substantiate this conclusion. The b_{20} ion results from loss of both leucine and phenylalanine (FL), while the b_{18} ion results from loss of the cysteine possessing the geranylgeranyl group along with the three terminal amino acids (C+gg SFL).

A.



B.



C.

Fragment	Residues	Predicted mass	z^a	Predicted Ion	Observed Signal
loss of gg	47-68	2378.8	2+	1189.9	1189.8
loss of H_2O	47-68+gg	2633.2	2+	1317.1	1317.6
b_{21}	47-67	2520.1	2+	1260.5	1261.2
b_{20}	47-66	2372.9	2+	1186.9	1186.6
b_{18}	47-64	1911.2	2+	956.1	955.8
b_{17}	47-63	1812.1	2+	906.5	907.1
b_{14}	47-60	1458.6	1+	1458.6	1457.9
y_{19}	50-68+gg	2327.8	2+	1164.4	1164.6
y_{13}	56-68+gg	1809.3	1+	1809.3	1810.2
y_{11}	58-68+gg	1594.1	1+	1594.1	1594.1
y_9	60-68+gg	1349.8	1+	1349.8	1349.8
y_8	61-68+gg	1193.6	1+	1193.6	1193.7
y_6	63-68+gg	968.4	1+	968.4	969.1

FIGURE 8: (A) Sequence of γ_5 amino acids 47−68 identifying the possible b and y ions observed. (B) MS/MS/MS spectrum of C-terminal y_{22} fragment ion of the 7502.5 dalton protein, *m/z* 1326.0 from Figure 5 selected, average of 70 scans. (C) Table of predicted and observed ion signals: $^a z$ = charge state of fragment.

DISCUSSION

These data indicate that the late γ_5 isoform associated with G_o and G_i proteins, the principal G proteins in bovine brain, is geranylgeranylated, but neither proteolytically processed nor carboxymethylated. The predicted $[M+H]^+$ ion of such a γ_5 isoform is 7502, with N-terminal and C-terminal acid hydrolysis fragments of *m/z* 4870 and *m/z* 2651, respectively. These predictions agree with our measurement of the intact protein molecular weight by MALDI mass spectrometry ($[M+H]^+$, *m/z* 7500 ± 1, $n = 20$), with the ESI molecular weight of 7502.5 ± 0.5 ($[M+H]^+$, *m/z* 7503.5 ± 0.5), and with the observed D−P fragments of *m/z* 4868 and *m/z* 2651 (Figure 4B).

These results were unexpected and are significant for several reasons. First, they identify a new pattern of C-terminal processing for G protein γ subunits. Second, since we also found γ_5 with the predicted C-terminal processing pattern, this is the first demonstration of heterogeneous processing of G protein γ subunits. These results are important because there are a large number of proteins processed by isoprenylation, many of them involved in intracellular signaling pathways (*21, 23*). These results also have functional implications because the complex C-terminal modifications of γ subunits play an important role in G protein interactions with membranes (*37*) and with other proteins. For example, the A1 adenosine receptor prefers γ subunits with a geranylgeranyl group (*38*), whereas rhodopsin may prefer γ subunits with a farnesyl group (*39*). Furthermore, the C-terminal processing of γ is required for high-affinity interactions of $\beta\gamma$ dimers with α subunits or for $\beta\gamma$ dimers to regulate effector enzymes such as adenylyl cyclase (*40*−*42*). Additionally, carboxymethylation of γ, which may be a receptor-regulated modification (*43*), may increase membrane association of $\beta\gamma$ dimers (*44*). All of these results indicate that C-terminal processing of G protein γ subunits is an important determinant of signaling efficacy and specificity, and that variations in the processing pattern at this site will have functional significance.

