Yeast Glutamine-fructose-6-phosphate Aminotransferase (Gfa1) Requires Methionine Aminopeptidase Activity for Proper Function*

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The amino acid methionine is used almost universally to initiate protein synthesis. In eubacteria and in the organelles of eukaryotes, the initiator methionine is modified by the addition of a formyl group, which is subsequently removed, cotranslationally, by peptide deformylase (1–3). Thus, following peptide deformylase action, nearly all nascent proteins begin with an initiating methionine residue. However, a large subset of mature proteins have the initiator methionine removed, a process that occurs co-translationally and is catalyzed by the enzyme methionine aminopeptidase (MetAP).¹

There are two types of MetAPs, designated as type 1 and type 2 (4, 5). Although not an absolute rule, prokaryotes, which include eubacteria and archaeabacteria, will typically express a single isoform of MetAP, whereas eukaryotes typically express both MetAP1 and MetAP2. Additionally, organellar versions of MetAP have been identified in multiple organisms, including plants, fruit flies, and humans (6, 7).

MetAP activity is essential, as demonstrated by several deletion experiments, including deletion of the single MetAP in

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¹ The abbreviations used are: MetAP, methionine aminopeptidase; Gfa1, glutamine-fructose-6-phosphate aminotransferase; Ub, ubiquitin.

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and resistance to methyl mercury (19). The hexosamine biosynthesis pathway is also suggested to contribute to the negative effects of high glucose in mammalian cells (17).

Aminotransferases consist of two domains, a glutaminase domain, responsible for hydrolysis of glutamine to glutamate, and a synthase domain, responsible for amination of a substrate (20). Gfa1 is part of the glutaminase domain (20). Gfa1 is one of the more intriguing N-terminal nucleophile aminotransferases with respect to MetAP activity for several reasons. These include its requirement for yeast viability, the fact that the α-amino group of the catalytic N-terminal cysteine is exposed by the activity of MetAP, and the fact that the Gfa1 N-terminal sequence is conserved from yeast to humans.

In the reaction mechanism, the cysteine α-amino group serves as a general base catalyst. Experiments with a Gly0 mutant of E. coli Gfa1 demonstrate the importance of the presence of the cysteine α-amino group as revealed by a >100-fold decrease in enzyme activity (20). The conservation of the N-terminal sequence of Gfa1 suggests that the importance of the cysteine α-amino group is also conserved; however, this has not been evaluated, nor has the impact on the function of the enzyme in vivo.

The identification of MetAP substrates that contribute to the dependence of the cell on MetAP activity for normal growth and viability and that further require MetAP activity to perform their essential functions is a long-standing goal of our laboratory. Gfa1, for the aforementioned reasons, is one such candidate. We used the yeast system to evaluate the extent to which Gfa1 relies on MetAP action to perform its essential function. The results indicated that failure to expose the α-amino group of the N-terminal cysteine results in decreased enzyme activity and slow growth. Further, in yeast, either isoform of MetAP appeared sufficient to process Gfa1. These results validated and slow growth. Further, in yeast, either isoform of MetAP appeared sufficient to process Gfa1. These results validated the coding sequence for yeast ubiquitin was amplified using the primers 5′-ATGCTTGGTATCTTGTGATGC-3′ and 5′-CTTATCGACGGTAACA GAT-3′. The PCR product was cloned into PCRII-TOPO (Invitrogen), digested with EcoRI, and then subcloned to p26GPD. To create ubiquitin-Cys-Gfa1 (Ub-cys-Gfa1), the coding sequence for yeast ubiquitin was amplified using genomic DNA as a template with the primers 5′-ATGCCAGGTATTGGTTGATGC-3′ and 5′-ATAATTTGCAGTAACAAAGATACCC AACAACCCCTTACGCTAGCA-3′. The resulting PCR product shares 27 bp of overlap at the 3′ end with the 5′ end of a PCR product generated by the primers 5′-ATGGCTTGGTATCTTGTGATGC-3′ and 5′-CTTATCGACGGTAAACA GAT-3′. The PCR product was gel-purified, and then equimolar amounts were annealed by heating at 95 °C for 3 min followed by cooling on ice for 2 min, in 1× PCR buffer. Then, the annealed PCR products were mixed with dNTPs in 1× PCR buffer, with the primers 5′-ATGCCAGGTATTGGTTGATGC-3′ and 5′-ATAATTTGCAGTAACAAAGATACCC AACAACCCCTTACGCTAGCA-3′. Fusion PCR product was generated with Phusion high fidelity polymerase (Finnzymes, Espoo, Finland), TOPO-PA cloned to PCRII-TOPO, digested with SphI/Xhol, and then subcloned to p26GPD. A similar strategy was used to create a Ub-Met-Gfa1 fusion protein. The sequences of all constructs were confirmed using the automated DNA sequencing facility at St. Louis University.

