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

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**Methionine aminopeptidase (MetAP) catalyzes the co-translational processing of initiator methionine from nascent proteins. A cellular requirement for MetAP activity is likely due to dysfunction of MetAP substrates that require methionine removal for proper protein function. Glutamine-fructose-6-phosphate aminotransferase (Gfa1) is an essential enzyme in yeast that catalyzes the first and rate-limiting step in hexosamine biosynthesis. The α-amino group of Gfa1 Cys-1 has been proposed to act as a nucleophile in the catalytic mechanism. We used two mutational strategies to evaluate whether removal of initiator methionine, catalyzed by MetAP, is required for Gfa1 function. Our results demonstrate that exposure of the α-amino group of Cys-1 is required for normal Gfa1 function as failure to do so results in decreased enzyme activity and slow growth. Further, either isoform of MetAP in yeast is sufficient for Gfa1 processing *in vivo*. These results are the first demonstration of an endogenous yeast protein that requires the exposure of the α-amino group by MetAP action for normal function. Additionally, Gfa1 will be a relevant target in therapeutic or physiological applications in which MetAP activity is inhibited.**

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, co-translationally, 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 archaebacteria, 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 *Escherichia coli* (8) and *Salmonella typhimurium* (9) and deletion of both MetAP1 and MetAP2 in *Saccharomyces cerevisiae* (5), all of which result in lethality. Additionally, deletion of MetAP1 (*map1*Δ) in yeast results in a severe slow growth phenotype and decreased processing of a model protein substrate *in vivo* (10, 11). Deletion of yeast MetAP2 (*map2*Δ) has little or no effect on cell growth and model protein substrate processing. Despite the lack of phenotype observed with deletion of MetAP2 in yeast, MetAP2 has been demonstrated to be important for the growth of endothelial cells, some tumor cells, and some microorganisms (12–14). Thus, the relative importance of either MetAP isoform may be cell type-specific.

The importance of MetAP2 for the growth of endothelial cells and some cancer cells has made human MetAP2 an attractive target for cancer therapy, both by inhibition of angiogenesis and by direct inhibition of tumor cell growth. Recently, inhibition of growth of some cancer cells by treatment with bengamides, which are analogs of natural marine products that inhibit both MetAP1 and MetAP2 with similar potency, has potentially implicated MetAP1 in the growth of cancer cells (15). The specific MetAP substrates that may be important targets for cancer therapy have yet to be identified.

Failure to remove initiator methionine may have several effects on proteins that are detrimental to protein function. Methionine removal is required for subsequent N-terminal modifications, including *N*-myristoylation and *N*-acetylation, the lack of which may alter protein stability, localization, or functional interactions. Multiple examples have demonstrated that inappropriate retention of initiator methionine can, in some circumstances, result directly in decreased protein stability, albeit in unpredictable ways (11, 16). Another possibility is that removal of initiator methionine may be required to expose a mature N-terminal residue involved in catalysis. An example is the case of some N-terminal nucleophile aminotransferases, which have a nucleophilic N-terminal cysteine.

To date, specific characterization of downstream targets of MetAP that directly require methionine removal for function has been elusive. The identification of these targets will be important not only in understanding why MetAP activity is essential but also in identifying targets for treatment of cancer, both via anti-angiogenesis strategies and through direct inhibition of tumor cell growth.

The essential yeast gene *GFA1* codes for glutamine fructose-6-phosphate aminotransferase, which catalyzes the first and rate-limiting step in the hexosamine biosynthesis pathway. Also known as glucosamine-6-phosphate synthase, the enzyme catalyzes the formation of glucosamine-6-phosphate and glutamate from fructose-6-phosphate and glutamine (17). The hexosamine biosynthesis pathway is responsible for generating the precursors for *N*- and *O*-linked glycosylation. Further, Gfa1 and the hexosamine biosynthesis pathway are involved in yeast in the biosynthesis of chitin, a cell wall component (18),

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

TABLE I
*Yeast strains*

| Strain | Genotype | Reference |
|---|---|---|
| BY4743 | *MATα/MATa ura3Δ0/ura3Δ0 his3Δ1/his3Δ1 met15Δ0/met15Δ0 lys2Δ0/lys2Δ0 gfa1::KanMX/GFA1* | Ref. 26 |
| BY4741 | *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0* | Ref. 26 |
| BY4741 | *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 map1::KanMX* | Ref. 26 |
| BY4741 | *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 map2::KanMX* | Ref. 26 |
| BD2000 [a] | *ura3Δ0 leu2Δ0 his3Δ1 gfa1::KanMX* | This work |

[a] The mating type and methionine or lysine requirement of this strain were not determined.

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 N-terminal nucleophile family of aminotransferases, which have an N-terminal catalytic cysteine as 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 exposure of the cysteine α-amino 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 likely 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 Gfa1 as a downstream MetAP target that requires methionine removal for proper function.

## EXPERIMENTAL PROCEDURES

*General Methods*—Yeast transformation was performed by the lithium acetate procedure (21). Protein assays were performed according to the method of Bradford (22) using bovine serum albumin as a standard. UV measurements were made on a DU-70 spectrophotometer (Beckman).