The possibility that this unusual γ subunit is a processing intermediate with little physiological significance is unlikely. First, this novel γ_5 isoform is more abundant in our G protein preparations than the fully processed γ_5 by both immunoblotting (Figure 1B,C) and MALDI analysis (Figure 3). Second, we do not find analogously modified γ_2 and γ_3 subunit isoforms, even though they are of much greater abundance in bovine brain than is γ_5. Third, the γ subunits analyzed were purified from detergent extracts of membrane preparations, implying their functional expression in bovine brain. Finally, this isoform is consistently present in bovine brain G protein, having been seen in 10 HPLC separations made from 6 independent G protein preparations. All of these observations argue that this is a specific processing pattern important for the function of this γ subunit isoform.

Based upon quantitative immunoblotting, the novel γ_5 protein reported here accounts for at least 40% of the γ_5 immunoreactivity in our G protein preparations, whereas γ_5 with the predicted processing pattern accounts for only 5% (Figure 2). Two major G protein γ subunits in preparations of brain G_o/G_i are γ_2 and γ_3. These are processed at the C-terminus in the same way as the early γ_5 protein. This would seem to indicate that the purification methods used to isolate brain G proteins do not select against heterotrimers containing γ subunits with this type of modification pattern. This suggests then that our late γ_5 protein represents the predominant γ_5 isoform associated with the G_o and G_i proteins in bovine brain. It is important to note, however, that our preparations do not contain significant amounts of G_s, G_q, or G_z which are also found in bovine brain, but in lower amounts than the G_i and G_o proteins. We therefore cannot exclude the possibility that normally processed γ_5 is preferentially associated with one of those G proteins and represents a greater fraction of the total γ_5 in brain than suggested by our quantitative comparison. There is evidence that G protein heterotrimers do contain specific combinations of α and γ subunit isoforms (*25, 45, 46*). The significance of this is not entirely clear, but it will be interesting to determine if such selective associations are in part due to different patterns of γ subunit processing.

C-terminal processing involving prenylation affects a large and diverse set of proteins. This modification is usually an all or none event including C-terminal proteolysis and carboxymethylation (*21*). The only physiologically observed exceptions to this previously reported are the α and β subunits of rabbit skeletal muscle glycogen phosphorylase kinase, which retain the C-terminal sequences CAMQ and CLVS, respectively, after farnesylation (*47*). Our results also indicate that C-terminal proteolysis does not necessarily follow prenylation and that this alternative processing pattern occurs with geranylgeranylated as well as farnesylated proteins. It is interesting to speculate that this processing pattern might be sequence dependent, although the determinants of this are not yet clear. A similar processing pattern

has been observed for mutant Ras constructs with the C-terminal sequences CVYM and CVGM instead of CVIM (wild type), which are farnesylated without further processing (*48*). Among Gγ subunits, the C-terminal sequence in γ_5, CSFL, is unique, possessing an aromatic amino acid which is similar to the CVYM Ras mutants. However, the relationship of these sequences to the other Ras mutant (*48*) and those of the phosphorylase kinase subunits (*47*) is not obvious.

Recently, it was shown in yeast that disruption of a gene coding for the protease that removes the three C-terminal amino acids of prenylated proteins results in the redistribution of Ras2p to internal membranes (*49*). This suggests, at least in yeast, that proteolytic processing of prenylated proteins is involved in determining their cellular distribution. A similar phenomenon may also occur in mammalian cells. For example, the γ_5 subunit is preferentially localized to focal adhesions, while other γ subunits are not (*13*). Interestingly, those experiments indicate that in addition to specific association of γ_5 with focal adhesions, there is also a low level of diffuse γ_5 staining throughout the cell, similar to that of other Gγ subunits (*13*). Based upon these observations in yeast (*49*) and mammalian cells (*13*), and on the identification here of two different γ_5 processing patterns, we suggest that the differential processing of γ subunits could provide a component of the cellular mechanisms targeting G proteins to different locations in the cell. Testing this hypothesis will require an understanding of the cellular and molecular requirements for differential processing of γ subunits.

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