Sequence Dependence and Differential Expression of Gγ5 Subunit Isoforms of the Heterotrimeric G Proteins Variably Processed after Prenylation in Mammalian Cells*

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Between 1 and 2% of proteins coded for in the human genome, including all G protein γ subunits, are predicted to be prenylated. Subsequently, prenylated proteins are proteolytically cleaved at the C terminus and carboxymethylated. These reactions are generally obligatory events required for functional expression of prenylated proteins. The biological role of prenyl substrates has made these reactions significant targets for anticancer drug development. Understanding the enzymology of this pathway will be key to success for this strategy. When Gγ1, -2, -4, -10, -11, -12, and -13 were expressed in HEK293 cells they were completely processed according to the current understanding of the prenylation reaction. In contrast, Gγ5 was processed to two forms; a major one, fully processed as predicted, and a major one that was prenylated without further processing. When the Cα₂X motif of Gγ5, CSFL, was exchanged for that of Gγ2, CAIL, Gγ5 was completely processed. Conversely, Gγ2-SFL was incompletely processed. Differential processing of Gγ5 was found due to the presence of an aromatic amino acid in its Cα₂X motif. Retrieving endogenous Gγ subunits from HEK293 or Neuro-2a cells with FLAG-Gβ constructs identified multiple Gγ subunits by mass spectrometry in either cell, but in both cases the most prominent one was Gγ5 expressed without C-terminal processing after prenylation. This work indicates that post-prenylation reactions can generate multiple products determined by the C-terminal Cα₂X motif. Within the human genome 10% of predicted prenylated proteins have aromatic amino acids in their Cα₂X sequence and would likely generate the prenylation pattern described here.

One to two percent of proteins coded for in the human genome are thought to be substrates for prenylation (1–6). These include a number of proteins affecting cell growth, including the Ras and Rho proteins, numerous other small GTPases, many phosphatases and kinases, the nuclear lamins A and B, and all of the Gγ subunits of the heterotrimeric G proteins involved in cell signaling. The role of prenylation in protein function appears to be multiple and complex; it affects membrane localization, intracellular trafficking, and protein-protein interactions. In general, prenylated proteins require correct processing to mediate their biological functions (7–10).

Protein prenylation is catalyzed by one of three prenyl transferases that adds either a farnesyl or a geranylgeranyl group to a Cys residue four amino acids from the C terminus of target proteins (1, 5). The prenyl group transferred is primarily, but not exclusively (11), determined by the C-terminal amino acid of the protein where a geranylgeranyl group is transferred in the case of Leu and sometimes Phe, and a farnesyl is generally transferred in the case of Ser, Met, Asn, Ala, and sometimes Cys (5, 12). Following prenylation, the last three amino acids are cleaved by a specific protease (13). In yeast, there are two prenyl transferases, Rce1 and Afc1/Ste24 (13), each with fairly broad specificity (14). In vertebrates, Rce1 appears responsible for most proteolysis of prenyl proteins (15, 16), whereas the specificity of the homolog of Afc1/Ste24, Zmpste24, is more limited, targeting in particular prelamin A (17). Finally, the new C terminus of the protein is carboxymethylated (5, 6) and mediated by a specific carboxymethylase (18).

The involvement of prenylated proteins in the regulation of cell growth, their relatively low number, and the specialized enzymology of this modification have resulted in all three steps in this enzymatic cascade being targeted for production of anticancer drugs (5, 19, 20), as well as for treatment of other diseases where prenylation or prenylated proteins play a role (21). Prenyl transferase inhibitors are currently in Phase III clinical trials, with both advantages and disadvantages. They appear to be promising for treatment in a number of tumors, but they have lower efficacy, particularly for solid tumors, than first envisioned (19), and alternative strategies have been suggested for their use, such as adjunct treatment with other cancer drugs (22). One issue with the further development of these drugs is that their relevant substrates have turned out to be ambiguous and difficult to determine. For example, it is uncertain that the Ras proteins for which the drugs were originally designed are related to the pharmacological effects of these drugs (23). Another issue is that the complex enzymology involved seems to include effects of inhibitors causing a switch in the biological targets of different enzymes (24).

