

Evidence of a Dominant Negative Mutant of Yeast Methionine Aminopeptidase Type 2 in *Saccharomyces cerevisiae*

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Abstract Eukaryotic methionine aminopeptidase type 2 (MetAP2, MetAP2 gene (*MAP2*)), together with eukaryotic MetAP1, cotranslationally hydrolyzes initiator methionine from nascent polypeptides when the side chain of the second residue is small and uncharged. In this report, we took advantage of the yeast (*Saccharomyces cerevisiae*) *map1* null strain's reliance on MetAP2 activity for the growth and viability to provide evidence of the first dominant negative mutant of eukaryotic MetAP2. Replacement of the conserved His¹⁷⁴ with alanine within the C-terminal catalytic domain of yeast MetAP2 eliminated detectable catalytic activity against a peptide substrate in vitro. Overexpression of MetAP2 (H174A) under the strong *GPD* promoter in a yeast *map1* null strain was lethal, whereas overexpression under the weaker *GAL1* promoter slightly inhibited *map1* null growth. Deletion mutants further revealed that the N-terminal region of MetAP2 (residues 2–57) is essential but not sufficient for MetAP2 (H174A) to fully interfere with *map1* null growth. Together, these results indicate that catalytically inactive MetAP2 is a dominant negative mutant that requires its N-terminal region to interfere with wild-type MetAP2 function. *J. Cell. Biochem.* 94: 656–668, 2005. © 2004 Wiley-Liss, Inc.

Key words: methionine aminopeptidase; angiogenesis; TNP-470; p67; protein processing

Eukaryotes [Housman et al., 1970] and archaeobacteria [Ramesh and RajBhandary, 2001] initiate the translation of endogenous mRNA with a methionine-bound initiator tRNA (Met-tRNA_i^{Met}), whereas eubacteria [Adams and Capecchi, 1966; Clark and Marcker,

1966], mitochondria [Bianchetti et al., 1977], and chloroplasts [Lucchini and Bianchetti, 1980] initiate mRNA translation with an N^α-formylated methionine-bound initiator tRNA (f-Met-tRNA_f^{Met}) [Adams, 1968; Housman et al., 1972; Ball and Kaesberg, 1973]. As a result, the primary structure of the majority of nascent polypeptides, with the exception of a few proteins that initiate translation from rare non-AUG codons [Hann et al., 1988; Prats et al., 1989; Kopke and Leggatt, 1991; Shirako, 1998; Arnaud et al., 1999; Kobayashi et al., 2002], begins with an initiating N-terminal methionine (Met_{init}) (eukaryotes and archaeobacteria) or N^α-formylated N-terminal methionine (f-Met_{init}) (eubacteria, mitochondria, and chloroplasts).

Methionine aminopeptidase (MetAP, EC 3.4.11.18, product of the MetAP gene (*MAP*)) cotranslationally hydrolyzes Met_{init} and f-Met_{init} from nascent polypeptides when the second residue in the primary structure is small and uncharged (e.g., Ala, Cys, Gly, Pro, Ser, Thr, Val) [Boissel et al., 1985; Tsunasawa et al., 1985; Flinta et al., 1986; Ben-Bassat et al., 1987; Huang et al., 1987; Hirel et al., 1989; Moerschell

Abbreviations used: MetAP, methionine aminopeptidase; *MAP*, MetAP gene; *map1Δ*, yeast *map1* deletion strain; *map2Δ*, yeast *map2* deletion strain; Met_{init}, translation initiating N-terminal methionine.

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et al., 1990]. MetAP additionally requires deformylation of f-Met_{init} before Met_{init} can be hydrolyzed in eubacteria, mitochondria, and chloroplasts [Adams, 1968; Solbiati et al., 1999].

Two major types of MetAP (MetAP1 and MetAP2) were originally identified as cytosolic proteins [Arfin et al., 1995; Li and Chang, 1995] that are structurally similar, but share little sequence homology [Lowther and Matthews, 2000]. Typically, eubacteria express only a type 1 MetAP [Chang et al., 1989], whereas archaeobacteria express only a type 2 MetAP [Arfin et al., 1995; Li and Chang, 1995]. It has recently been shown, however, that the genome of the cyanobacterium *Synechocystis* sp. has *MAP1* and *MAP2* isoforms, as well as a novel *MAP3* isoform [Atanassova et al., 2003].

All eukaryotes examined to date express cytosolic forms of MetAP1 and MetAP2 [Arfin et al., 1995; Li and Chang, 1995]. Eukaryotic MetAP1 has been shown to associate with the 60S ribosome subunit in yeast [Vetro and Chang, 2002]. Isoforms of MetAP1 also localize to the mitochondria and chloroplasts of plant cells and are likely to localize to the mitochondria of animal cells [Gigliione et al., 2000; Serero et al., 2003]. Eukaryotic MetAP2 was first identified as a eukaryotic initiation factor 2 (eIF2)-associated protein (rat p⁶⁷) involved in positively regulating the initiation scanning mRNA translation in animal cells [Ray et al., 1992] before being identified as a MetAP protein [Li and Chang, 1996]. Eukaryotic MetAP2 has also been shown to associate with the metastasis association protein, S100A4, in a Ca²⁺-dependent manner [Endo et al., 2002] as well as the caveolae-associated protein, flotillin [Liu and Liu, 2001].

All MetAPs share a conserved C-terminal catalytic domain [Arfin et al., 1995; Li and Chang, 1995]. Within the catalytic domain are five conserved amino acids (*Escherichia coli*: Asp⁹⁷, Asp¹⁰⁸, His¹⁷¹, Glu²⁰⁴, and Glu²³⁵) that bind up to two metal ion cofactors [Roderick and Matthews, 1993]. The major structural difference between the type 1 and type 2 MetAPs is an approximately 60-amino acid insert in the C-terminal catalytic domain of MetAP2 [Bazan et al., 1994; Arfin et al., 1995; Li and Chang, 1995].

Unlike bacterial MetAPs, eukaryotic MetAPs have an extended N-terminal region [Chang et al., 1992; Arfin et al., 1995; Li and Chang, 1995]. Within this N-terminal region, eukaryo-

tic MetAP1 has two zinc finger motifs [Chang et al., 1992] that are likely to be involved in maintaining the correct functional alignment of MetAP1 on the 60S ribosomal subunit [Vetro and Chang, 2002]. Eukaryotic MetAP2 has a highly charged region within its extended N-terminal consisting of a single polylysine block (yeast MetAP2) [Arfin et al., 1995; Li and Chang, 1995] or a polyaspartate block flanked by two polylysine blocks (mammalian MetAP2/p67) [Wu et al., 1993; Li and Chang, 1995]. These charged domains have been proposed to mediate the presumed association of yeast MetAP2 with the ribosomes [Li and Chang, 1995] or the association of mammalian MetAP2 with eIF2 [Wu et al., 1993].

