Physiologically Relevant Metal Cofactor for Methionine Aminopeptidase-2 Is Manganese

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ABSTRACT: The identity of the physiological metal cofactor for human methionine aminopeptidase-2 (MetAP2) has not been established. To examine this question, we first investigated the effect of eight divalent metal ions, including Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, on recombinant human methionine aminopeptidase apoenzymes in releasing N-terminal methionine from three peptide substrates: MAS, MGAQFSKT, and ^{3}H-MASK(biotin)G. The activity of MetAP2 on either MAS or MGAQFSKT was enhanced 15−25-fold by Co^{2+} or Mn^{2+} metal ions in a broad concentration range (1−1000 μM). In the presence of reduced glutathione to mimic the cellular environment, Co^{2+} and Mn^{2+} were also the best stimulators (∼30-fold) for MetAP2 enzyme activity. To determine which metal ion is physiologically relevant, we then tested inhibition of intracellular MetAP2 with synthetic inhibitors selective for MetAP2 with different metal cofactors. A-310840 below 10 μM did not inhibit the activity of MetAP2−Mn^{2+} but was very potent against MetAP2 with other metal ions including Co^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+} in the in vitro enzyme assays. In contrast, A-311263 inhibited MetAP2 with Mn^{2+}, as well as Co^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}. In cell culture assays, A-310840 did not inhibit intracellular MetAP2 enzyme activity and did not inhibit cell proliferation despite its ability to permeate and accumulate in cytosol, while A-311263 inhibited both intracellular MetAP2 and proliferation in a similar concentration range, indicating cellular MetAP2 is functioning as a manganese enzyme but not as a cobalt, zinc, iron, or nickel enzyme. We conclude that MetAP2 is a manganese enzyme and that therapeutic MetAP2 inhibitors should inhibit MetAP2-Mn^{2+}.

Methionine aminopeptidases (MetAPs)[1] are intracellular metalloenzymes responsible for removal of the N-terminal initiator methionine residue of nascent proteins (*1*, *2*). They are required for protein co- and/or posttranslational modifications and participate in cellular signal transduction and cell cycle progression by controlling protein subcellular localization and/or protein degradation. The importance of these enzymes in cell growth and tumor progression has been increasingly appreciated, especially since the identification of the type-2 enzyme (MetAP2) as a molecular target for the anti-angiogenesis/antitumor agent TNP-470 (*3*, *4*). The natural product bengamide and its derivatives can inhibit the enzyme activity of both MetAP1 and MetAP2, and this type of agent is under clinical development as a novel cytotoxic agent for cancer therapy (*5*).

MetAPs belong to a new class of proteases with a pita-bread fold that was first observed in the crystal structure of *Escherichia coli* MetAP1 (*6*). This family also includes aminopeptidase P, creatine amidinohydrolase (creatinase), and prolidase (*2*). Two types of MetAPs exist based on their amino acid sequences (*7*), and both are present in human cells (*8*). Human MetAP1 has not been fully characterized, while MetAP2 is a protein of dual functions: methionine aminopeptidase activity and protection of eIF-2α from inactivation. MetAP2 was first cloned by Li and Chang (*9*) as the human orthologue of rat eIF-2α associated protein (p67). Arfin et al. (*7*) also independently cloned human MetAP2 from a partial amino acid sequence of the purified porcine MetAP2 enzyme. Full-length human MetAP2 consists of 478 amino acids. The C-terminal portion contains the catalytic domain showing high amino acid identity with MetAP sequences from prokaryotes and yeast, while the N-terminal region has two basic poly-lysine motifs and an aspartic acid motif. N-terminal truncation of the highly charged domain, which was speculated to be involved in the binding of eIF-2α to prevent its phosphorylation and consequent inactivation (*10*), did not affect the enzyme activity in vitro (*11*). On the other hand, MetAP2 was able to protect eIF-2α from phoshorylation even when its enzyme activity was covalently inhibited by TNP-470 (*3*).

The physiological metal ions for MetAPs have not been established and are controversial. Like MetAPs of bacteria and yeast, human MetAP2 has been categorized as a cobalt dependent metalloenzyme, based on the observations that the purified enzymes show highest activity in the presence of cobalt as compared to that of other divalent metals ions

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[1] Abbreviations: MetAPs, methionine aminopeptidases; MetAP1, methionine aminopeptidase type 1; MetAP2, methionine aminopeptidase type 2; HMVEC, human neonatal dermal microvascular endothelial cell; GSH, glutathione.

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(*7*, *12*). A more recent study showed that Zn^{2+} was a superior cofactor to Co^{2+} for yeast MetAP1 because Co^{2+} did not stimulate yeast MetAP1 activity in the presence of physiological concentrations of reduced glutathione (*13*). Another study demonstrated that the physiologically relevant metal ion for *E. coli* MetAP1 was likely Fe^{2+} on the basis of whole cell metal analyses (*14*). In addition, Cosper et al. (*15*) provided structural evidence that *E. coli* MetAP1 is a mononuclear enzyme. Most recently, D'souza et al. (*16*) showed the kinetic and structural characterization of manganese-loaded MetAPs from *E. coli* and the hyperthermophilic archaeon *Pyrococuus furiosus*, implicating manganese as a metal cofactor for MetAPs. However, detailed studies on the effect of metal ions on human MetAP1 and MetAP2 have been lacking. Identification of physiological metal cofactors for MetAP2 is critical for discovery of small molecule therapeutic inhibitors since their potency may vary for different metal ions. In the current study, we extensively compare the effect of most common divalent metal ions on the activity of recombinant human MetAP1 and MetAP2 enzyme and use synthetic inhibitors selective for MetAP2 with different metal cofactors to show that human MetAP2 is a manganese enzyme.