An alternative strategy for future therapies using prenyl transferase inhibitors is the development as adjunct therapy drugs targeting the carboxymethylation and proteolysis steps (5). The mammalian Rce1 enzyme that catalyzes proteolysis of the C terminus of prenyl proteins is a unique metalloprotease

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primarily targeting prenylated proteins (25). In general, it is thought to target most or all naturally occurring prenylated proteins, and a recent review identified no significant or important exceptions to this (5). Deletion of this enzyme is lethal during embryonic development or shortly after birth (15), indicating the importance of this reaction. Undoubtedly, the successful development of Rce1 as a therapeutic target, as has proved true for the farnesyl/geranylgeranyl transferase inhibitors (5, 19, 23, 24), will depend upon correctly understanding the substrate specificity and diversity of Rce1. Previously, we found that Gγ5 associated with purified bovine brain G proteins is largely unprocessed by either proteolysis or carboxymethylation after prenylation (26). The significance of this observation has remained unclear, however, and this processing pattern has been perceived as likely explained by isolation of a precursor or an abnormally processed protein. Here, we have investigated the origin and significance of this unprocessed protein after expression of G protein subunits in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—FLAG-Gγ and FLAG-Gβ constructs in pcDNA3 were recently described (27) and corresponded to human (γ1, γ12, and γ13), bovine (γ2) or rat (γ3) sequences. FLAG-tagged γ5 and γ2 were further modified using the QuickChange kit (Stratagene) to generate chimeras by swapping the three C-terminal residues of each isoform: Gγ5-AIL and Gγ2-SFL. Finally, a series of FLAG-tagged γ5 constructs were generated with QuickChange substituting codons for all combinations of aromatic amino acids into the aliphatic positions within the Ca3a2X motif (Table 1). The sequence of all constructs was verified by complete DNA sequencing of the final insert.

**Cell Culture and Transfection**—HEK293 and Neuro-2a cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium (MediaTech) supplemented with 1-glutamine, pyruvate, non-essential amino acids, and 10% fetal bovine serum. One day prior to transfection, cells were plated onto 100-mm diameter dishes (3.5 × 10^6 cells/dish). Co-transfection with cDNA for all three subunits (αi1, 3 μg; βg, 3 μg; and FLAG-γ, 12 μg), was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Post-transfection cells (40–48 h) were rinsed twice with phosphate-buffered saline without calcium or magnesium (MediaTech) and scraped into 1.5-ml microcentrifuge tubes with 1 ml of phosphate-buffered saline. Cells were pelleted by centrifugation (800 × g, 10 min, 4 °C), and the supernatant was removed prior to snap freezing in liquid nitrogen.

**Immunoprecipitation**—Frozen cell pellets were thawed on ice before addition of 600 μl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM MgCl2, 10 μM GDP, 5 mg/ml DNase I, 10 μg/ml RNase A, 1% protease mixture mix 1 (Calbiochem), 1% cholate) and incubation for 1 h at 4 °C in a rotating mixer. Lysate was then centrifuged for 15 min (16,000 × g, 4 °C), and the supernatant transferred to a new tube. FLAG monoclonal antibody-conjugated agarose beads (Sigma, 10 μl of 50% slurry) were added, and the mixture was incubated for 3 h while rotating at 4 °C. Beads were precipitated by 10-s centrifugation at 300 rpm in a microcentrifuge, the supernatant removed, and the beads were washed twice with cold Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl) and twice with cold H2O. Bound proteins were eluted with 70% acetonitrile/0.1% trifluoroacetic acid (50 μl).

**MALDI-MS**—Eluted FLAG-tagged proteins were concentrated by vacuum spin drying to <1 μl volume and resuspended with 5 μl of 50% acetonitrile/0.1% trifluoroacetic acid. The matrix (sinapinic acid, 10 mg/ml) was solubilized with acetonitrile-trifluoroacetic acid. Protein mass standards (Calibration mixture 3, Applied Biosystems) were mixed with the matrix, and both matrix alone and matrix with calibrant were pre-spotted for each sample and allowed to fully air dry. Each protein sample (1 μl) was mixed with matrix (1 μl), and 0.5 μl of protein-matrix mixture was applied to both the matrix and calibration mixture dried spots to obtain spectra with internal and external calibration. MALDI² was performed on an Applied Biosystems Voyager DE-STR Biospectrometry workstation in linear mode. Calibration was performed using the two closest neighbors bracketing the mass of the sample. Observed values are reported with mass to charge ratio (m/z) of singly protonated proteins (M + H)⁺.