Gfa1 Activity Assays—Assays were performed as described previously (23), with some modifications. Yeast cells were grown in 50–100 ml of growth medium (SD – Ura for plasmid carrying strains, YPD for endogenous Gfa1 activity) to an \(A_{600\text{ nanomoles}}\) of 1.0. The cells were washed once with water and then resuspended in 4 ml of breaking buffer (200 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). 4 g of glass beads were added, and the cells were broken by vortexing eight times for 30 s each with 30-s intervals on ice. The supernatants were centrifuged at 18,000 rpm in an SS-34 rotor and then concentrated to 100–200 \(\mu\)l by using Centricon Plus-20, 10,000 molecular weight cut-off spin columns. The resulting extract was then applied to a Bio-Spin 6 column pre-equilibrated in Gfa1 assay buffer (60 mM potassium phosphate, pH 7.0, 1 mM EDTA). The eluent was mixed 1:1 with 2× Gfa1 assay solution (30 mM glutamine, 30 mM fructose-6-phosphate in 1× Gfa1 assay buffer). Reactions were continued for 30–60 min and were terminated by boiling for 2 min. The protein precipitate was separated by centrifugation. The supernatant was then assayed for glucosamine-6-phosphate using the AccQ-Tag method (Waters, Milford, MA). Portions of the extract eluted from the Bio-Spin 6 column were also subjected to Western blot analysis to determine relative expression levels of Gfa1.

Western Blotting—Polyclonal antibodies to the Gfa1 C terminus peptide were generated in rabbits by standard procedures (24). Antibodies were affinity-purified and used at a 1:1000 dilution for Western blotting. Proteins were separated by 7.5 or 10% SDS-PAGE and transferred to nitrocellulose filters overnight in the cold. Western blotting was performed according to the ECL protocol (Amersham Biosciences).

N-terminal Sequencing—To immunoprecipitate Gfa1, cells were grown in 200-ml cultures to an \(A_{600\text{ nm}}\) of 1.0. Cell pellets were washed once with water, resuspended in 4 ml of radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1× Complete mini protease inhibitor mixture (Roche Applied Science), 1 mM EDTA), and broken with glass beads. The extracts were centrifuged, and the supernatant (6–8 mg) was precleared with protein-G agarose beads. The beads were washed four times with radioimmune precipitation buffer. Bound Gfa1 was eluted by heating at 95 °C for 3 min followed by cooling on ice for 2 min, 1× PCR buffer. The resulting PCR product was TOPO-TA cloned into PCRII-TOPO, digested with SphI/Xhol, and then subcloned to p26GPD. A similar strategy was used to create a Ub-Met-Gfa1 fusion protein. The sequences of all constructs were confirmed using the automated DNA sequencing facility at St. Louis University.

Gfa1 Function Is Dependent upon Methionine Aminopeptidase

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<th>Strain</th>
<th>Genotype</th>
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*The mating type and methionine or lysine requirement of this strain were not determined.