MATERIALS AND METHODS

Cloning and Expression of Human MetAP2 and MetAP1. MetAP2 cDNA was amplified from total RNA of human neonatal dermal microvascular endothelial cells (Clonetics, San Diego, CA) by RT-PCR with the following two oligonucleotide primers: 5′- - -ATT AAT AGA TCT TTG GAC AAG AGG CAC CAT CAC CAT CAC CAT GCG GGC GTG GAG GAG GTA GCG GCC T- - -3′ and 5′- - -ATT AAT CTC GAG GAA TTC TTA ATA GTC ATC TCC TCT GCT GAC AAC T- - -3′. Access RT-PCR kit from Promega (Madison, WI), 0.5 μg of RNA per 50 μL, and 1 μM primers were used in the one tube RT-PCR reaction according to manufacturer's instruction. The amplified DNA product was cloned into pCR-Blunt vector (Invitrogen, San Diego, CA), and its (pCR-Blunt-MetAP2) sequence was confirmed by DNA sequencing. MetAP2 cDNA was cut from pCR-Blunt-MetAP2 with Bgl II and EcoR I and ligated to a baculovirus transfer vector pAcGP67B (Pharmingen, San Diego, CA) cut with BamH I (generating compatible ends to that of Bgl II) and EcoR I. The final expression vector pAcGP67B-MetAP2 is shown in Figure 1A.

Human MetAP1 cDNA sequence was reported in the literature as mRNA for KIAA0094 (*17*), which has complete 3′-end sequence of potential MetAP1 but not the defined 5′-end starting codon. To clone full-length MetAP1 cDNA, human fetal liver marathon-Ready cDNA library (Clontech, Palo Alto, CA) was used to do 5′-RACE with a MetAP1 primer, 5′- - -CGT TAA AAT TGA GAC ATG AAG TGA GGC CGT- - -3′, which is complimentary to the 3′ end of MetAP1 coding sequence. The PCR products were cloned into pT-Adv cloning vector (Clontech, Palo Alto, CA) and sequenced. The clone with most extended 5′-end sequence had 40 bp additional sequence as compared to the KIAA0094 sequence. No new ATG codon upstream of the ATG codon (position 26) in KIAA0094 sequence was found, indicating that KIAA0094 may already have the full-length MetAP1 coding sequence. The MetAP1 coding sequence was then further PCR amiplified with the following two primers: 5′- - -ATT AAT GGA TCC A GCG GCC GTG GAG ACG CGG GTG T- - -3′ and 5′- - -ATT AAT CTC GAG GAA TTC TTA AAA TTG AGA CAT GAA GTG AGG CCG T- - -3′, which was cleaved with BamH I and EcoR I and cloned into the baculovirus transfer vector pAcGP67B as shown in Figure 1A.

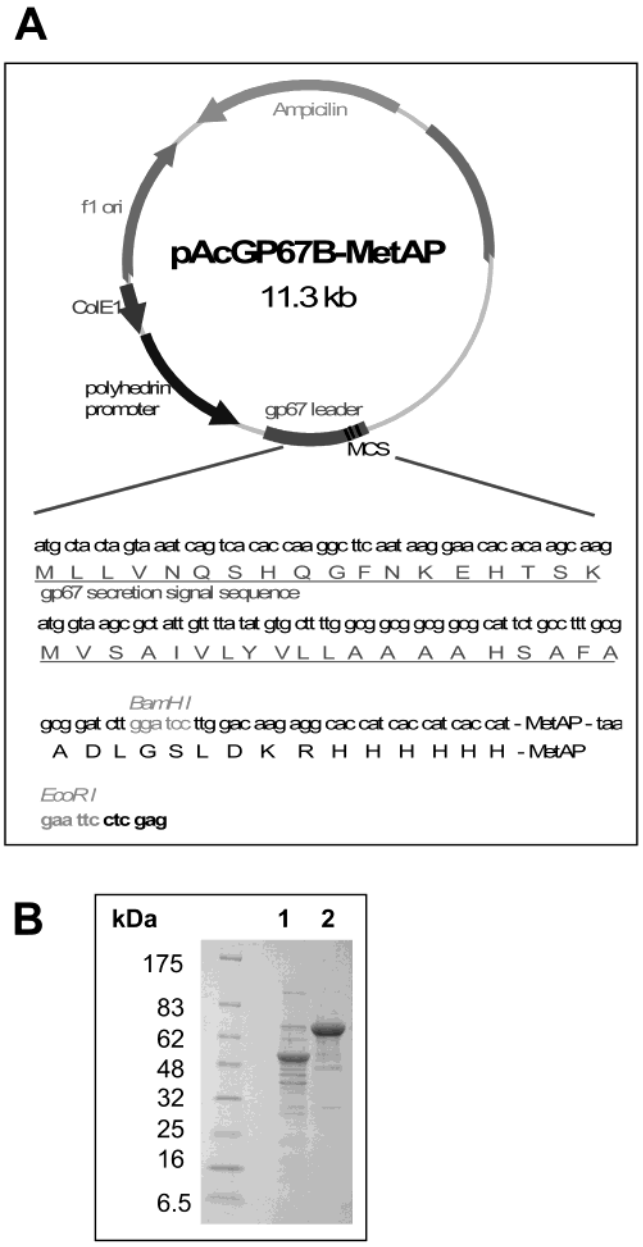


FIGURE 1: Recombinant human MetAP1 and MetAP2. Both human MetAP1 and MetAP2 were cloned by RT-PCR from endothelial cell total RNA, and coding sequences were then cloned in a baculovirus transfer vector pAcGP67B with a N-terminal 6-His tag (A). Both proteins were expressed and secreted by this system. MetAP1 and MetAP2 purified by one-step nickel column were analyzed by SDS−PAGE as shown in a Coommassie blue stained gel (B). Both enzymes showed methionine aminopeptidase activity.

Both MetAP2 and MetAP1 recombinant transfer vectors were transfected with BaculoGold (Pharmingen, San Diego, CA) into insect sf9 cells, and recombinant viruses were obtained. Recombinant MetAP2 and MetAP1 were expressed and secreted into the culture medium of sf9 cells infected

with the recombinant viruses. The active enzymes were purified in a similar manner as outlined below.