**Immunoblots**—One microliter of concentrated eluted protein sample was dried down and resuspended in SDS sample buffer. Samples were separated by SDS-PAGE on 8–16% gradient Tris-HCl gels (Bio-Rad Criterion gel) and transferred to 0.1 μm of nitrocellulose. Membranes were incubated with FLAG polyclonal antibody (1:50,000, Sigma) for 1 h, washed four times with Tris-buffered saline/Tween 20, and incubated with

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**TABLE 1**

| Construct | Primer sequence (forward) |
|-----------|---------------------------|
| Gγ2       | GG GAG AAG AAG TTC TCC TCT ACT CAT CTT TTA GTC TGC TCG |
| Gγ2-SFL   | GG GAG AAG AAG TTT TTC TCT TCC TTG TTT TGG TAA CAT CAT CAG |
| Gγ5       | -C AGA CCC CAG AAA GTC TGC TCC TCT TCT TGG TAG TGA TCA GAT CTT |
| Gγ5-AIL   | -- -- -C CAG AAA GTC TGC TCC TCA TCT TTA TGG TAG TCA TAT CAT |
| Gγ5-SIL   | -C AGA CCC CAG AAA GTC TGC TCC TTA TTA TGG TAG TAC TAT CAT |
| Gγ5-SYL   | -C AGA CCC CAG AAA GTC TGC TCC TTA TTA TGG TAG TAC TAT CAT |
| Gγ5 (VIL) | -C AGA CCC CAG AAA GTC TGC TCC TTA TTA TGG TAG TAC TAT CAT |
| Gγ5 (WIL) | -C AGA CCC CAG AAA GTC TGC TCC TTA TTA TGG TAG TAC TAT CAT |
| Gγ5 (FIL) | -C AGA CCC CAG AAA GTC TGC TCC TTA TTA TGG TAG TAC TAT CAT |

Indicated are the forward primers used for site-directed mutagenesis using QuikChange to prepare Gγ5 sequences for the corresponding regions.
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secondary anti-rabbit polyclonal antibody (1:20,000) for 30 min. ECL was performed using the Pierce West-Femto SuperSignal, and images were captured on a Bio-Rad FluorImager.

RESULTS

Mass spectrometry (MS) has proven to provide an effective approach for characterizing the post-translational modifications of the Gγ subunits of the heterotrimeric G proteins purified from brain (11, 28–30). Here we have used MS to characterize the processing of expressed and endogenous Gγ subunits in cultured cells. Gγ subunits were expressed as FLA(FLAG)-tagged proteins in HEK293 cells and isolated by immunoprecipitation on anti-FLAG beads after detergent solubilization of cell pellets. Although expression and recovery after FLAG immunoprecipitation were variable, as evaluated on FLAG immunoblots (Fig. 1, IP), all the expressed proteins generated robust MALDI-TOF MS signals (Fig. 2). Samples from cells expressing FLAG-Gγ1 (Fig. 2A), FLAG-Gγ2 (Fig. 2E), FLAG-Gγ4 (Fig. 2B), FLAG-Gγ12 (Fig. 2C), and FLAG-Gγ13 (Fig. 2D) all generated MS spectra containing a single very prominent mass in the 8- to 10-kDa mass range characteristic of singly charged FLAG-Gγ proteins. In contrast, precipitates from control pcDNA-transfected cells generated no appreciable signal in this mass range (data not shown). The average masses observed in repeated independently isolated samples were characteristic of the Gγ isoform expressed, had errors (S.D.) of 1.6 Da or less, and were within 1.1 Da or less (113 parts per million; range 45–190 ppm) of the predicted mass of the correct subunit isoform modified as expected from past studies of prenylated proteins (Fig. 2). Thus, FLAG-Gγ1 had a mass compatible with that of a farnesylated protein, whereas all of the others had masses compatible with geranylgeranylated proteins, as predicted by their C-terminal Ca1a2X sequence. Additionally, all of the masses were compatible with those of a FLAG-tagged protein with an acetylated N terminus.