MetAP activity is essential for cellular growth and viability. Deletion of the single *MAP* gene in *E. coli* [Chang et al., 1989] and *Salmonella typhimurium* (described as *pepM* [peptidase M]) [Miller et al., 1989] or of both *MAP* genes (*MAP1* and *MAP2*) in the yeast *Saccharomyces cerevisiae* is lethal [Li and Chang, 1995]. Furthermore, treatment of both a primary and immortalized human cell line with LAF389, a reversible inhibitor of MetAP1 and MetAP2 activity, causes extensive cell death at concentrations of LAF389 that significantly inhibit the activities of both MetAP1 and MetAP2 in vitro [Towbin et al., 2003]. In contrast, deletion of *MAP1* or *MAP2* alone in yeast results in a slow-growth phenotype [Chang et al., 1992; Li and Chang, 1995]. The slow-growth phenotype of the yeast *map1* knockout strain (*map1*Δ), however, is significantly more severe than that of the yeast *map2* knockout strain (*map2*Δ) [Chang et al., 1992; Li and Chang, 1995] and is consistent with the finding that MetAP1 is primarily responsible for cellular Met_{init} hydrolysis activity in yeast [Chen et al., 2002]. Thus, the growth rate of yeast roughly correlates with the level of cellular Met_{init} hydrolysis activity [Chen et al., 2002]. The more dominant role of MetAP1 in cellular Met_{init} hydrolysis activity likely results, in part, from the greater sensitivity of MetAP2 to product inhibition by cytosolic concentrations of methionine [Dummitt et al., 2003] and insufficient expression levels of MetAP2 under normal growth conditions [Chen et al., 2002].

Like MetAP1 in yeast, mammalian MetAP1 appears to be predominantly responsible for cellular Met_{init} hydrolysis activity in animal cells [Turk et al., 1999]. The growth of vascular

endothelial cells (VECs) and other animal cell types [Ingber et al., 1990] as well as *Plasmodium falciparum* and *Leishmania donovani* [Zhang et al., 2002], however, is sensitive to the selective inhibition of MetAP2 catalytic activity by the angiogenesis inhibitors TNP-470 and ovalicin [Griffith et al., 1997; Sin et al., 1997]. Thus, the physiological role of eukaryotic MetAP2 in cellular Met_{init} hydrolysis activity remains unclear. A dominant negative mutant [Herskowitz, 1987] of eukaryotic MetAP2 would, therefore, be a useful tool to further dissect the physiological role of MetAP2 in the cellular Met_{init} hydrolysis activity of both lower and higher eukaryotes.

Many examples of catalytically inactive proteins that behave as dominant negative mutants have been reported [Herskowitz, 1987]. Given that cellular Met_{init} hydrolysis activity roughly correlates with growth rate in yeast [Chen et al., 2002], and that the yeast *map1Δ* strain relies on the activity of wild-type MetAP2 alone, we tested whether catalytically inactive yeast MetAP2 could act as a dominant negative mutant in yeast *map1Δ*. In this report, we provide evidence of the first dominant negative mutant of MetAP2 and further, we show that the N-terminal region of MetAP2 is required for the dominant negative effect.

MATERIALS AND METHODS

Materials

All materials were from Sigma (St. Louis, MO) unless otherwise stated. Restriction enzymes were from Promega (Madison, WI).

Yeast Strains

All yeast strains used in this report are isogenic and differ only at the *MAP1* or *MAP2*

loci. Yeast *map1Δ* (*map1::HIS3*) [Klinkenberg et al., 1997] and *map2Δ* (*map2::URA3*) [Li and Chang, 1995] were derived from W303-1A (Table I).

Bacterial Culture and Transformation

Bacteria were cultured and handled using established techniques [Ausubel et al., 1992]. Bacteria were cultured in Luria-Bertani (LB) broth (1% bacto-tryptone (BD Difco, Franklin Lakes, NJ), 0.5% yeast extract (Difco), and 1% NaCl). Transformations were carried out using the Z-Competent *E. coli* transformation Kit (Zymo Research, Orange, CA) according to manufacturer's protocol. Plasmid DNA was isolated using silica gel-based spin columns (Qiagen, Valencia, CA) and purified using an agarose gel extraction kit (Qiagen).

Yeast Culture and Transformation

General handling procedures for yeast were followed [Ausubel et al., 1992]. Unless otherwise specified, yeast strains were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose). Synthetic dropout media contained yeast nitrogen base without amino acids (YNB; Difco), appropriate amino acids to give the desired dropout mixture, and either 2% glucose (SD medium) or 2% galactose (SG medium). DNA transformations were performed by the lithium-acetate method [Ito et al., 1983] using a kit from BIO 101 (Vista, CA) following the manufacturer's protocol.

DNA Constructs

pGPD-HA-map2 (H174A). The wild-type yeast *MAP2* gene containing an N-terminal hemagglutinin (HA) epitope tag (YPYDVP-DYA) was subcloned from pXL-PE1A [Li and Chang, 1995] into p425*GPD* or p426*GPD*

TABLE I. Yeast Strains Used in This Study

Strain	Genotype	Reference
YHC001 (<i>map1Δ</i>)	MAT α ade2-1 can1-100 ura3-1 leu 2-3, 112 trp 1-1, his 3-11, 15 <i>map1::HIS3</i>	Klinkenberg et al. [1997]
XLW002 (<i>map2Δ</i>)	MAT α ade2-1 can1-100 ura3-1 leu 2-3, 112 trp 1-1, his 3-11, 15 <i>map2::URA3</i>	Li and Chang [1995]
<i>map1Δ</i> /p <i>GAL1</i>	YHC001/p425 <i>GAL1</i>	This study
<i>map1Δ</i> /p <i>GAL1</i> - <i>MAP2</i>	YHC001/p425 <i>GAL1</i> /HA- <i>MAP2</i>	This study
<i>map1Δ</i> /p <i>GAL1</i> -H174A	YHC001/p425 <i>GAL1</i> /HA- <i>map2</i> (H174A)	This study
<i>map1Δ</i> /p <i>GAL1</i> - Δ 2-57/ H174A	YHC001/p425 <i>GAL1</i> / <i>map2</i> (Δ 2-57/H174A)	This study
<i>map1Δ</i> /p <i>GPD</i>	YHC001/p426 <i>GPD</i>	This study
<i>map1Δ</i> /p <i>GPD</i> - Δ 58-421	YHC001/p426 <i>GPD</i> /HA- <i>map2</i> (Δ 58-421)	This study
<i>map2Δ</i> /p <i>GPD</i>	XLW002/p425 <i>GPD</i>	This study
<i>map2Δ</i> /p <i>GPD</i> - <i>MAP2</i>	XLW002/p425 <i>GPD</i> /HA- <i>MAP2</i>	This study
<i>map2Δ</i> /p <i>GPD</i> -H174A	XLW002/p425 <i>GPD</i> /HA- <i>map2</i> (H174A)	This study

map1Δ, yeast *map1* deletion strain; *map2Δ*, yeast *map2* deletion strain.