*Vectors and Strains*—Yeast strains used in this study are listed in Table I. BY4743 (*gfa1::KanMX/GFA1*), BY4741 (*map1::KanMX*), and BY4741 (*map2::KanMX*) were obtained from ATCC. A haploid yeast strain carrying a null allele of *gfa1* was constructed by sporulation and subsequent tetrad dissection of BY4743. *gfa1*Δ cells were selected by resistance to G418 coupled with glucosamine auxotrophy. *gfa1*Δ cells were maintained in medium supplemented with 5 mg/ml glucosamine. *GFA1* coding sequence was obtained by PCR from yeast genomic DNA using the primers 5′-GCGAACTAGTA TGTGTGGTATCTTTGGTTACTG-3′ and 5′-CGCAGAATTCTTATTCGACGGTAA CAGATTTAGCC-3′, adding an SpeI site at the 5′ end and an EcoRI site at the 3′ end. The resulting PCR product was subcloned into p426GPD, a high copy plasmid that places the gene of interest under the strong glucose-6-phosphate dehydrogenase promoter. Gfa1-Ala0, a Gfa1 mutant with an alanine insertion between the initiation codon and Cys-1 of the mature wild type protein was created by standard PCR mutagenesis, using the primers 5′-ATGGCTTGTGGTATCTTTGGTTACTGC-3′ and 5′-CTTATTCGACGGTAACA GAT-3′. The PCR product was TOPO-TA subcloned into PCR2.1-TOPO (Invitrogen), digested with EcoRI, and then subcloned to p426GPD. 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′-ATGCAGATTTTCGTCAAGACTT-3′ and 5′-ATAATTGCAGTAACCAAAGATACC ACAACCACCTCTTAGCCTTAGCA-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′-TGTGGTATCTTTGGTTACTGC-3′ and 5′-CTTATTCGACGGTAACAGAT-3′, which amplifies the entire *GFA1* coding sequence with the exception of the AUG initiation codon. The two PCR products were 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′-ATGCAGATTTTCGTCAAGACTT-3′ and 5′-CTTATTCGACGGTAACAGAT-3′. Fusion PCR product was generated with Phusion high fidelity polymerase (Finnzymes, Espoo, Finland), TOPO-TA cloned to PCR2.1-TOPO, digested with SpeI/XhoI, and then subcloned to p426GPD. 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 $A_{600\ nm}$ 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 μl final volume (10–20 mg/ml) with 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 $A_{600\ nm}$ of 1.0. Cell pellets were washed once with water, resuspended in 4 ml of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1× Compete 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 for 1 h. The supernatant was then mixed with 30 μg of anti-Gfa1 antibody for 1 h. Gfa1-antibody complexes were collected by incubation for 1 h with protein-G agarose beads. The beads were washed four times with radioimmune precipitation buffer. Bound Gfa1 was eluted by boiling in sample buffer. The eluent was separated by 7.5% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. Proteins were visualized by staining with Coomassie Blue. N-terminal sequencing was performed by Midwest Analytical (St. Louis, MO).

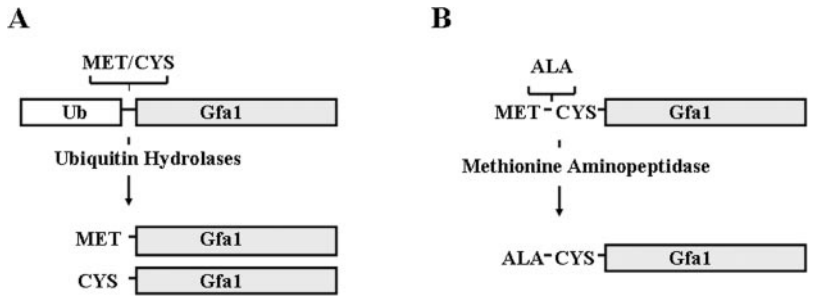


FIG. 1. **Mutational analysis of Gfa1.** Outlined are the two mutational strategies utilized in this study to evaluate the importance of exposure of the Gfa1 Cys-1 α-amino group. *A*, fusion of ubiquitin to the N terminus of a protein allows for engineering of mature proteins with unique N termini by simply manipulating the identity of the residue at the fusion junction, which is a co-translational cleavage site for cellular ubiquitin hydrolases. In this case, fusion PCR was used to create ubiquitin fusion proteins with codons specifying for methionine or cysteine at the fusion junction. *B*, insertion of a codon specifying for alanine between the initiator methionine codon and the normal second codon for cysteine in Gfa1 results in a mature protein that will be a substrate for methionine aminopeptidase. Thus, the methionine will be removed; however, the extra alanine residue will serve the purpose of blocking the cysteine α-amino group, analogous to the inappropriate retention of methionine.

*Generation of Growth Curves*—Cells were grown overnight in SD −Ura and then diluted in 25 ml of total volume to an $A_{600\ nm}$ of ~0.1. Growth was then continued, and aliquots were taken at 1-h intervals for determination of $A_{600\ nm}$ Experiments were repeated at least three times. Shown is a representative growth curve.