In contrast to the other Gγ isoforms, the most prominent mass after expression of FLAG-Gγ5 was substantially different from that predicted according to accepted understanding of prenyl processing. The predicted FLAG-Gγ5 should have a mass of 8294.6, but the most prominent signal had an m/z ratio of 8628.0 ± 1.1 (Fig. 2G, peak a). This corresponds to the pre-
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predicted mass of 8628.0 for the FLAG-tagged version of a Gγ5 protein prenylated with a geranylgeranyl moiety but lacking the expected C-terminal proteolysis and carboxymethylation. Such a Gγ5 protein was previously characterized in bovine brain G proteins (26). Just as in that case, there was a minor peak (Fig. 2G, peak b) at 8294.4 m/z that was compatible with the protein processed as predicted. The similarity of the spectrum in Fig. 2G to that previously found for bovine brain G protein Gγ5 suggests that differential proteolysis of the C terminus of Gγ5 is a characteristic of the processing of this protein in mammalian cells. An initial hypothesis was that this processing pattern depended directly on the C-terminal Ca1a2X prenylation sequence of the protein. To test this possibility, we expressed a chimeric FLAG-Gγ5 protein containing the Ca1a2X sequence from Gγ2 (CAIL) instead of its parent sequence (CSFL). This chimeric FLAG-Gγ5-AIL protein generated a single prominent peak (Fig. 1H) characteristic of the predicted processed Gγ5 protein, which was geranylgeranylated with proteolysis of AIL and carboxymethylation of the new C terminus. In contrast, expression of a chimeric FLAG-Gγ2-SFL construct generated multiple peaks (Fig. 2F), the most prominent of which (peak a) was within 1 Da (33 ppm) of the geranylgeranylated protein lacking C-terminal proteolysis and carboxymethylation. Another mass (peak d), as with Gγ5, was compatible with correctly processed Gγ2, suggesting that the -SFL sequence of Gγ5 is weakly processed by Rce1.

Whereas expressed FLAG-Gγ5 has a primary mass indicative of prenylation without further processing (Figs. 2G and 3A), the FLAG-Gγ5-AIL chimera is processed normally (Figs. 2H and 3B). This indicates that the Gγ5 Ca1a2X sequence determines the susceptibility of the protein to proteolysis by Rce1. Gγ5 has the hydrophilic amino acid Ser at position a1 of its Ca1a2X sequence instead of an aliphatic amino acid. To determine if this Ser determines susceptibility to proteolysis we tested a mutant FLAG-Gγ5-AFL construct (Fig. 3C), which was also found to be insensitive to proteolysis by Rce1. Thus, the presence of a hydrophilic amino acid at position a1 does not seem to account for differential processing by Rce1; a conclusion supported also by the observation that two other Gγ subunits, γ4 and γ13, which are processed normally (Fig. 2, B and D), contain a threonine in that position. More significantly, the Ca1a2X sequence of Gγ5 differs from that of all other Gγ subunit isoforms in that it contains an aromatic amino acid at position a2 (CSFL) instead of a canonical aliphatic amino acid. In contrast to unmodified Gγ5 (Fig. 3A) ending in -SFL, the -SIL mutant (Fig. 3D) was correctly processed, as was the protein ending in the native Gγ2-AIL sequence (Fig. 3B). This indicates that it is the Phe in the Gγ5 Ca1a2X motif that is responsible for poor proteolytic processing by Rce1. Replacing the Phe at a2

**FIGURE 3.** MALDI-TOF spectra of FLAG-Gγ5 isoforms containing variant C-terminal prenyl processing signals. HEK293 cells were transfected with FLAG-Gγ cDNAs, and detergent lysates were prepared and used to recover