[Mumberg et al., 1995] using the Hind III and Xho I sites. The codon for His¹⁷⁴ was replaced with a codon for Ala in *HA-MAP2* using the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's protocol with the following mutagenic primers: forward: 5'-CAA CCA TTG TGC TGC AGC TTT CAC ACC CAA TGC AG-3'; reverse: 5'-CTG CAT TGG GTG TGA AAG CTG CAG CAC AAT GGT TG-3'.

Transformants were plated on Gibco agarose containing isopropyl thio- β -D-galactoside (IPTG; Gold Biotechnologies, St. Louis, MO) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal; Gold Biotechnologies). Plasmid DNA from positive blue colonies was isolated and sequenced by an automated sequencer (PE/Applied Biosystems 377). *HA-map2* (*H174A*) was then subcloned into p425*GPD*, p426*GPD*, or p425*GAL1* [Mumberg et al., 1994] at Hind III/Xho I and sequenced on both strands.

pGAL1-map2 (Δ 2-57/H174A). Residues 2–57 of yeast HA-MetAP2 (*H174A*) were deleted by PCR-mediated mutagenesis from p425*GPD*/*HA-map2* (*H174A*) using the following primers: forward: 5'-GCG CAA GCT TAT GAT TGA ATT ACT GTT TCC AGA TGG AAA G-3'; reverse: 5'-GCG CCT CGA GTC AGT AGT CAT CAC CTT TCG AAA CG-3'.

Amplified *map2* (Δ 2-57/*H174A*) was then gel-purified (Qiagen), subcloned into p425*GAL1* using Hind III/Xho I, and sequenced.

pGPD-HA-map2 (Δ 58-421). The N-terminal region of HA-MetAP2 was selectively amplified up to residue 57 from pXL-PE1A/*HA-MAP2* [Li and Chang, 1995] using the following primers: forward: 5'-CGG CAA GCT TAT GTA CCC ATA C-3'; reverse: 5'-CGC CTC GAG TCA CTT CAC ATT CGT TTT CTT CTT C-3'.

The amplified N-terminal fragment, HA-2-57, was then gel-purified (Qiagen), subcloned into p426*GPD* using Hind III/Xho I, and sequenced.

Extraction and Purification of HA-Tagged Yeast MetAP2

The purification of HA-tagged wild-type and mutant yeast MetAP2 from a haploid yeast *map1* Δ strain [Klinkenberg et al., 1997] was performed as previously described [Li and Chang, 1995] with slight modifications. A 1-liter yeast culture of *map1* Δ /p*GAL1-H174A* was grown aerobically at 30°C in SG/Leu⁻ medium. Cells were collected at an ABS₆₀₀ \sim 1–2 by centrifugation at 1,500g for 5 min. The pellet

was rinsed with buffer XL (10 mM HEPES [pH 7.4], 1.5 mM MgCl₂, 10% glycerol [v/v] plus 100 mM NaCl and fresh protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.7 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by vortexing (five cycles of 1 min vortexing, 1 min on ice) with acid-washed glass beads (0.5 mm diameter, Biospec, Bartelsville, OK). The lysate was cleared at 10,000g for 20 min at 4°C. Cleared lysate was applied to a Protein-Sepharose G column (Pharmacia) previously rinsed and pre-equilibrated with buffer XL and an anti-hemagglutinin epitope (YPYDVPDYA) monoclonal antibody (BabCO). After loading, the column was washed with buffer XL until ABS₂₈₀ < 0.01. The HA-tagged enzyme was then eluted from the immunoaffinity column with 1 mg/ml of free HA polypeptide in buffer XL. After extensive dialysis against buffer XL, each purified protein was assayed for MetAP activity using 2 mM of Met–Gly–Met–Met as described [Li and Chang, 1995].

Assay of MetAP Catalytic Activity In Vitro

The catalytic activity of HA-tagged wild-type MetAP2 and MetAP2 (*H174A*) was compared by a precolumn derivitization HPLC assay [Zuo et al., 1994]. Briefly, purified enzyme in buffer H supplemented with 200 mM KCl and 1 mM CoCl₂ was preincubated for 5 min at 37°C, then 2 mM MGMM peptide substrate was added. The reaction was incubated at 37°C and terminated by the addition of 1 mM EDTA, followed by boiling for 5 min. The concentration of released methionine in the supernatant was then determined by HPLC using the AccQ-Tag method according to manufacturer's protocol [Cohen and Michaud, 1993].

Yeast Growth Assay

For qualitative growth rate comparison (growth plates), strains transformed with Leu⁺ *GAL1* promoter plasmids were grown to mid-logarithmic phase in 5 ml of 2% raffinose minimal media lacking leucine and \sim 2 \times 10⁵ cells were streaked onto SD/Leu⁻ or SG/Leu⁻ plates, then incubated for 4 days at 30°C. Cell counts were determined by culture turbidity at 600 nm (described as ABS₆₀₀ for convenience), where ABS₆₀₀ of 1 is \sim 3.0 \times 10⁷ cells/ml. For quantitative growth rate comparison, growth curves were obtained for each culture and

TABLE II. Yeast Strain Growth Rates

Strain	Doubling time (h)
<i>map1Δ</i>	
Galactose	
p <i>GAL1</i>	6.2 ± 0.3
p <i>GAL1-MAP2</i>	4.1 ± 0.1
p <i>GAL1-H174A</i>	8.5 ± 0.4
p <i>GAL1-Δ2-57/H174A</i>	6.4 ± 0.2
Glucose	
p <i>GPD</i>	4.8 ± 0.1
p <i>GPD-Δ58-421</i>	5.9 ± 0.1
<i>map2Δ</i>	
Glucose	
p <i>GPD</i>	2.2 ± 0.1
p <i>GPD-MAP2</i>	3.4 ± 0.4
p <i>GPD-H174A</i>	2.5 ± 0.2

