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Methionine Aminopeptidases Type I and Type II Are Essential to Control Cell Proliferation

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Abstract The dependence of cell growth on methionine aminopeptidase (MetAP) function in bacteria and yeast is firmly established. Here we report experimental evidence that the control of cell proliferation in mammalian cells is directly linked and strictly dependent on the activity of both MetAP-1 and MetAP-2. The targeted downregulation of either methionine aminopeptidase MetAP-1 or MetAP-2 protein expression by small interfering RNA (siRNA) significantly inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) (70%–80%), while A549 human lung carcinoma cell proliferation was less inhibited (20%–30%). The cellular levels of MetAP-2 enzyme were measured after MetAP-2 siRNA treatment and found to decrease over time from 4 to 96 h, while rapid and complete depletion of MetAP-2 enzyme activity was observed after 4 h treatment with two pharmacological inhibitors of MetAP-2, PPI-2458 and fumagillin. When HUVEC and A549 cells were treated simultaneously with MetAP-2 siRNA and PPI-2458, or fumagillin, which irreversibly inhibit MetAP-2 enzyme activity, no additive effect on maximum growth inhibition was observed. This strongly suggests that MetAP-2 is the single critical cellular enzyme affected by either MetAP-2 targeting approach. Most strikingly, despite their significantly different sensitivity to growth inhibition after targeting of either MetAP-1 or MetAP-2, HUVEC, and A549 cells, which were made functionally deficient in both MetAP-1 and MetAP-2 were completely or almost completely inhibited in their growth, respectively. This closely resembled the observed growth inhibition in genetically double-deficient *map1map2* yeast strains. These results suggest that MetAP-1 and MetAP-2 have essential functions in the control of mammalian cell proliferation and that MetAP-dependent growth control is evolutionarily highly conserved. J. Cell. Biochem. 95: 1191–1203, 2005.

Key words: cell proliferation; enzyme inhibition; HUVEC; MetAP-1; MetAP-2

The methionine aminopeptidases type I (MetAP-1) and type II (MetAP-2) are two members of a class of metallopeptidases that selectively catalyze the removal of the initiator methionine residue from nascent polypeptide chains, a central step in protein maturation [Keeling and Doolittle, 1986; Arfin et al., 1995; Bradshaw and Yi, 2002]. This cotranslational processing step is the most frequently occuring protein modification and is important in the regulation of a number of cellular processes such as protein turnover, protein targeting, and cell proliferation. Correspondingly, MetAPs have a ubiquitous phylogenetic distribution. Prokaryotes express a single MetAP isoform, with a remarkably different expression pattern between eubacteria and archaebacteria: eubacteria express the type I MetAP, while archaebacteria express the type II MetAP [Keeling and Doolittle, 1986; Bradshaw and Yi, 2002]. Despite these differences, *MetAP*s are essential genes in all prokaryotes, since MetAP deletion is lethal [Chang et al., 1989; Miller et al., 1989].

In eukaryotes, both MetAP isoforms are expressed, thus providing two alternative pathways for the removal of the initiator methionine during protein processing [Arfin et al., 1995; Bradshaw and Yi, 2002]. Although MetAP-1 and MetAP-2 share the same general substrate specificity, selective differences have evolved in response to the more complex regulation of cellular organization and signaling in eukaryotic

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cells. The *Saccharomyces cerevisiae* (*S.c.*) *MAP1* and *MAP2* genes, homologs of the mammalian *MetAP-1* and *MetAP-2* genes, have conserved a significant degree of functional redundancy, most apparent in the regulation of cell growth [Chang et al., 1992; Li and Chang, 1995a; Chen et al., 2002]. Genetic studies have shown that both *map1* and *map2* null strains are viable, but have a slow-growth phenotype which is more severe in the *map1* null strain, while the *map1map2* double-null strain is lethal [Li and Chang, 1995a]. These results confirm that MAP function is essential for yeast cell growth and that this activity cannot be rescued by a *MAP* independent pathway. Therefore, like prokaryotic MetAPs, yeast MAPs have emerged as potential new drug targets [Luo et al., 2003].