FLAG-tagged proteins for MALDI-TOF MS as described under "Experimental Procedures." Masses reported are based on measurements with internal calibrants and are reported as mean ± S.D. of data from three independent experiments. Also indicated in each panel are the cDNA insert and the predicted masses for the proteolytically processed and unprocessed proteins of the Gγ variant. A, normal Gγ5; FLAG-Gγ5-(SFL); B, FLAG-Gγ5-(AIL); C, FLAG-Gγ5-(FIL); D, FLAG-Gγ5-(SIL); E, FLAG-Gγ5-(SYL); F, FLAG-Gγ5-(SWL); G, FLAG-Gγ5-(WIL); H, FLAG-Gγ5-(YIL); and I, FLAG-Gγ5-(WIL). The y axis indicates relative signal intensity.
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with either Tyr (Fig. 3E) or Trp (Fig. 3F) also generated a \( \gamma_5 \) protein resistant to Rce1 proteolysis. Finally, the effect of an aromatic amino acid in the \( \alpha_2 \) position but also resulted from any aromatic amino acid in the \( \alpha_1 \) position (Fig. 3, G–I). Thus, any aromatic amino acid (Phe, Tyr, or Trp) at either the \( \alpha_1 \) or \( \alpha_2 \) position in the Ca1a2X sequence resulted in expression of a protein that was prenylated without further C-terminal processing. Interestingly, most (but not all) of these point mutants showed more complete suppression of the C-terminal processing than was even found in the case of the unmodified -SFL sequence. Most, in fact, lacked any evidence of production of a processed protein.

The data in Figs. 2 and 3 were generated with \( \gamma \) constructs containing a FLAG epitope tag. To substantiate that the results obtained were not dependent upon the epitope tag, an alternative expression strategy was used whereby untagged \( \gamma \) constructs were co-expressed with FLAG-G\( \beta_1 \) (Fig. 4). In the case of \( \gamma_2 \), once again, FLAG immunoprecipitation generated a single prominent peak with a singly charged \( m/z \) of 7750.1 consistent with the predicted N- and C-terminal modifications (Fig. 4C) previously observed for native bovine brain \( \gamma_2 \) (31). Co-expression of \( \gamma_5 \) with FLAG-G\( \beta_1 \) resulted in recovery of a primary peak of 7502.1 corresponding to prenylated \( \gamma_5 \) without further C-terminal processing, but with correct N-terminal processing (Fig. 4C), as reported previously (26). A second, less prominent peak, was also observed at 7168.7 that corresponded to the fully processed \( \gamma_5 \) with a predicted mass of 7168.4, as also had been previously observed (26). In fact, all of the remaining \( \gamma \) constructs characterized in Fig. 2, when expressed as untagged proteins in the presence of G\( \beta_1 \), generated a primary mass very consistent with that predicted for both correct N- and C-terminal processing (Fig. 4C). These results substantiate correct processing of multiple forms of expressed \( \gamma \) isoforms and their functional formation of a \( \gamma \) dimer based upon co-immunoprecipitations with FLAG-G\( \beta_1 \).

Although the data in Fig. 4 indicated preferential association of expressed FLAG-G\( \beta_1 \) with co-expressed \( \gamma \) constructs, the spectra obtained often had minor masses that seemed to be compatible with predicted masses of processed \( \gamma \) isoforms. When FLAG-G\( \beta_1 \) isoforms were expressed without an associated \( \gamma \) construct in HEK293 cells, mass spectra of FLAG immunoprecipitation samples showed numerous peaks in the \( \gamma \) subunit range, at least for G\( \beta_1 \)–G\( \beta_4 \) (Fig. 5, A–D). In contrast, as reported by others (32) and as found in vitro (27), FLAG-G\( \beta_3 \) failed to form stable dimers with any proteins in this mass range (Fig. 5E). The distinct signals found associated with G\( \beta_1 \)–G\( \beta_4 \) were generally consistent with one another and had average masses compatible with expression of multiple \( \gamma \) proteins processed as predicted, including, in particular, \( \gamma_5 \), \( \gamma_{10} \), \( \gamma_{11} \), and \( \gamma_{12} \) (Fig. 5G). Of these, the one that appeared to be differentially associated with G\( \beta_8 \) isoforms was \( \gamma_11 \), which appeared to be associated with G\( \beta_1 \) and G\( \beta_4 \) (Fig. 5, A and D) but not with G\( \beta_2 \) and G\( \beta_3 \) (Fig. 5, B and C). In addition, there were also weaker but consistently observed signals compatible with \( \gamma_7 \), \( \gamma_8 \), and three masses that did not correspond to any known human \( \gamma \) variant. Most notable about these experiments, however, in all of the samples (Fig. 5, A–D), the most prominent signal was that corresponding to the unprocessed \( \gamma_5 \)-SFL protein characterized as the primary variant of expressed \( \gamma_5 \). In all cases too, however, there was a mass characteristic of the fully processed \( \gamma_5 \) protein, generally at lower intensity.