Quantitative growth rate comparison of *map1Δ* transformant strains. Growth curves were obtained for each strain, and doubling times calculated from log phase growth. We have consistently observed that *map1Δ* grows more slowly in galactose compared to glucose (*map1Δ/p425GAL1* vs. *map1Δ/p425GPD*). Data are reported as mean doubling times ± SD derived from propagated error (n = 3).

doubling times were calculated from log phase growth (Table II). A 50-ml seed culture of each strain from SG/Leu⁻ or SD/Ura⁻ plates was grown overnight in SG/Leu⁻ or SD/Ura⁻ until ABS₆₀₀ ~ 0.1. Each culture was resuspended in 1 ml of SG/Leu⁻ or SD/Ura⁻ media to give ABS₆₀₀ ~ 5.0. The ABS₆₀₀ of each 1 ml culture was measured and diluted to an ABS₆₀₀ of ~ 0.2 in 5 ml of SG/Leu⁻ or SD/Ura⁻ media. Each 5 ml culture was then grown aerobically at 30°C and the ABS₆₀₀ was measured in triplicate at 1-h intervals.

Demonstration of MetAP2 (H174A) Lethality

A yeast *map1Δ* strain (YHC001) was co-transformed with the wild-type yeast *MAP1* gene in a single-copy vector under 1 kb of the wild-type *MAP1* promoter (pRS416/*MAP1*) [Vetro and Chang, 2002] and either the *HA-map2* (*H174A*) gene in a Leu⁺ vector under the *GPD* promoter (p*GPD-HA-map2* (*H174A*)) or vector alone (p*GPD*). For SD/Leu⁻/Ura⁻ plates, each strain was grown in SD/Leu⁻/Ura⁻ media for 48 h, then 5 μl from each culture was plated on SD/Leu⁻/Ura⁻ plates and incubated for 5 days at 30°C. For 5'-fluoro-orotic acid (5'-FOA) plates, each strain was grown in SD/Leu⁻ media for 48 h to allow for loss of the Ura⁺ pRS416 plasmid, then 5 μl of pRS416-depleted cells (ABS₆₀₀ ~ 2.0) were plated on SD/Leu⁻ plates containing 1 mg/ml 5'-FOA and incubated for 5 days at 30°C.

Generation of Polyclonal Antibodies Against Yeast MetAP2

A standard procedure was adapted [Harlow and Lane, 1988]. An oligopeptide corresponding to a conserved 10-amino acid sequence at the C-terminus of MetAP2 (CKEVVSKGDDY) was obtained (Research Genetics, Carlsbad, CA). An N-terminal cysteine was included in the peptide for coupling to the carrier protein, maleimide-activated keyhole limpet hemocyanin (KLH). The peptide and KLH were conjugated at 2 mg peptide/2 mg KLH according to the manufacturer's protocol (Pierce, Rockford, IL). The KLH-peptide immunogen was mixed with an equal volume of Freund's complete adjuvant (Difco), and 400 μl of this emulsion was injected intramuscularly into each thigh of a host rabbit. Two booster injections of the same amount of antigen emulsified in incomplete Freund's adjuvant (Sigma) were given at weeks 4 and 8. Blood was collected from the ear prior to the initial injection and 10 days after each boost. Blood samples stood at room temperature for 4 h before being placed at 4°C overnight. Coagulated blood was cleared by centrifugation at 3,000g for 10 min. MetAP2 antibodies were then purified using cyanogen bromide-activated thiol-sepharose (Sigma) coupled with the peptide antigen. Antisera specificity and titer from each collection was examined by immunoblot against immunoaffinity-purified epitope-tagged yeast MetAP2 [Li and Chang, 1995].

Polyacrylamide Gel Electrophoresis and Western Blots

SDS-PAGE [Laemmli, 1970] was performed on 10% polyacrylamide gels. Total protein concentration of crude extracts was determined by Bradford assay [Bradford, 1976] using reagents from Bio-Rad. Gels were wet-transferred overnight to a nitrocellulose membrane and blocked for 1 h with Tris-buffered saline solution containing 0.2% Tween-20 (TBST) plus 5% nonfat dry milk. All Western blots were performed following the ECLTM detection protocol (Amersham, Piscataway, NJ). Membranes were incubated with rabbit anti-yeast MetAP2 polyclonal antibodies (1/500) in TBST plus 1% nonfat dry milk for 1 h at room temperature. Membranes were then incubated with goat anti-rabbit horseradish peroxidase conjugated antibodies (1/6,000, Sigma) for 30 min at room

temperature and exposed to X-ray film (Molecular Technologies, St. Louis, MO).

RESULTS

Many catalytically inactive proteins interfere with the function of their corresponding wild-type proteins in a concentration-dependent manner when co-expressed within the same cell (dominant negative mutants) [Herskowitz, 1987]. Since yeast growth and viability are sensitive to levels of cellular Met_{init} hydrolysis activity [Chang et al., 1992; Li and Chang, 1995; Chen et al., 2002], we took advantage of the yeast *map1Δ* strain's reliance on wild-type MetAP2 activity for growth and viability to test whether catalytically inactive MetAP2 could act as a dominant negative mutant.

Yeast MetAP2 (H174A) Is Catalytically Inactive

Replacement of the conserved His²³¹ residue in mammalian MetAP2 eliminates detectable MetAP2 activity in vitro [Griffith et al., 1998]. Thus, it was likely that a His¹⁷⁴ mutant of yeast MetAP2 would also be inactive. Using the wild-type yeast *MAP2* gene containing an N-terminal HA epitope tag [Li and Chang, 1995], the codon for His¹⁷⁴ was replaced with the codon for alanine. The resultant mutant gene, *HA-map2* (*H174A*), was then subcloned into a multi-copy vector under the strong yeast glyceraldehyde 3-phosphate (*GPD*) promoter (*pGPD-HA-map2* (*H174A*)).