The serum-free culture media with expressed MetAP1 or MetAP2 was diluted with an equal volume of cold water and loaded to a hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer, pH 6.7, and MetAP2 or MetAP1 activity was eluted with a gradient of potassium phosphate buffer (10−400 mM). The pooled fractions containing MetAP2 or MetAP1 activity were pooled and diluted 5-fold with cold water and loaded to a cation exchange S20 column (BioRad, Hercules, CA) equilibrated with 10 mM Hepes buffer pH 7.4, 10 mM NaCl. MetAP2 or MetAP1 was eluted with a salt gradient (10−500 mM NaCl) and finally purified on a Sephacryl S-100 gel filtration column with 10 mM Hepes buffer, pH 7.4, 150 mM NaCl.

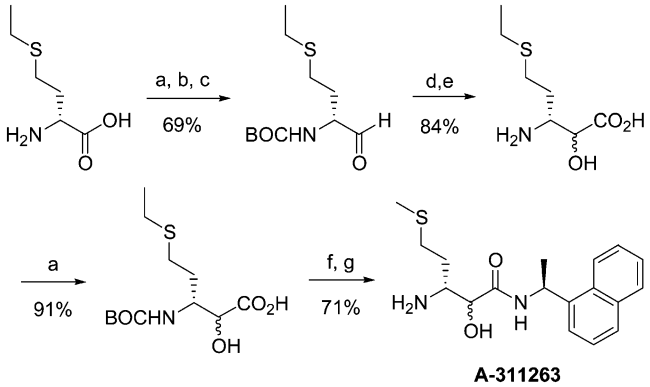
Peptide Substrates for MetAPs. MetAP peptide substrate MAS (methionine-alanine-serine) was purchased from Bachem (King of Prussia, PA). The octapeptide substrate, MGAQFSKT, based on the N-terminal sequence of human MARCKS protein, was synthesized and HPLC purified by Research Genetics (Huntsville, AL).

^{3}H-Methionine-alanine-serine-lysine(biotin)-glycine-amide (^{3}H-MASK(biotin)G) was synthesized in our laboratory on an Applied Biosystems Synergy peptide synthesizer using standard FMOC chemistry. Fmoc-L-Lys(biotinyl)-OH was purchased from BACHEM. Fmoc-L-[methyl-^{3}H]methionine was synthesized by reacting Fmoc-Cl with L-[methyl-^{3}H]-methionine (Amersham) in a 1:1 dioxane/10% Na_2CO_3(aq) mixed solvent. The peptides were deprotected and cleaved from the resin using a trifluoroacetic acid solution containing water, thioanisole, and ethanedithiol (900:25:50:25 μL) as scavengers. The peptides were purified by HPLC using a Waters C18 Symmetry column (7.8 × 300 mm, 7 μm). A gradient of acetonitrile/water (0.1% TFA) was used going from 3 to 15% acetonitrile in 20 min and a flow rate of 2 mL/min. Radioactive ^{3}H-MASK(biotin)G peptide had specific activity of 25 000 dpm/nmol.

Activity Assays for MetAP1 and MetAP2. A coupled-enzyme chromogenic assay was developed to measure methionine aminopeptidase activity by monitoring the production of free methionine with L-amino acid oxidase and horseradish peroxidase. Assays were performed in 96-well microtiter plates. Enzyme preparations were diluted in assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl), and 10 μL enzymes were introduced into each well of the plate. A mixture (90 μL) of 0.1 mg/mL L-amino acid oxidase (Sigma Catalog No. A-9378), 0.1 mg/mL horseradish peroxidase (Sigma Catalog No. P-8451), 0.1 mg/mL *ortho*-dianisidine (Sigma Catalog No. D-1954), and 0.5 mM peptide substrate was added to each well. The reactions were carried out at room temperature, and absorbance at 450 nm (A_{450}) was measured every 20 s over a period of 20 min using an automatic plate reader (Molecular Devices, CA). The rate in mOD/min, calculated for each well, was converted to nM methionine releases per minute per nM MetAP1 or MetAP2 in the reaction (nM/min/nM).

Another assay for methionine aminopeptidase activity used the peptide substrate ^{3}H-methionine-alanine-serine-lysine-(biotin)-glycine-amide. MetAP2 or MetAP1 was incubated with 0.1 mM radioactive peptide in the assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) for 20 min at room temperature, and released ^{3}H-methionine was measured by scintillation counting (Beckman LS1801, Beckman Coulter, Fullerton, CA) after removal of the uncleaved biotinylated peptide substrate with Streptavidin-agarose (Pierce, Rockford, IL).

Scheme 1: Synthesis of A-311263[a]



[a] Reagents and conditions: (a) BOC-ON, Et_3N, dioxane/H_2O; (b) REDAL, toluene, 0−25 °C; (c) SO_3 pyridine, Et_3N, DMSO; (d) $NaHSO_3$, KCN, EtOAc/H_2O; (e) 6M HCl, dioxane/H_2O, reflux, 18 h; (f) EDCl, HOBT, NMM, (S)-1-(1-naphthyl)ethylamine; (g) HCl/dioxane.

Effect of Metals on MetAP1 and MetAP2 Activities. The chromogenic assay with either the 3-mer MAS peptide or the 8-mer MGAQFSKT peptide was used to study the effect of metals on MetAP1 and MetAP2 activities. Calcium chloride (Ca^{2+}), cobalt chloride (Co^{2+}), cupric chloride (Cu^{2+}), ferrous chloride (Fe^{2+}), magnesium chloride (Mg^{2+}), manganese chloride (Mn^{2+}), nickel chloride (Ni^{2+}), or zinc chloride (Zn^{2+}) was included in the assay at final concentrations of 0, 0.1, 1, 10, 100, 1000, and 10 000 μM in the assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl). Since glutathione affects the chromogenic assay, the radioactive assay with ^{3}H-methionine-alanine-serine-lysine(biotin)-glycine-amide peptide was used in assays with 5 mM reduced glutathione.