To determine the generality of the approach used in HEK293 cells to retrieve endogenous \( \gamma \) proteins with FLAG-G\( \beta \) constructs, similar experiments were carried out with Neuro-2a cells, a mouse neuroblastoma cell line (33). In this case, a signal corresponding to G\( \beta_2 \) was found that was not present in HEK293 cells and is indicative of the neural origin of these cells (33). Even in this case, however, the other signals identified corresponded to those for \( \gamma_5 \) and the most prominent signal.

![FIGURE 4. MALDI-TOF spectra of expressed untagged \( \gamma \) constructs recovered from cells co-transfected with FLAG-G\( \beta_1 \). HEK293 cells were transfected as described under “Experimental Procedures” with untagged \( \gamma \) constructs and FLAG-G\( \beta_1 \), and detergent lysates were prepared and used to recover FLAG-G\( \beta_1 \) and associated \( \gamma \) proteins for MALDI-TOF as described under “Experimental Procedures.” Masses reported are based on measurements with internal calibrants and are reported as mean ± S.D. of data from three independent experiments. A, MALDI spectrum for cells transfected with G\( \gamma_2 \) and FLAG-G\( \beta_1 \); B, MALDI spectrum for cells transfected with G\( \gamma_5 \) and FLAG-G\( \beta_1 \); and C, summary of data from cells transfected with FLAG-G\( \beta_1 \) and different \( \gamma \) isoforms. Structural notation is as described in the legend of Fig. 1.](image)
FIGURE 5. MALDI-TOF spectra of endogenous Gγ recovered from HEK293 cells or Neuro-2a cells in association with FLAG-Gβ isoforms. Cells were transfected as described under “Experimental Procedures” with FLAG-Gβ1 and processed as described in legends of Figs. 1–3. A–E, HEK293 cells; F, Neuro-2a cells; A and F, transfection with FLAG-Gβ1; B, FLAG-Gβ2; C, FLAG-Gβ3; D, FLAG-Gβ4; and E, FLAG-Gβ5. G, summary of mass estimates for the masses numbered under each spectra giving mean mass observed, ± S.D. of estimates, possible Gγ assignment, and predicted mass of likely Gγ isoform processed as predicted.
was for the Gγ5-SFL variant. These results indicate that variable processing of Gγ5 is a general phenomenon in mammalian cells, that the proteolytically unprocessed form is the predominant variant expressed in cells and that, at least as assayed by cells, the proteolytically unprocessed form is the predominant Cys-a1-a2-