A yeast *map1Δ* strain, YHC001, was transformed with *pGPD-HA-map2* (*H174A*). Steady state levels of wild-type MetAP2 in the *map1Δ* strain and in the parental wild-type strain (W303-1A) are similar (data not shown). No colonies were obtained after two consecutive transformations of *map1Δ* with ~0.1 μg of *pGPD-HA-map2* (*H174A*), whereas ~170 colonies were obtained with ~0.1 μg of plasmid DNA vector alone (data not shown). In contrast, transformation of *map1Δ* with ~0.1 μg *pGAL1-HA-map2* (*H174A*) under the relatively weaker, galactose-regulated yeast galactokinase (*GAL1*) promoter [Mumberg et al., 1994] gave ~150 colonies (data not shown) and allowed for the purification of *HA-MetAP2* (*H174A*) after galactose activation.

The relative catalytic activities of wild-type MetAP2 and MetAP2 (*H174A*) were compared in vitro. Wild-type MetAP2 and MetAP2 (*H174A*), each having an N-terminal hemagglutinin (HA)-tag (YPYDVPDYA), were immu-

nopurified from *map1Δ* to minimize background aminopeptidase activity resulting from endogenous wild-type MetAP2. Unlike wild-type MetAP2, MetAP2 (*H174A*) displayed no detectable activity against a peptide substrate (MGMM) in vitro (Fig. 1). These results indicate that replacement of conserved His¹⁷⁴ with alanine disrupts the catalytic activity of yeast MetAP2.

Yeast MetAP2 (H174A) Expression Under the *GPD* Promoter Is Lethal in Yeast *map1Δ*

The hydrolysis of N-terminal methionine by MetAP is essential for normal cellular growth [Chang et al., 1992] and viability in yeast [Li and Chang, 1995]. Also, the level of cellular Met_{init} hydrolysis activity in yeast roughly correlates with growth rate [Chen et al., 2002]. Therefore, if a mutant of MetAP2 interferes with wild-type MetAP2 function in a yeast *map1Δ* strain (expresses only wild-type MetAP2), a decreased growth rate or lethal phenotype is expected.

The observation that transformants of yeast *map1Δ* could only be obtained when MetAP2 (*H174A*) is expressed under the *GAL1* promoter, but not the relatively stronger *GPD* promoter, indicates that MetAP2 (*H174A*) is lethal at

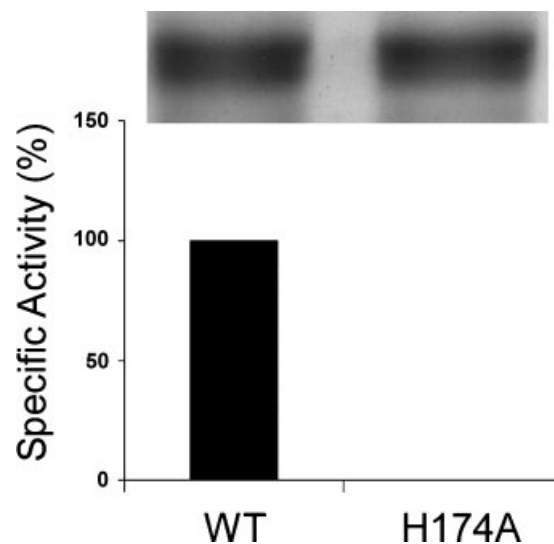


Fig. 1. Yeast MetAP2 (*H174A*) is a dysfunctional catalyst. N-terminal HA-tagged wild-type MetAP2 and MetAP2 (*H174A*) were immunopurified from a haploid yeast *map1Δ* strain with mouse anti-hemagglutinin epitope (YPYDVPDYA) monoclonal antibodies. Approximately 2 μg of purified wild-type MetAP2 (wt) and MetAP2 (*H174A*) were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. Specific activity for wild-type MetAP2 was 11.0 ± 0.5 U/mg protein (100%), whereas MetAP2 (*H174A*) had no detectable activity.

high concentrations in *map1* Δ . We tested this hypothesis by first transforming *map1* Δ with a Ura⁺ plasmid encoding the *MAP1* gene to increase the cellular Met_{init} hydrolysis activity of *map1* Δ , followed by transformation with a Leu⁺ plasmid encoding MetAP2 (H174A) under the *GPD* promoter or a vector control. Each strain was then plated on 5'-FOA to test whether *map1* Δ could survive the loss of the *MAP1* gene in the presence of overexpressed MetAP2 (H174A). As expected, the *map1* Δ strain containing vector alone was viable on 5'-FOA (Fig. 2C), albeit slower growing than when plated on medium lacking 5'-FOA (Fig. 2A) due to the loss of the *MAP1* encoding plasmid. In contrast, no colonies were obtained from *map1* Δ overexpressing MetAP2 (H174A) on the 5'-FOA plate, even after 5–7 days of incubation (Fig. 2D), whereas colonies of *map1* Δ co-expressing wild-type MetAP1 and MetAP2 (H174A) were observed in the absence of 5'-FOA (Fig. 2B). These results demonstrate that overexpression of MetAP2 (H174A) under the *GPD* promoter is lethal in the *map1* Δ strain.

Yeast MetAP2 (H174A) Expression Under the *GAL1* Promoter Inhibits the Growth Rate of Yeast *map1* Δ

Given that the growth rate of yeast roughly correlates with the level of cellular Met_{init} activity [Chen et al., 2002], the growth rate of *map1* Δ overexpressing MetAP2 (H174A) under the *GAL1* promoter was compared with the growth rates of *map1* Δ alone or *map1* Δ overexpressing wild-type yeast MetAP2 under *GAL1*. Colonies of equal size were observed on

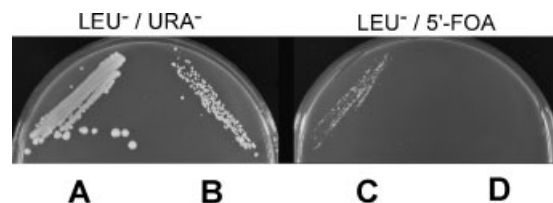


Fig. 2. Yeast MetAP2 (H174A) is lethal in yeast *map1* deletion strain (*map1* Δ). A haploid yeast *map1* Δ strain was co-transformed to leucine and uracil prototrophy with the wild-type yeast *MAP1* gene under 1 kb of its wild-type promoter and either vector alone (A) or *HA-map2* (H174A) under the *GPD* promoter (B). *MAP1* plus vector (C) and *MAP1* plus *HA-map2* (H174A) (D) were grown in SD/Leu⁻ for 48 h to displace the pRS416 plasmid, then plated on SD/Leu⁻ + 1 mg/ml of 5'-FOA to select for cells that have not retained pRS416 (URA³⁺). Plates were incubated for 5–7 days at 30°C to ensure that *map1* Δ cells overexpressing MetAP (H174A) were dead.

glucose plates where expression of MetAP2 (H174A) and wild-type MetAP2 under the *GAL1* promoter is repressed (Fig. 3A). Colonies of *map1* Δ overexpressing wild-type MetAP2 were the largest in the presence of galactose, which suggested that the expression of wild-type MetAP2 under *GAL1* has the greatest effect on the growth rate of *map1* Δ (Fig. 3B, WT). This finding is consistent with our previous observation that overexpression of wild-type MetAP2 can almost completely complement the slow-growth phenotype of the *map1* Δ strain [Li and Chang, 1995]. A quantitative comparison of growth rates confirmed that *map1* Δ overexpressing wild-type MetAP2 had the fastest growth rate in galactose (4.1 ± 0.1 h, Fig. 4 and Table II).