## 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 *gfa1*Δ cells can be maintained by supplementing the medium with glucosamine (23). We generated haploid *gfa1*Δ 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 to a high copy yeast expression vector, which was then used to transform *gfa1*Δ 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

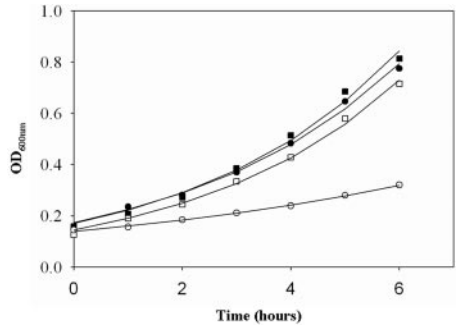


FIG. 2. **Growth of *gfa1*Δ expressing various Gfa1 constructs.** *gfa1*Δ cells were transformed with high copy overexpression plasmids encoding wild type Gfa1 (■), Ub-Cys-Gfa1 (●), Ub-Met-Gfa1 (□), or Gfa1-Ala0 (○) and grown as detailed under "Experimental Procedures."

was unable to complement the *gfa1*Δ 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

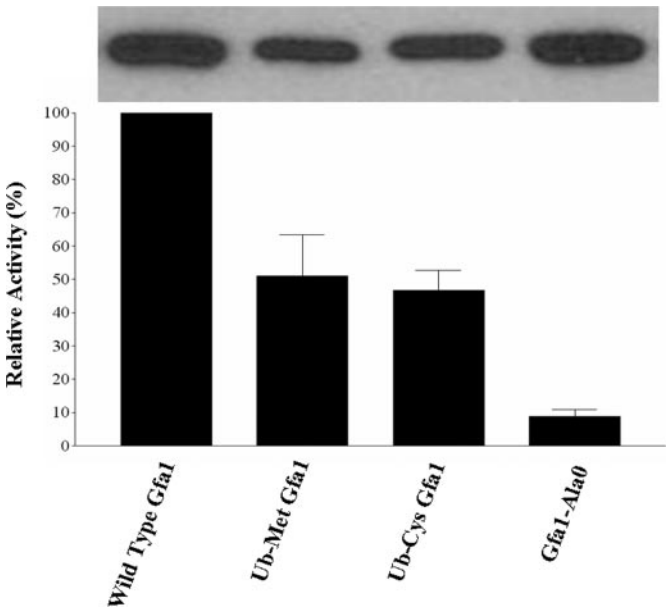


FIG. 3. **Expression and activity levels of Gfa1 variants.** Shown at the *top* is a representative Western blot using Gfa1-specific antibodies, from *gfa1*Δ cells expressing the indicated Gfa1 proteins. The graph shows relative Gfa1 activity levels measured from total cell extracts.

TABLE II
*N-terminal sequence of Gfa1 mutants*

| Protein expressed | N-terminal sequence |
|---|---|
| Gfa1 wild type [a] | Not Determined |
| Ub-Met-Gfa1 [b] | *X*GIFG |
| Ub-Cys-Gfa1 [b] | *X*GIFG |
| Gfa1-Ala0 [b] | A*X*GIF |

[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 both in terms of cell growth and in terms of *in vivo* protein processing of a model substrate (11). Secondly, a model substrate with cysteine at the mature N terminus was only 3% processed in the presence of endogenous MetAP2 alone (11). The processing of peptides with penultimate cysteine by MetAP cannot be assayed *in vitro*, presumably because the cobalt required for the assay interacts with the cysteine. We used Gfa1 activity assays in isogenic wild type, *map1*Δ, or *map2*Δ strains to infer the extent of initiator methionine processing of Gfa1. We measured endogenous Gfa1 activity because the overexpression of Gfa1 in wild type cells resulted in a significant growth defect (data not shown). Surprisingly, the Gfa1 activity level was the same in all three strains, indicating that likely either MetAP enzyme is sufficient to process Gfa1 *in vivo* (Fig. 4).

## DISCUSSION

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

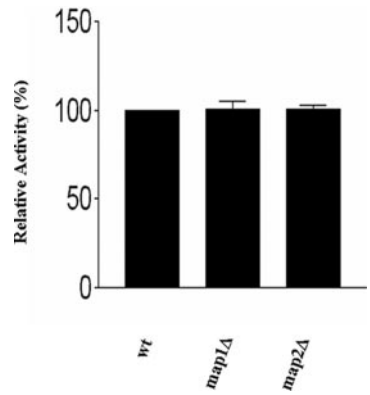


FIG. 4. **Either MetAP isoform is sufficient to process Gfa1 *in vivo*.** Gfa1 activity was determined in total cell extracts from isogenic *map1* or *map2*Δ strains relative to a wild type (*wt*) strain.

MetAP activity for function. Although we excluded effects on Gfa1 as contributing to the slow growth of the *map1*Δ 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 bengamides (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.

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