| Accession number | Ca_a2X sequence | Predicted prenyl Gp | Description |
|------------------|------------------|---------------------|-------------|
| Hs.523628        | CAYL             | GG                  | Death-associated protein; mitochondrial 28 S ribosomal protein |
| Hs.223782        | CFPP             | GG                  | Extraembryonic, spermatogenesis, homeobox 1-like |
| Hs.30875         | CYFL             | GG                  | Hypothetical protein LOC284033 |
| Hs.191540        | CLVL             | GG                  | Cohen syndrome 1 protein isoform 4 |
| Hs.407709        | CFKL             | GG                  | DRE1 protein |
| Hs.517666        | CFVF             | GG                  | Cytochrome b, reductase membrane-bound isoform |
| Hs.554749        | CSFL             | GG                  | Guanine nucleotide-binding protein (G protein), γ5 |
| Hs.523529        | CVGF             | GG                  | Predicted: similar to formin-binding protein 2 |
| Hs.534990        | CWVF             | GG                  | Predicted: hypothetical protein XP_498894 |
| Hs.554966        | CYLF             | GG                  | Predicted: similar to involucrin |
| Hs.301345        | CFAF             | GG                  | Predicted: hypothetical protein XP_498499 |
| LOC441457        | CTFE             | GG                  | Predicted: hypothetical protein XP_499191 |
| LOC440222        | CPWL             | GG                  | Predicted: hypothetical protein XP_498593 |
| LOC440227        | CPWL             | GG                  | Predicted: hypothetical protein XP_498594 |
| XP_497522        | CSYL             | GG                  | Predicted: similar to 60 S ribosomal protein L7a |
| Hs.449875        | CWDL             | GG                  | Predicted: hypothetical protein XP_498847 |
| Hs.151216        | CFNF             | GG                  | Hypothetical protein PP1057 |
| Hs.504517        | CYLA             | Far                 | Hypothetical protein DKFPz4p34F054 |
| Hs.459632        | CEYS             | Far                 | Sarcoglycan α; -sarcoglycan |
| Hs.195298        | CLWS             | Far                 | Hypothetical protein MGC33345, zinc finger |
| Hs.111501        | CFPS             | Far                 | Hypothetical protein FLJ32421 |
| Hs.552608        | CIYS             | Far                 | Nei-like 2 |
| Hs.293818        | CQFS             | Far                 | Sialidase 4; neuraminidase 4 |
| Hs.414300        | CWPS             | Far                 | Lamin R2 |
| Hs.515092        | CVYM             | Far                 | Predicted: hypothetical protein XP_211983 |
| Hs.449297        | CYSS             | Far                 | RAB37, member of the Ras oncogene family |
| Hs.351413        | CSFM             | Far                 | Hypothetical protein LOC283932 |
| Hs.621392        | CWEM             | Far                 | Chemokine-like factor superfamily 1 isoform 6 |
| Hs.554909        | CSFQ             | Far                 | 1-mfa domain-containing protein isoform p40 |
| Hs.462392        | CFPS             | Far                 | Hypothetical protein FLJ20308 |
| Hs.413801        | CYYA             | Far                 | Proteasome activator subunit 4; 200 kDa |
| Hs.128814        | CNWA             | Far                 | Eosinophil cationic cytochrome GPI deacetylase |
| Hs.229988        | CNFM             | Far                 | AAA1 protein isoform V |
| Hs.487951        | CFPS             | Far                 | Zinc finger protein 517 |
| Hs.521942        | CWAS             | Far                 | Cyclin G2 |
| Hs.521291        | CFPS             | Far                 | Myoβ family inhibitor |
| Hs.554795        | CKWM             | Far                 | PlexinC1; receptor for virally encoded semaphorin |
| Hs.534729        | CWLQ             | Far                 | Predicted: similar RIKEN cDNA R203096012 |
| Hs.120196        | CCFS             | Far                 | Predicted: hypothetical protein XP_379181 |
| Hs.529122        | CYQQ             | Far                 | β-1,4-Galactosyltransferase 7 |
| Hs.455109        | CTFS             | Far                 | Collagen, type V |
| Hs.235368        | CFSS             | Far                 | |

**DISCUSSION**

The studies reported here were designed primarily to elucidate the requirements of the prenylation pathway for variable processing of the Gγ5 subunit isoform. In addition, however, these studies have established a number of other findings related to Gγ5 or to general questions about the expression, processing, and function of Gβ and Gγ subunits in intact cells. These observations include the following: 1) These studies extend the use of MS to studying processing of Gγ subunits in cultured cells either by capturing them directly or by capturing them indirectly by their association with Gβ subunits. Part of the power of this approach is to be able to evaluate completely the processing of these proteins. 2) These studies verify that many Gγ isoforms, including Gγ1, -2, -4, -12, and -13, are expressed and processed in cells as predicted by past studies of the processing of prenylated proteins (Fig. 2). These proteins are also processed as predicted at the N terminus as to, for example, whether or not they are acetylated (Fig. 4). 3) For all of the Gγ isoforms studied, essentially all of the expressed Gγ made in the cell either forms a functional dimer with Gβ or is
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During neural development and Gγ5, previous studies suggest that Gγ5 signals for endogenous Gγ5, is preferentially expressed in a neuro-related source, as bovine brain (26). This may be related to recent studies suggesting that they are, they appear to be preferentially substrates of Afc1p/Ste24p rather than Rce1p (14). The apparently greater conservation of Gγ5, which ends in CSFL (26) and is remarkable compared with most other prominent Ca1a2X substrates in its inclusion of an aromatic amino acid. This residue appears to define the insensitivity of this protein to proteolytic processing after prenylation (Fig. 3). The Gγ5 Ca1a2X sequence is both unique to Gγ5 sequences in containing an aromatic amino acid and is highly evolutionarily conserved throughout the vertebrate phylum (6). This is a strong argument for the importance of this variation in prenyl processing for this protein. In addition, there are at least 44 proteins or predicted proteins in the human genome (Table 2) that are potentially modified in a similar way. These proteins belong to many different classes of proteins found in varying cellular compartments and organelles, suggesting that the functional role of this processing would relate to specific protein-protein interactions rather than general targeting to a common cellular site (7–10).