**Gfa1 Function Is Dependent upon Methionine Aminopeptidase**

**TABLE I.** BY4743 (Beckman).

**Yeast strains**

<table>
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Gfa1 Function Is Dependent upon Methionine Aminopeptidase

**RESULTS**

To evaluate whether removal of initiator methionine and subsequent exposure of the α-amino group of N-terminal cysteine is required for Gfa1 to perform its essential function, we used two strategies (Fig. 1). The first involved insertion of a codon specifying for alanine between the initiator methionine codon and the codon for mature N-terminal cysteine. The resulting Gfa1-Ala0 mutant will be a substrate for MetAP and will have an alanine residue at the mature N terminus, blocking the cysteine α-amino group, mimicking the effect of the failure to process initiator methionine, independent of MetAP activity. The second strategy was to utilize the ubiquitin fusion protein approach (25). When ubiquitin is fused to the N terminus of a protein, ubiquitin hydrolases will cleave the attached ubiquitin co-translationally (25). Taking advantage of this system allows for the creation of proteins with unique N termini by simply manipulating the identity of the residue at the ubiquitin-protein junction. We created two ubiquitin fusion proteins with codons specifying for either cysteine or methionine at the fusion junction by fusion PCR in an attempt to create mature Gfa1 proteins with either methionine or cysteine residues at the N terminus.

Although GFA1 is an essential gene in yeast, the lethal phenotype is conditional. Haploid gfa1Δ cells can be maintained by supplementing the medium with glucosamine (23). We generated haploid gfa1Δ cells by sporulation and tetrad dissection of a diploid strain heterozygous for the GFA1 gene followed by selection of glucosamine auxotrophs. To assay the ability of the various mutant forms of Gfa1 to perform the essential function, each Gfa1 mutant was cloned into a high copy yeast expression vector, which was then used to transform gfa1Δ cells. Cells were then assayed for growth in medium lacking glucosamine. All of the plasmids supported cell viability in medium lacking glucosamine, whereas a vector control was inviable (data not shown). The growth of strains expressing wild type Gfa1, Ub-Cys-Gfa1, or Ub-Met-Gfa1 were essentially indistinguishable, whereas the growth of cells expressing the Gfa1-Ala0 mutant was significantly slower (Fig. 2). These results indicated that exposure of the α-amino group of N-terminal cysteine was not required for the essential function of Gfa1; however, the difference in results obtained between cells expressing the Gfa1-Ala0 mutant versus Ub-Met-Gfa1 was unexpected and precluded further conclusions. The addition of an epitope tag at the C terminus of Gfa1 resulted in a protein that was unable to complement the gfa1Δ lethal phenotype. Thus, to compare the expression levels of different Gfa1 mutants, we developed Gfa1-specific polyclonal antibodies. Western blotting with these antibodies demonstrated that expression of the Gfa1-Ala0 mutant was as high as wild type Gfa1 (Fig. 3), thus ruling out decreased Gfa1 expression as the reason for the slow growth of this strain.

To determine whether the growth rate of the cells correlated with cellular Gfa1 activity, we assayed activity in the various strains. The activity from strains expressing Ub-Cys-Gfa1 versus Ub-Met-Gfa1 was approximately equal, although less than in cells expressing wild type Gfa1. This decrease likely results from a lower expression level of both ubiquitin fusion proteins revealed by Western blotting (Fig. 3). The blot also shows that ubiquitin is processed from both ubiquitin fusion proteins as expected. In agreement with a reduced growth rate, cells expressing Gfa1-Ala0 had dramatically reduced Gfa1 activity, less than 10% of the activity from cells expressing wild type Gfa1 (Fig. 3).