Colonies of *map1* Δ transformed with MetAP2 (H174A) were observed on both glucose and galactose plates (Fig. 3, H174A). Colonies of *map1* Δ with vector alone on galactose plates, however, were larger than colonies of *map1* Δ overexpressing MetAP2 (H174A), indicating that expression of MetAP2 (H174A) under

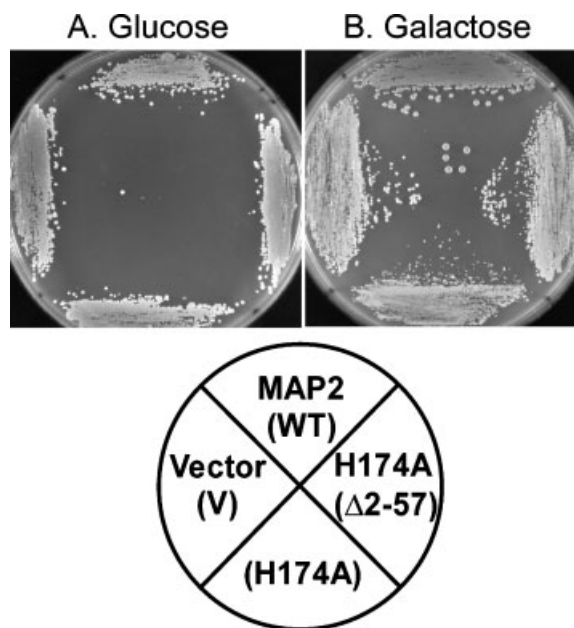


Fig. 3. Growth plate comparison of *map1* Δ transformant strains. A haploid yeast *map1* Δ strain was transformed to leucine prototrophy with p425 *GAL1*/*MAP2* wild-type (WT), p425 *GAL1*/*HA-map2* (H174A), p425 *GAL1*/*map2* (Δ 2-57/H174A), or p425 *GAL1* vector alone (V). Each strain was grown to mid-logarithmic phase at 30°C in minimal synthetic raffinose medium lacking leucine. Approximately 2×10^5 cells (by ABS₆₀₀) were then streaked onto minimal synthetic plates lacking leucine and supplemented with (A) glucose or (B) galactose. Plates were incubated for 4 days at 30°C.

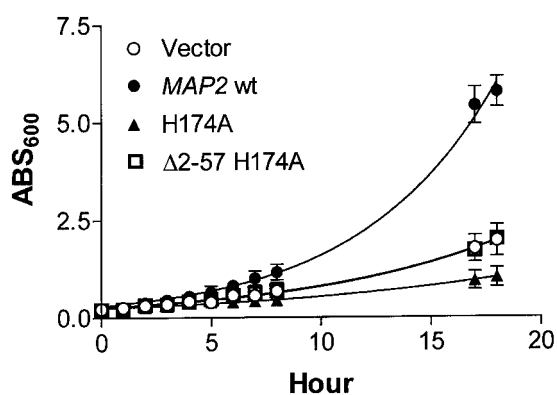


Fig. 4. Growth rate comparison of *map1* Δ transformant strains. A 50 ml seed culture (SG/Leu⁻) of *map1* Δ alone or expressing wild-type MetAP2 (MAP2), MetAP2 (H174A), or truncated MetAP2 (Δ 2-57 H174A) was grown overnight from SD/Leu⁻ plates until $ABS_{600} \sim 0.1$. Each culture was resuspended in 1 ml of SG/Leu⁻ to give $ABS_{600} \sim 5.0$. The ABS_{600} of each 1 ml culture was measured and diluted to an ABS_{600} of 0.2 in 5 ml of SG/Leu⁻. Culture were then grown aerobically at 30°C and the ABS_{600} was measured in triplicate at 1 h intervals

GAL1 decreased the growth rate of *map1* Δ (Fig. 3B, H174A and V). A quantitative comparison of growth rates in the presence of galactose confirmed that *map1* Δ overexpressing MetAP2 (H174A) under *GAL1* grew more slowly than *map1* Δ , a vector control (8.5 ± 0.4 vs. 6.2 ± 0.3 h, Fig. 4 and Table II). These findings indicate that overexpression of MetAP2 (H174A) under the *GAL1* promoter inhibits the growth of *map1* Δ . Co-expression of wild-type MetAP2 in the multi-copy plasmid was also found to protect *map1* Δ from MetAP2 (H174A) lethality under the *GPD* promoter (data not shown). Together, these findings indicate that MetAP2 (H174A) is a dominant negative mutant.

Yeast MetAP2 (H174A) Requires Its N-Terminal Region for the Dominant Negative Effect in *map1* Δ

To test whether the N-terminal region of MetAP2 (H174A) is required for the dominant negative effect, codons for the HA tag and residues 2–57 of *map2* (H174A), which include the polylysine block (41–57), were removed from *HA-map2* (H174A) by PCR-mediated mutagenesis. Truncated *map2* (Δ 2-57/H174A) was then expressed in *map1* Δ in a multi-copy vector under the *GAL1* promoter (p425*GAL1*) to assess its effect on *map1* Δ growth.