Synthesis of a Triazole MetAP2 Inhibitor A-310840 (3-((2-Naphthylmethyl)sulfanyl)-4H-1,2,4-triazole). To a suspension of 3-mercapto-1,2,4-triazole (0.18 g, 1.8 mmol) and cesium carbonate (0.72 g, 2.2 mmol) in 5 mL of *N*,*N*-dimethylformamide was added 2-(bromomethyl)naphthalene (0.38 g, 1.7 mmol). The mixture was heated at 40 °C for 16 h. The volume was reduced by rotary evaporation, and the remaining mixture was shaken with water and methylene chloride and then filtered. The layers of the filtrate were separated, and the organic phase was dried over magnesium sulfate. Filtration and solvent removal gave a white solid (0.143 g).

MS (DCI/NH_3) *m*/*e* 242 $(M + H)^+$, 259 $(M + NH_4)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 14.08 (s, 1H), 8.57 (bds, 1H), 7.88 (m, 4H), 7.49 (m, 3H), 4.51 (s, 2H); Anal. Calcd for $C_{10}H_{20}N_4S$: C, 64.70; H, 4.59; N, 17.41. Found: C, 64.93; H, 4.58; N, 17.25.

Synthesis of Bestatin MetAP2 Inhibitor A-311263 ((2RS,-3R)-3-Amino-2-hydroxy-5-ethylthio)pentanoyl-((S)-(−)-(1-naphthyl)ethyl)amide). The compound was prepared from D-ethionine (Aldrich Chemical Co.) by the route shown in Scheme 1.

Spectroscopic data for A-311263: MS (ESI + Q1MS) *m*/*e* 347 $(M + H)^+$, 693 $(2M + H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75−7.47 (m, 7H), 6.55 (br, 1H), 4.82−4.75

(m, 1H), 4.15 (d, 1H), 3.60−3.33 (br m, 3H), 2.68−2.34 (m, 3.6H), 2.18 (q, 0.4H), 1.87−1.68 (m, 2H), 1.58−1.53 (m, 3H), 1.13 (t, 0.6H), 0.98 (t, 0.4H). Anal. Calcd for $C_{19}H_{27}N_2O_2S\bullet HCl$: C, 59.59; H, 7.11; N, 7.32. Found: C, 59.54; H, 7.13; N, 7.16.

Cellular MetAP2 Activity Assay. HMVEC cells from Clonetics were grown in EGM2 medium as described (*8*). Cells in T75 flasks at ∼70% confluence were changed to 15 mL EGLM (labeling medium, also from Clonetics) without added methionine. Fumagillin or A-310840 was added to the culture at final concentrations of 1 nM (Fumagillin) or 0.5, 2, and 10 μM (A-310840 or A-311263). The cells and inhibitors were incubated for 4 h before ^{35}S-methionine (Amersham) was added to final 1 mCi/flask. The cells were allowed further incubation for 2 h and then washed, trypsinized, and lysed in 0.5 mL RIPA buffer. The lysate samples having the same amount of radioactivity were loaded onto a 1 mL column of Reactive Blue 72-agarose (Sigma) equilibrated with 20 mM Tris/HCl, pH 7.5, 150 mM NaCl. The columns were washed with 30 mL of equilibration buffer to get a baseline count of radioactivity and then washed with 1 mL of 100 nM MetAP2 in washing buffer three times at room temperature. The unprocessed initiator methionine (MetAP2 cleaved radioactivity) was the difference between the last 3 mL washing buffer counts and the 3 mL MetAP2 eluted counts.

HMVEC Proliferation Assay. Human neonatal dermal microvascular endothelial cells (HMVEC) and the growth medium EGM2 were purchased from Clonetics (San Diego, CA), and the cells were grown according to the manufacturer's instructions. Cell proliferation assay was performed in 96-well plates using cells between passages 6 and 12. Cells were seeded at 3000 cells/well and allowed to attach for 4 h. MetAP2 inhibitors, diluted in culture medium, were added in quadruplicate wells, and the cells were incubated for 3 days before MTS reagents (Promega, Madison, WI) were added to quantify the live cells in each well (*8*).

Cell Permeability of MetAP2 Inhibitors. HMVEC cells were grown in complete EGM2 medium to 70% confluence in T175 flasks. A-311263 or A-310840 was added into the culture medium at a final concentration of 10 μM. The cells were incubated at 37 °C for 4 h. At the end of incubation, the medium was removed, and cells were washed quickly with 30 mL of PBS. The cells were then lysed in 1 mL of water by scraping, and the cytosol fraction was obtained after centrifugation at 10 000g × 10 min. A separate flask of cells was detached by trypsinization to measure the total cell volume (20 μL). A-311263 and A-310840 concentrations in the cytosolic fractions were determined by LC-MS using YMC-basic column and 30% ACN, 0.1% acetic acid mobile phase.

RESULTS AND DISCUSSION

Recombinant Human MetAP1 and MetAP2. MetAP1 and MetAP2 enzymes each with an N-terminal six histidine tag were expressed and secreted by baculovirus infected sf9 cells. Both recombinant MetAP1 and MetAP2 showed enzymatic activity, cleaving a tripeptide MAS substrate after purification on the nickel column. The culture medium of normal sf9 cells or cells infected with wild-type baculovirus did not have activity cleaving MAS peptide. Figure 1 shows the expression vector construct (Figure 1A) and a Coommassie blue stained SDS−PAGE gel (Figure 1B) of MetAP1 and MetAP2 purified with nickel-agarose columns. MetAP1 had an expected electrophoretic mobility for a 47 kDa protein, while MetAP2 had mobility of 67 kDa instead of theoretical 54 kDa. The unusual electrophoretic behavior of MetAP2 was documented in the literature when it was identified as p67 (*10*), probably because of its elongated motifs of acidic and basic residues (*7*). MetAP1 and MetAP2 are known to be metalloenzymes (*7*, *12*), and the presence of nickel ion may affect the activity of these enzymes. We therefore developed a scheme to purify MetAP1 and MetAP2 in the absence of added metals to >95% purity. Recombinant MetAP1 and MetAP2 expressed and purified without added exogenous metal ions in the culture medium or in purification buffers still showed significant methionine aminopeptidase activity as seen in a coupled enzyme assay. To minimize the baseline activity of MetAP1 and MetAP2 without added metal ions, both enzymes were treated with 5 mM EDTA followed by extensive dialysis as described before (*12*). These enzyme preparations were used for all following studies.