Prior to our identification of the processing pattern of Gγ5 (26) and its sequence dependence described here, past studies were inconclusive that this variation in prenyl processing would be relevant to major prenyl substrates. Based upon analysis of 3- and 4-amino acid peptides, many of those with aromatic amino acids a1 and a2 were thought likely to be substrates of a purified rat liver Rce1-like activity (41). This is not the case for Gγ5 where any aromatic amino acid at either the a1 or a2 position prevents processing (Fig. 3). Although other endogenous prenyl substrates containing an aromatic amino acid have not yet been characterized, a mutant Ras protein containing Phe at a2 is poorly processed by Rce1, although it is also poorly prenylated (7), and small Ca1a2X peptide sequences containing aromatic amino acids are often prenylation inhibitors (42).

In yeast, aromatic amino acids at a1 or a2 also often decrease prenylation, which complicate in this case too rigorous analysis of the effects of these residues on the proteolytic step. Nevertheless, several Ca1a2X sequences that contain aromatic amino acids do not appear to be substrates of either Afc1p/Ste24p or Rce1p (14). Other Ca1a2X sequences with aromatic amino acids, however, often do appear to be cleaved, but to the degree that they are, they appear to be preferentially substrates of Afc1p/Ste24p rather than Rce1p (14). The apparently greater role of Rce1 in targeting most prenyl substrates in vertebrates (15, 16) would then be compatible with general lack of processing of Gγ5 and other prenyl proteins containing an aromatic amino acid in their Ca1a2X sequence. From this, it is interesting to speculate that the small but consistent amount of fully-pro-
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processed Gγ5 we see in cells could result from the activity of Zmpste24, which seems to otherwise target somewhat selectively prelamin A (17).

In all cases where we have observed the variant processing of Gγ5 there are two components: one unprocessed as the major one and a second processed as predicted, including C-terminal proteolysis, and always nearly in the same ratio. This is true of purified bovine brain G proteins (11, 26), heterologously expressed Gγ5 without (Fig. 4B) or with (Fig. 1G) an epitope tag, and endogenous Gγ5 expressed in HEK293 cells or Neuro-2a cells (Fig. 5). This expression ratio appears to be evolutionarily conserved also, because even a conservative substitution with, for example, a Tyr (Fig. 3E) would eliminate production of the minor form, and this does not appear to be evolutionarily observed (cited from above). Thus, not only does prenylation of the minor form, and this does not appear to be efficiently processed by Rce1, but it also seems to be one designed to generate two forms of the protein with differential C-terminal processing, pointing to endogenous heterogeneity of prenyl substrates by design.

Another observation from these studies is that, at least as assayed by MALDI-MS, Gγ5 (and the Gγ5-SFL) in particular, is one of the more abundant Gγ subunits expressed in cells, at least in two diverse cell lines such as embryonic kidney cells (HEK293) and brain neuroblastoma (Neuro-2a). This is also true of NIH3T3 cells. These observations are consistent with the previous finding that Gγ5 is one of the most abundant and widely expressed mRNAs in cells. In MALDI-MS analysis of Gγ5 subunits in purified G proteins, signal intensities are roughly related to the amount of protein present, including for Gγ5 (11, 28–30). Nevertheless, it is likely that part of the predominance of the signal intensity in cells for Gγ5 (Fig. 5) is its abundance and part how readily it can be ionized compared with some other Gγ isoforms.

The studies reported here complement our recently reported proteomic analysis of the variation of Gγ proteins found in purified brain G proteins and, in particular, variation in prenyl processing of these proteins (11). Those studies demonstrated that the normal variation in expression of prenylated proteins is far greater than generally appreciated and nearly all of the observed Gγ isoforms are expressed as variably prenylated proteins to the level that they could be characterized in these samples. The current work indicates that, for those prenylated proteins containing an aromatic amino acid within their Cα1a2X motif, there is also likely variation in the proteolytically processed variants of the proteins observed. This variation would likely affect the efficacy or consequences of treatment with drugs targeting this reaction. In addition, the realization of a previously unrecognized interaction of aromatic amino acids with the Cα1a2X motif may provide information important for the development of more effective therapeutics targeting these reactions.

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