Colonies of *map1* Δ overexpressing truncated MetAP2 (Δ 2-57/H174A) under *GAL1* were of the same size as *map1* Δ with vector alone (Δ 2-57, V,

Fig. 3B). This observation is in contrast to the smaller colonies of *map1* Δ overexpressing full-length MetAP2 (H174A) (Fig. 3B). The growth rate of *map1* Δ overexpressing MetAP2 (Δ 2-57/H174A) was similar to *map1* Δ with vector alone (6.4 ± 0.2 vs. 6.2 ± 0.3 h, respectively) and greater than *map1* Δ overexpressing full-length MetAP2 (H174A) (8.5 ± 0.4 h) (Fig. 4 and Table II). These results indicate that overexpression of MetAP2 (Δ 2-57, H174A) under the *GAL1* promoter has no effect on the growth rate of *map1* Δ . Furthermore, while overexpression of the HA-2-57 fragment alone under the *GPD* promoter decreased the growth rate of *map1* Δ (Table II), it was not lethal like full-length H174A (Fig. 2D). Taken as a whole, these findings indicate that residues 2–57 are essential, but not fully sufficient, for growth inhibition of the *map1* null yeast strain by MetAP2 (H174A).

Steady State Levels of Each MetAP2 Construct Are Comparable

To rule out differences in expression, the relative steady state levels of each MetAP2 construct were compared. Wild-type MetAP2, MetAP2 (H174A), and truncated MetAP2 (H174A) were expressed at similar levels in *map1* Δ under the *GAL1* promoter (Fig. 5). A second fragment (~ 45 kDa), similar in size to MetAP2 (Δ 2-57/H174A), was also observed with MetAP2 (H174A) but not with wild-type MetAP2 (Fig. 5, lanes 1, 3, and 4). Given that our antibodies were generated against the C-terminal region of MetAP2, the second smaller fragment is likely a C-terminal truncation of MetAP2 (H174A). Because the truncated

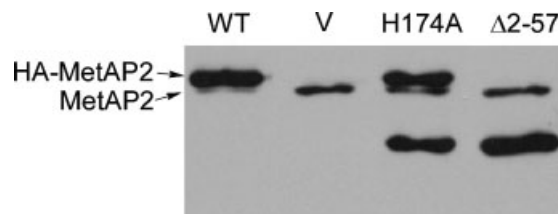


Fig. 5. The steady state levels of each MetAP2 construct under *GAL1* are comparable in *map1* Δ . Each *map1* Δ transformant strain expressing wild-type HA-MetAP2 (WT), vector only (V), HA-MetAP2 (H174A), or MetAP2 (Δ 2-57/H174A) was grown to $ABS_{600} \sim 1.0$ (25 ml SG/Leu⁻), and a crude extract was obtained. Approximately 1.2 μ g of total protein was loaded into each lane. Proteins were visualized by Western blot using primary rabbit anti-MetAP2 polyclonal antibodies and secondary goat anti-rabbit polyclonal HRP-conjugated antibodies. Endogenous wild-type MetAP2 is identified as MetAP2.

MetAP2 ($\Delta 2-57$) has no dominant negative effect, it is highly unlikely that this smaller fragment is the cause of the dominant negative effect in this mixture.

Overexpression of MetAP2 (H174A) Under *GPD* has a Minimal Effect on Yeast *map2 Δ* Growth Rate

Given the overlapping substrate specificities of MetAP1 and MetAP2, we tested whether MetAP2 (H174A) could interfere with MetAP1 activity in vivo. Since the H174A mutant will bind substrates with little ability in processing the N-terminal Met, overexpressing the H174A mutant may compete for similar sets of substrates with MetAP1 and prevent them from being processed by MetAP1 in vivo. Both the H174A mutant and wild-type MetAP2 were expressed under the *GPD* promoter in a yeast *map2 Δ* strain and their respective growth rates were compared to *map2 Δ* (expresses only wild-type MetAP1) with vector alone.

Overexpression of MetAP2 (H174A) under the *GPD* promoter slightly inhibited the growth rate of yeast *map2 Δ* compared to vector alone (2.5 ± 0.2 vs. 2.2 ± 0.1 h, Table II). Overexpression of wild-type MetAP2 under the *GPD* promoter, however, also decreased the growth rate of *map2 Δ* relative to vector alone (3.4 ± 0.4 vs. 2.2 ± 0.1 h, Table II). Thus, overexpression of MetAP2 (H174A) and wild-type MetAP2 slightly decreases the growth rate of yeast in the presence of wild-type MetAP1. Given that both wild-type and mutant MetAP2 affect the growth rate of yeast, it is likely that growth inhibition in the presence of wild-type MetAP1 is occurring by some mechanism unrelated to the inhibition of cellular Met_{init} activity.

DISCUSSION

In this study, evidence of the first dominant negative mutant of eukaryotic MetAP2 is reported. Our findings show that catalytically inactive yeast MetAP2 (H174A) interferes with wild-type MetAP2 function in a concentration-dependent manner. Furthermore, residues 2–57 of its N-terminal region are essential, but not sufficient, for this interference.

His¹⁷⁴ is strictly conserved in all MetAPs sequenced to date [Li and Chang, 1995; Tahirov et al., 1998; Giglione et al., 2000]. Previous studies have reported a similar disruption of catalytic function by replacement of the homologous

residue in human MetAP2 with Arg (H231R) [Griffith et al., 1998] or in *E. coli* MetAP with Ala (H79A) [Lowther et al., 1999a]. The imidazole nitrogen (N ϵ 2) of the homologous residue in human MetAP2, His²³¹, has also been identified as the covalent attachment site for the fumagillin class of angiogenesis inhibitors (TNP-470 and ovalicin) [Griffith et al., 1998; Liu et al., 1998].

A role for conserved His¹⁷⁴ in catalysis has been proposed based on the crystal structures of *E. coli* MetAP bound to a series of phosphorous-based transition-state analogues. Homologous His⁷⁹ of *E. coli* MetAP is thought to stabilize a tetrahedral intermediate by hydrogen-bond interactions with the nitrogen of the scissile peptide bond [Lowther et al., 1999b]. Given the nature of the substrate-binding pocket in *E. coli* MetAP, His⁷⁹ was further proposed to act as a primary substrate-binding determinant [Lowther et al., 1999b]. If so, we predict that mutating this histidine may alter the substrate specificity of MetAP2. Given that the MetAP2 (H174A) mutant is inactive, however, we were unable to test this model. Nonetheless, our findings are consistent with previous observations that this conserved residue is essential for MetAP2 catalytic activity.

Possible Mechanisms of MetAP2 (H174A) Dominant Negative Effect

There are at least three plausible mechanisms for the dominant negative phenotype exhibited by the overexpression of MetAP2 (H174A) in *map1 Δ* . First, it is possible that overexpression of MetAP2 (H174A) inhibits *map1 Δ* growth by a mechanism separate from wild-type MetAP2 catalytic function. This possibility is unlikely, however, because co-expression of wild-type yeast MetAP2 in a multi-copy vector (YEP352) protects *map1 Δ* from MetAP2 (H174A) overexpression under the *GPD* promoter (data not shown). Furthermore, overexpression of wild-type yeast MetAP2 in yeast *map1 Δ* almost completely restores normal growth [Li and Chang, 1995] and cellular Met_{init} hydrolysis activity [Chen et al., 2002]. Thus, an increase in cellular Met_{init} hydrolysis activity within *map1 Δ* protects against MetAP2 (H174A) inhibition.