Effect of Metal Ions on the Enzymatic Activities of MetAP1 and MetAP2. Two peptide substrates were used in the coupled enzyme chromogenic assays for methionine aminopeptidase activity. These were the tripeptide MAS and the octapeptide MGAQFSKT of the natural N-terminal sequence of human MARCKS protein, a myristoylated alanine rich protein kinase C substrate (*18*). The baseline activity of MetAP2, in the absence of added exogenous metals, was minimal and was not significantly different for the two substrates (3.1 nM/min/nM turnover of MAS vs 4.1 nM/min/nM turnover of MGAQFSKT). MetAP1 had 14-fold higher baseline activity (14.0 nM/min/nM turnover) on the shorter MAS peptide than that on the longer peptide MGAQFSKT (0.94 nM/min/nM turnover).

MetAP2 activity was stimulated by the addition of metal ions. Both Co^{2+} and Mn^{2+} stimulated MetAP2 activity on MAS and MGAQFSKT substrates 10−20-fold (Figure 2A,B). Co^{2+} showed maximum stimulation at 10 μM, while Mn^{2+} reached its maximum effect at 1 μM. At higher concentrations all the metal ions, except Mn^{2+}, showed inhibitory activity on the enzymes. MetAP1 had higher baseline activity with MAS substrate, and stimulation of its activity by Co^{2+} and Mn^{2+} was about 2-fold (Figure 3A). When MGAQFSKT was used as the substrate, MetAP1 was stimulated 5-fold by Co^{2+} and 3-fold by Mn^{2+} (Figure 3B). These data show Co^{2+} and Mn^{2+} are the best cofactors for the purified MetAP enzymes.

To study the effect of metal ions in the presence of physiologically relevant reduced glutathione (GSH) (*13*), we used an assay measuring radioactive ^3H-methionine release from the peptide ^3H-methionine-alanine-serine-lysine(biotin)-glycine-amide because GSH affected the coupled enzyme chromogenic assay. The baseline activity of MetAP2 without added exogenous metals under these assay conditions was very low, 0.33 nM/min/nM. In the presence of 5 mM glutathione, both Co^{2+} and Mn^{2+} stimulated MetAP2 activity by 30−40-fold (Figure 4A). At low concentrations (0.1−1 μM), Zn^{2+} and Ca^{2+} also enhanced MetAP2 activity 5−10-fold. MetAP1 showed high baseline activity (20 nM/min/nM). The effect of metal ions on MetAP1 activity in the presence of 5 mM glutathione (Figure 4B) was different from

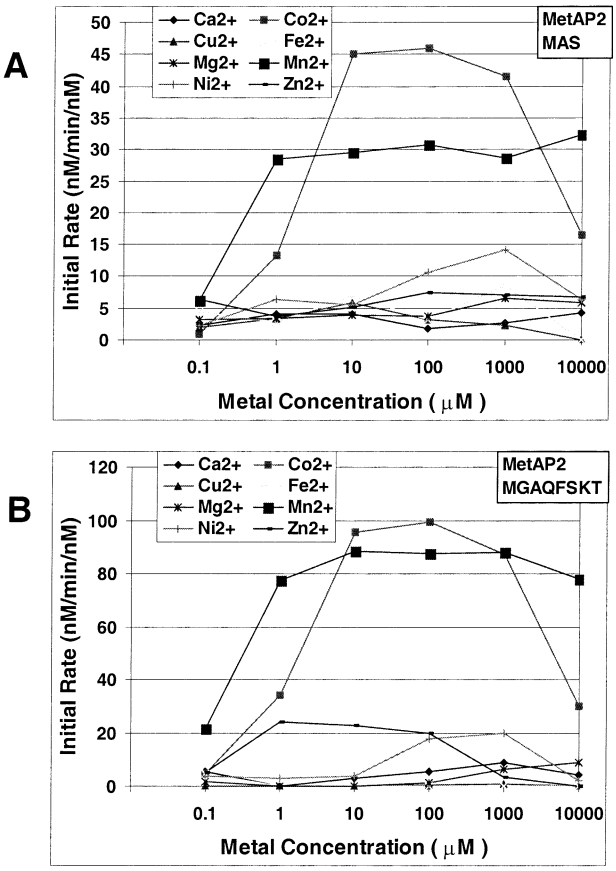


FIGURE 2: Metal dependence of MetAP2 activity. MetAP2 activity was measured by a coupled enzyme chromogenic assay as described in Materials and Methods with a short 3-mer peptide substrate MAS (panel A, 60 nM MetAP2) and a longer 8-mer peptide substrate MGAQFSKT of MARCKS protein (panel B, 30 nM MetAP2). Both peptides were used at 0.5 mM final concentrations. The initial rate for MAS with MetAP2 without added metal was 3.1 nM/min/nM enzyme (defined in Materials and Methods) and that for MGAQFSKT with MetAP2 without added metal was 4.1 nM/min/nM enzyme. Co^{2+} and Mn^{2+} showed highest stimulation of MetAP2 activity, while Ni^{2+} and Zn^{2+} showed minor stimulation. The data are from one of two independent experiments with similar results. Concentrations of metal ions are in log scale.