These results were unexpected. The simplest explanation for the lack of effect on Gfa1 activity seen with the Ub-Met-Gfa1 mutant is that the methionine is still processed, despite the ubiquitin fusion approach. We performed N-terminal sequencing on the three mutant proteins. The results are shown in Table II. As expected, N-terminal sequencing of Ub-Cys-Gfa1 and Gfa1-Ala0 indicated cysteine and alanine, respectively, as the N-terminal residues of the mature proteins. Surprisingly, the sequencing results from the Ub-Met-Gfa1 fusion protein also indicated that cysteine was the mature N-terminal residue of this protein. No laddering effect was apparent in the chromatograms. Although we did not test it, the enzyme most likely to process the methionine is MetAP; thus, it appears that MetAP can still cleave the N-terminal methionine residue at
MetAP activity for function. Although we excluded effects on Gfa1 as contributing to the slow growth of the map1Δ yeast strain, it is nevertheless likely one factor leading to a cellular requirement for MetAP activity for normal growth. Additionally, Gfa1 activity will likely be modulated in physiological and therapeutic applications in which MetAP activity is inhibited. One such application currently is inhibition of tumor cell growth by inhibiting the combined activities of MetAP1 and MetAP2 using benzamides (15). Whether Gfa1 will be relevant in endothelial cell growth or other situations featuring modulation of MetAP activity awaits further characterization of the relative roles of MetAP isoforms in various cell types.

We propose that the alanine insertional strategy outlined in this report can be used to generally test other potentially important downstream MetAP targets. Although alanine insertion does not directly mimic methionine retention, it is probably the closest approximation possible, due to the persistence of methionine processing using the ubiquitin fusion approach demonstrated in this report. The commercial availability of heterozygous deletions of all of the essential yeast genes should greatly facilitate these endeavors, and the yeast system is ideal for quick screening of potential candidates by complementation analysis. Likely candidates will include myristoylated proteins, acetylated proteins, and other proteins similar to Gfa1 that in some way depend upon their mature N-terminal residue for function, such as yeast 5′-phosphoribosyl-1-pyrophosphate (PRPP) aminotransferase, encoded by the ADE4 gene. Screening of these candidates will rapidly identify those MetAP substrates that rely upon MetAP activity for their functions, to more specifically explain the cellular requirement for MetAP activity, and potentially may lead to identification of substrates important for the growth of mammalian tumor and/or endothelial cells.

REFERENCES

FIG. 4. Either MetAP isoform is sufficient to process Gfa1 in vivo. Gfa1 activity was determined in total cell extracts from isogenic map1Δ or map2Δ strains relative to a wild type (wt) strain.

FIG. 3. Expression and activity levels of Gfa1 variants. Shown at the top is a representative Western blot using Gfa1-specific antibodies, from gfa1Δ cells expressing the indicated Gfa1 proteins. The graph shows relative Gfa1 activity levels measured from total cell extracts.

Table II

<table>
<thead>
<tr>
<th>Protein expressed</th>
<th>N-terminal sequence</th>
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</tr>
<tr>
<td>Ub-Met-Gfa1</td>
<td>XGIFG</td>
</tr>
<tr>
<td>Ub-Cys-Gfa1</td>
<td>XGIFG</td>
</tr>
<tr>
<td>Gfa1-Ala0</td>
<td>AXGIF</td>
</tr>
</tbody>
</table>

a The predicted wild type N-terminal sequence of Gfa1 is CGIFG.

b Cysteine is not detected by standard N-terminal sequencing, so the symbol X replaces cycles that gave no peak and are assumed to represent cysteine.

some point after ubiquitin is cleaved. Together, these results indicate that exposure of the α-amino group of N-terminal cysteine of Gfa1 is required for full activity and normal cell growth. Considering that these proteins are overexpressed, the effect on growth is likely underestimated.

Based on several lines of evidence, we thought it likely that MetAP1 would be the major enzyme responsible for processing Gfa1. MetAP1 is clearly the dominant isoform in yeast cells and for normal growth of yeast cells. Gfa1 is thus far the first endogenous yeast protein shown to be directly reliant upon

Our results demonstrate a requirement for exposure of the α-amino group of N-terminal cysteine for full activity of Gfa1 and for normal growth of yeast cells. Gfa1 is thus far the first endogenous yeast protein shown to be directly reliant upon