The second possibility is that MetAP2 (H174A) is competing with wild-type MetAP2 for substrate-binding during translation. Considering that yeast MetAP1 and MetAP2 share an overlapping set of substrates in vivo, one would predict that the H174A mutant will also interfere

with MetAP1 function in a similar manner. Consistent with this prediction, overexpression of MetAP2 (H174A) under the *GPD* promoter slightly decreases the growth rate of *map2Δ* or *map1Δ* co-expressing wild-type MetAP1. The overexpression of wild-type MetAP2 under the *GPD* promoter, however, also slightly decreases the growth rate of *map2Δ*, which is inconsistent with this mechanism, as wild-type MetAP2 is expected to increase cellular Met_{init} hydrolysis activity [Chen et al., 2002]. This suggests that both wild-type MetAP2 and MetAP2 (H174A) slightly inhibit growth in the presence of wild-type MetAP1 through a similar mechanism that is unrelated to MetAP1 activity. It remains possible, however, that MetAP1 and MetAP2 share some cellular factor or ribosome binding site that is essential for normal cellular function and may involve the N-terminal region of each protein. Interestingly, we previously reported a catalytically inactive, dominant negative mutant of yeast MetAP1 that interferes with wild-type MetAP2 function [Klinkenberg et al., 1997]. The instability of the MetAP1 mutant protein after deletion of its extended N-terminal region, however, precluded any conclusions about the mechanism of its effect on wild-type MetAP2 [Klinkenberg et al., 1997].

The final and most likely possibility is that MetAP2 (H174A) is directly competing with wild-type MetAP2 for association with the ribosomes or some other cellular factor that MetAP2 requires for normal function in vivo. Ribosome profiles show that yeast MetAP2 associates with the ribosomes (Vetro, unpublished results) in a pattern similar to wild-type yeast MetAP1 (40S, 60S, 80S) [Vetro and Chang, 2002]. Furthermore, unlike wild-type MetAP2, overexpression of truncated wild-type MetAP2 ($\Delta 2-57$) under the *GPD* promoter cannot rescue the yeast *map1Δ* slow-growth phenotype (data not shown). These findings are consistent with the apparent concentration-dependent effect of MetAP2 (H174A) in *map1Δ* and the requirement for the N-terminal region, which includes a putative protein/nucleic acid interaction domain (polylysine block). We are currently actively engaged in studying whether ribosome association is required for normal functioning of MetAP2 function in vivo, and whether the N-terminal region of MetAP2 mediates an association with the ribosomes or unidentified factor(s) important for normal MetAP2 function in vivo.

Possible Role of the N-Terminal Region in Yeast MetAP2 Function

Polylysine blocks have been found in several proteins involved in protein synthesis, including yeast eukaryotic initiation factor 2 β (eIF2 β) [Pathak et al., 1988], yeast elongation factor 3 [Chakraborty, 1999], and human *N*-myristoyltransferase [Glover et al., 1997]. In most cases, these blocks, together with adjacent acidic residues, are believed to be responsible for protein–protein and protein–nucleic acid interactions, and facilitate the activity of various factors in protein synthesis. Polylysine blocks found in human myristoyltransferase [Glover et al., 1997] and yeast EF-3 [Chakraborty, 1999] have also been reported to likely play an important role in ribosomal association.

The charged regions within the extended N-terminal of eukaryotic MetAP2 have been proposed to mediate ribosome binding in yeast [Li and Chang, 1995] and an association with eIF2 in animal cells [Ray et al., 1992]. The charged N-terminal region in mammalian MetAP2 has also been shown to be required for normal MetAP2-mediated protection of eIF2 α from phosphorylation by eIF2 kinases [Datta et al., 2001, 2003; Datta and Datta, 2003]. Given that eIF2 association is required to block eIF2 phosphorylation, it is likely these charged regions mediate the association of mammalian MetAP2 with eIF2. Thus, it is possible that the polylysine block of yeast MetAP2 is analogously involved in mediating the association of MetAP2 with the ribosomes or unidentified cellular factor(s) that are required for normal function in vivo. This might also explain our observation that overexpression of the N-terminal region alone under the *GPD* promoter slightly decreases the growth rate of *map1Δ* as the N-terminal region is able to compete for the ribosomes or some unidentified cellular factor(s).

Eukaryotic MetAP2 and Angiogenesis

There is recent evidence that the activity of mammalian MetAP2 in VEC is an important factor in blood vessel growth and formation (angiogenesis). Two potent angiogenesis inhibitors, TNP-470 and ovalicin, were found to selectively target and irreversibly inhibit mammalian MetAP2 in proliferating VEC [Griffith et al., 1997; Sin et al., 1997]. Concentrations of TNP-470 that fully inactivate MetAP2 in VEC

also correlate with concentrations that inhibit VEC cell growth [Turk et al., 1999]. The exact role of MetAP2 in angiogenesis remains unclear, although it appears that VEC undergoes p53-dependent growth arrest as cellular MetAP2 catalytic activity is decreased [Yeh et al., 2000; Zhang et al., 2000].

Recent findings regarding the possible essential role of human MetAP2 activity in angiogenesis has triggered a race to find potent new MetAP2-specific inhibitors. These inhibitors will allow us to examine the specific role of MetAP2 in angiogenesis and angiogenesis-related pathologies as well as possibly develop new anti-cancer drugs. As an alternative approach to accomplish these goals, we may be able to find new ways to modulate MetAP2 function in vivo, such as anti-sense, RNAi, or knockout approaches. Given that MetAP2 is a bi-functional protein involved in the regulation of protein synthesis [Ray et al., 1992], these approaches may be unduly complicated. Thus, a catalytically inactive but otherwise structurally sound mutant of MetAP2 would preserve MetAP2's role in protein synthesis while dissecting the mechanisms of MetAP2 function in VEC. We also have preliminary evidence that a catalytically inactive mutant of mammalian MetAP2 inhibits VEC growth in vitro (Ying Fei, unpublished results). A dominant negative mutant of MetAP2 should have great potential for these purposes, and we are actively researching these possibilities.

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