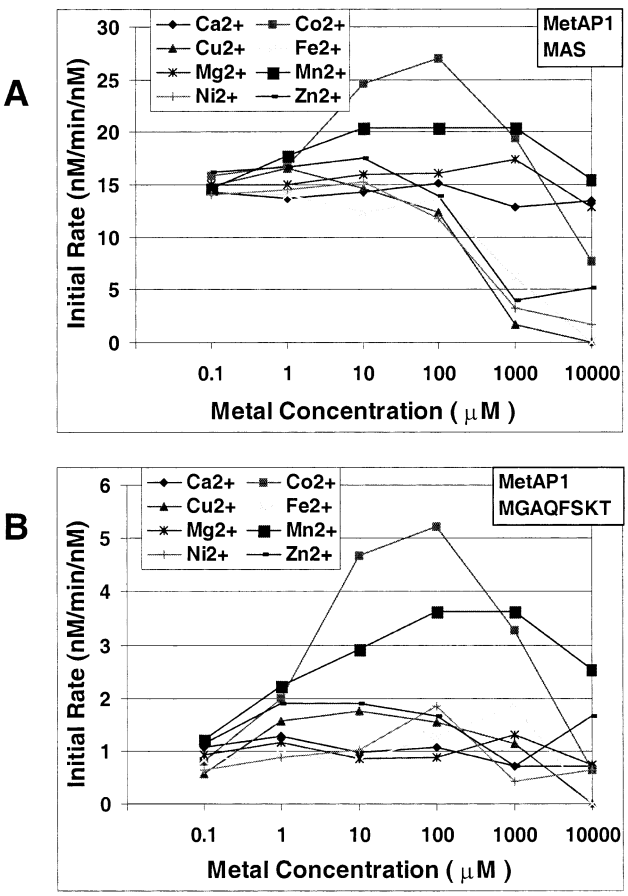


FIGURE 3: Metal dependence of MetAP1 activity. MetAP1 activity was measured by a coupled enzyme chromogenic assay as described in Materials and Methods with a short 3-mer peptide substrate MAS (panel A, 120 nM MetAP1) and a longer 8-mer peptide substrate MGAQFSKT of MARCKS protein (panel B, 500 nM MetAP1). Both peptides were used at 0.5 mM final concentration. The initial rate for MAS with MetAP1 without added metal was 14.0 nM/min/nM enzyme and that for MGAQFSKT with MetAP1 without added metal was 0.94 nM/min/nM enzyme. Co^{2+} and Mn^{2+} showed highest stimulation of MetAP1 activity, while Zn^{2+} showed minor stimulation. The data are from one of the two independent experiments. Concentrations of metal ions are in log scale.

that without glutathione (Figure 3). Zn^{2+} showed best activity, while Co^{2+} and Fe^{2+} were also stimulatory. These results with MetAP1 are consistent with the observations by Walker and Bradshaw (*13*), who demonstrated that Zn^{2+} was a better cofactor for yeast MetAP1 than Co^{2+} in the presence of reduced glutathione.

MetAP2 Inhibitors with Selectivity for Metal Cofactors Show MetAP2 as a Manganese Enzyme. We have demonstrated that Co^{2+} and Mn^{2+} form highest active MetAP2 enzymes in purified systems. We also have discovered synthetic MetAP2 inhibitors that selectively inhibit MetAP2 complexes with different metal cofactors. To determine which metal ion is physiologically relevant, we then used these inhibitors to determine their effects on intracellular MetAP2 inhibition and on cell proliferation in human microvascular endothelial cells (HMVEC). A-310840 is a triazole MetAP2 inhibitor with potent inhibitory activity on MetAP2 with Co^{2+} (IC_{50} of 61 nM) and many other metal ions including Zn^{2+}, Fe^{2+}, and Ni^{2+} (IC_{50} of 26−69 nM), but it is 1000-fold less potent on MetAP2-Mn^{2+} enzyme with an IC_{50} of 58 μM (Table 1). A-311263 is a bestatin type MetAP2 inhibitor that has little preference for metal ions board to MetAP2, as it inhibits MetAP2-Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, or Ni^{2+} with IC_{50} of 55−149 nM (Table 1). Both A-310840 and A-311263 are able to permeate and accumulate in HMVEC cells (63−157 μM in cytosol vs 10 μM outside, Figure 5A, inset). To determine if these inhibitors inhibited cellular MetAP2 enzyme activity, we analyzed the N-terminal initiator methionine status of cellular proteins including GAPDH, a known MetAP2 substrate (*19*). A-310840 at concentrations up to 10 μM does not inhibit the processing of cellular protein initiator methionine in HMVEC (Figure 5A), while A-311263 blocks N-terminal initiator methionine processing in a dose-dependent manner (Figure 5A). Consistent with its inactivity on cellular MetAP2 enzyme inhibition, A-310840 does not inhibit the cell proliferation (Figure 5B). In contrast, A-311263 inhibits HMVEC proliferation in a dose response (Figure 5B) similar to that observed for cellular MetAP2 inhibition (Figure 5A). Fumagillin, a covalent MetAP2 inhibitor that inhibits MetAP2 by alkylating a histidine residue in the enzyme active site regardless of which metal ion, resulted in inhibition of cell

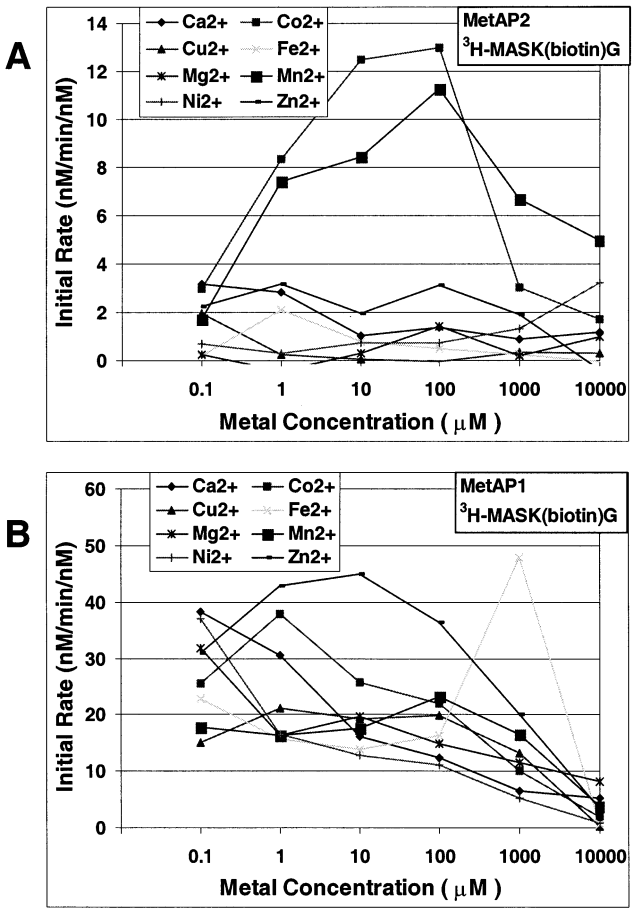


FIGURE 4: Metal dependence of MetAP2 and MetAP1 activity in the presence of glutathione. The activity of MetAP2 (panel A) and MetAP1 (panel B) was measured with a biotinylated radioactive peptide substrate ^{3}H-MASK(biotin)G (at 0.1 mM final concentration) as described in the Materials and Methods. The initial rate for MetAP2 (60 nM) was 0.33 nM/min/nM enzyme and that for MetAP1 (40 nM) was 20 nM/min/nM enzyme. Co^{2+} and Mn^{2+} showed maximal stimulation of MetAP2 activity, while Zn^{2+}, Co^{2+}, and Fe^{2+} showed stimulation of MetAP1 activity. The data are from one of the two separate experiments with similar results. Concentrations of metal ions are in log scale.

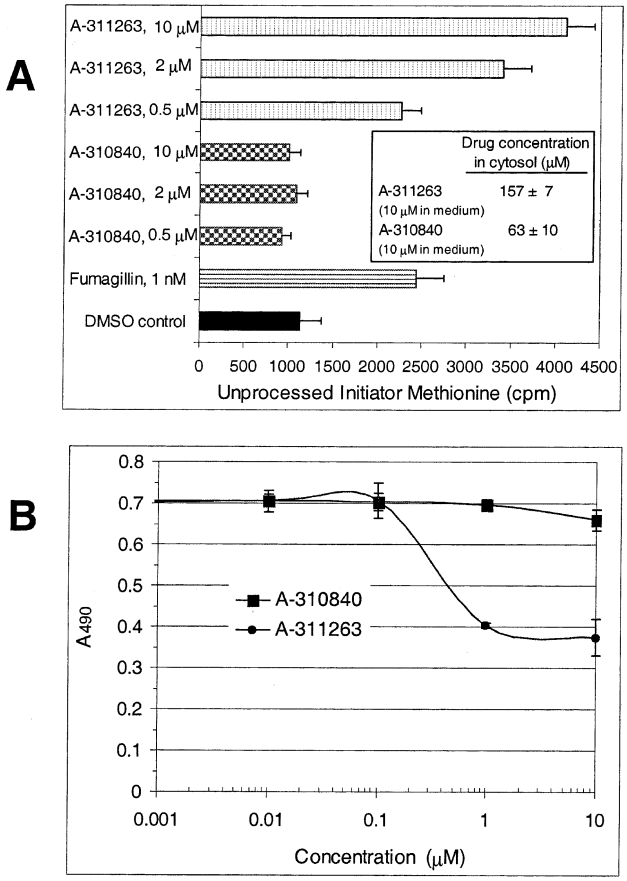
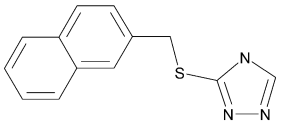
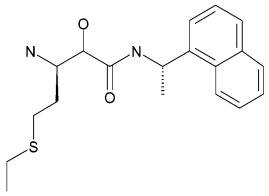


FIGURE 5: Cellular activity of inhibitors selective for MetAP2 with different metals. (A) Cellular MetAP2 activity is determined by labeling newly synthesized cellular proteins with ^{35}S-methionine in the presence of inhibitors in HMVEC followed by treating the isolated radioactive cellular proteins with recombinant MetAP2 to release unprocessed N-terminal initiator ^{35}S-methionine. A-310840 at concentrations below 10 μM does not inhibit cellular MetAP2 enzyme activity, while A-311263, in a dose-dependent manner, blocks cellular MetAP2 activity as shown by the unprocessed initiator ^{35}S-methionine of cellular proteins. The irreversible MetAP2 inhibitor fumagillin at 1 nM also inhibits cellular MetAP2. The inset shows that both compounds are able to permeate and concentrate in cytosol. Cells are treated with 10 μM inhibitors in the medium for 4 h, and intracellular compound concentrations are determined by LC-MS. (B) Effect of HMVE proliferation by MetAP2 inhibitors is determined by incubating cells with inhibitors for 3 days, and total viable cells are quantified by MTS reagents. The absorbance at 490 nm (A_{490}) represents live cell numbers. A-310840 at concentrations below 10 μM does not affect HMVEC proliferation, while A-311263 inhibited cell proliferation in the same dose ranges that inhibit cellular MetAP2 enzyme (A).

proliferation (IC_{50} of ~1 nM) (*8*) and accumulation of unprocessed N-terminal methionine of cellular proteins at 1 nM concentrations (Figure 5A). These data indicate that cellular MetAP2 is not functioning as a Co^{2+} enzyme. These data also exclude Zn^{2+}, Ni^{2+}, and Fe^{2+} as physiologically relevant metal ions for the MetAP2 enzyme because A-310840 is able to inhibit purified MetAP2 with these metal ions but not intracellular MetAP2. These data further suggest cellular MetAP2 uses manganese as its cofactor in physiological environment. However, direct evidence on the MetAP2 metal cofactor identity is still lacking. Purification of MetAP2 from a natural source without extra metal contamination and with chelating agents during the process should allow true metal ion identification. On the basis of characterization of the enzyme in vitro and results of metal selective MetAP2 inhibitors A-310840 and A-311263 in cellular MetAP2 enzyme inhibition and cell proliferation inhibition, we conclude that MetAP2 uses manganese as its physiological metal ion and that therapeutic MetAP2 inhibitors should inhibit MetAP2-Mn^{2+}.

Studies by others are consistent with our conclusion that manganese is the physiological metal cofactor for MetAP2. An earlier study of recombinant human MetAP2 showed that manganese, as well as cobalt, is able to stimulate the enzyme's activity although the authors conclude MetAP2 is a cobalt-dependent metalloenzyme (*12*). In a study on yeast MetAP1 (*13*) that argues for Zn^{2+} as the cofactor, manganese is also shown to stimulate the enzyme activity both in the absence and in the presence of glutathione. Manganese is shown to be concentrated in bacteria overexpressing *E. coli* MetAP1 by a factor of 2.2, similar to the increase of iron, and it also stimulated *E. coli* MetAP1 enzyme activity (*14*). In our studies, *E. coli* MetAP1 (kindly provided by Dr. B. Matthews) behaves similarly to human MetAP1, capable of

Table 1: Inhibition of MetAP2 Activity by Two Types of Inhibitors[a]

		IC_{50} (μM) for MetAP2 with metal ions				
		Zn^{2+}	Ni^{2+}	Co^{2+}	Fe^{2+}	Mn^{2+}
A-310840		0.031	0.043	0.069	0.026	57.8
A-311263		0.121	0.107	0.149	0.110	0.055

[a] The compounds at final concentrations of 0.001−100 μM were added to the coupled enzyme assay using the 8-mer peptide substrate MGAQFSKT of MARCKS protein as described in Materials and Methods. The IC_{50} values were the average of at least two separate experiments.

using Mn^{2+} and Co^{2+} as cofactors (data not shown) in the absence of glutathione. Human MetAP2 enzyme activity is fully stimulated by manganese at a concentration as low as 1 μM, and manganese has a broader window of activating concentrations (Figure 2A), suggesting that it is superior to cobalt as a MetAP2 cofactor. Most recently, D'souza et al. (*16*) describe the kinetic and structural characterization of manganese-loaded MetAPs from *E. coli* and the hyperthermophilic archaeon *P. furiosus*, demonstrating manganese can serve as a metal cofactor for these methionine aminopeptidases.

Manganese as an enzyme cofactor is not unprecedented. The concentration of Mn^{2+} in human plasma is 0.11 μM (*20*), and in rat hepatocytes Mn^{2+} concentrations are in the range of approximately 0.25−0.7 μM (*21*). Manganese is known to be a catalytically required cofactor in certain metalloenzymes involved in mammalian nitrogen and oxygen metabolism. In plants, manganese is an essential component of the oxygen-evolving complex of photosystem II in green plants (*22*). Arginase, manganese catalases, enolase, superoxide dismutase, and serine/threonine protein phosphatase-1 are just a few examples of well-studied manganese enzymes (*23*). In addition, aminopeptidase P from *E. coli* has been shown to contain a binuclear Mn^{2+} core (*24*). Aminopeptidase P hydrolyzes amino-terminal X-Pro peptide bonds (where X may be any amino acid). Its biological functions, amino acid sequence, and metal specificity are remarkably similar to human prolidase, an enzyme involved in proline recycling for collagen biosynthesis. Aminopeptidase P and prolidase structurally belong to the new protease family with a pita-bread fold (*25*), which includes methionine aminopeptidases. Identification of human MetAP2 as a manganese enzyme further expands the array of manganoenzymes.

Summary. MetAP2 is a novel molecular target for antiangiogenesis and anticancer therapy, and biochemical characterization of this metalloprotease will aid the development of specific inhibitors as potential therapeutic drugs. Identification of physiological metal cofactors for MetAP2 is critical because inhibitors differentiate this enzyme with different metal cofactors with potency varying up to 1000-fold. To identify the physiological relevant metal cofactor for methionine aminopeptidase, we first analyzed the effects of eight divalent metal ions on the purified recombinant human MetAP1 and MetAP2 and demonstrated that manganese, as well as cobalt, maximally stimulated MetAP1 and MetAP2 activity in the absence of physiologically relevant glutathione. Manganese and cobalt also were shown to be the best activators of MetAP2 enzyme activity when 5 mM glutathione was present in the assay. These in vitro biochemical data indicated that either manganese or cobalt was capable of being a MetAP2 cofactor. We next used synthetic inhibitors A-310840 and A-311263, selective for MetAP2 with different metal ions, in cell culture studies and showed that cobalt was not a physiological metal cofactor. A-310840 did not inhibit MetAP2 with manganese but was a potent inhibitor for MetAP2 with many other metal ions including cobalt, zinc, iron, and nickel, while A-311263 was an inhibitor of MetAP2 with manganese, as well as the above metals. A-310840 did not inhibit cellular MetAP2 activity, as measured by determining the cellular protein N-terminal initiator methionine status, and cell proliferation, even though it permeated cell membrane and accumulated inside the cells up to 60 μM concentration. In contrast, A-311263 inhibited cellular MetAP2 enzyme activity and cell proliferation in a similar concentration range. Therefore, we conclude MetAP2 functions as a manganese enzyme. Classification of MetAP2 as a manganese enzyme further expands the array of manganoenzymes in the new protease family with pita-bread fold, such as aminopeptidase P and prolidase.

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