The human *MetAP-1* gene was first identified among a group of 40 cDNAs isolated from the human immature myeloid cell line KG-1 [Nagase et al., 1995]. Its cellular functions, however, have not yet been firmly elucidated. MetAP-2 was originally discovered as the eukaryotic initiation factor-2 (eIF-2) associated 67-kDa polypeptide, and was also independently cloned from a human fetal brain cDNA library [Wu et al., 1993; Arfin et al., 1995; Li and Chang, 1995b]. The association with eIF-2, specifically the alpha subunit, was subsequently characterized to have an important non-catalytic function. The eIF-2α-MetAP-2 complex protects eIF-2α from inhibitory phosphorylation (POEP), thus MetAP-2 is a positive regulator of translation [Datta et al., 1988, 1989; Ray et al., 1988]. Despite the critical function of MetAPs in prokaryotes and lower eukaryotes, there is only limited information on their functions in mammalian cells, particularly their involvement in the regulation of cell proliferation. While there are no reports yet to directly link MetAP-1 to this process, the identification of MetAP-2 as the cellular target of fumagillin class molecules, and the significant growth inhibition observed in cells sensitive to MetAP-2 inhibition, suggested the direct involvement of MetAP-2 in the regulation of cell proliferation [Griffith et al., 1997, 1998; Sin et al., 1997; Turk et al., 1999]. More specifically, HUVEC proliferation has been shown to be exquisitely sensitive to MetAP-2 inhibition and MetAP-2 targeting drugs such as TNP-470 and PPI-2458 have advanced into clinical trials for oncology indications [Milkowski and Weiss, 1998]. A recent report, however, challenged the notion that MetAP-2 is involved in the control of cell proliferation and therefore suggested the involvement of alternative MetAPs, or MetAP unrelated targets, as the truly relevant targets of fumagillin class MetAP-2 inhibitors [Kim et al., 2004]. The discovery of a third MetAP (MAP1D) with strictly mitochondrial localization has recently been reported, but neither MAP1D-associated MetAP activity (in vitro or in vivo) nor any cellular functions have yet been demonstrated [Serero et al., 2003]. Here we report that the growth control of mammalian cells is directly linked and dependent on MetAP-1 and MetAP-2, and that the activity of both enzymes is essential to regulate this process.

MATERIALS AND METHODS

Materials

PPI-2458 and a biotinylated analog of PPI-2458 were synthesized at PRAECIS Pharmaceuticals, and fumagillin was obtained from Sigma. For cellular assays, both compounds were dissolved in ethanol (10 mM). [^{3}H]-thymidine was obtained from Amersham. The transfection reagents oligofectamine, TransIT-TKO, and fugene were purchased from InVitrogen, Mirus, and Roche, respectively. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and siRNAs duplexes were obtained from Dharmacon, Inc. (Lafayette, CO). The following siRNA targeting (sense) sequences were selected: MetAP-1 siRNA: 5′-AAAGGUACUUCUCAGAUUA-dTdT-3′, corresponding to bases 25 to 43 in the open reading frame (ORF) of the MetAP-1 mRNA, and MetAP-2 siRNA: 5′-GAAGAGAUUUGGAAUGAUU-dTdT-3′, corresponding to bases 487–505 in the ORF of the MetAP-2 mRNA. The control siRNA duplex sequence was: 5′-AUUAGACUCU UCAUGGAAA-dTdT-3′.

Saccharomyces cerevisiae (*S.c.*) Strains and Growth

Yeast strains used in this study were: wild-type strain W303-1A (MATa, ade 2-1 can1-100 ura3-1 leu2,3-112 trp1-1 his3-11,15); YHC001 map1::HIS3 (MATa, ade 2-1 can1-100 ura3-1 leu2,3-112 trp1-1 his3-11,15); and YHC002 map2::URA3 (MATa, ade 2-1 can1-100 ura3-1 leu2,3-112 trp1-1 his3-11,15) (Mediomics). Yeast cells were grown in YPD media (0.5% yeast extract, 1% peptone, 2% glucose) at 30°C overnight and diluted in YPD media to $OD_{600nm} =$

0.01. Yeast cultures were grown for a minimum of six generations in YPD supplemented with either vehicle (0.5% ethanol) or PPI-2458 at the indicated concentrations. The growth of yeast cultures containing PPI-2458 was calculated by the formula: OD_{600nm} culture (PPI-2458)/OD_{600nm} culture (vehicle) × 100%.

Cell Lysates and Western Blot Analysis

After treatment, cells were washed with phosphate-buffered saline, harvested in buffer A (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, and complete protease cocktail inhibitor (Roche)), solubilized for 30 min at 4°C, and centrifuged at 14,000*g*, 15 min. For Western blot analysis, 30 μg of cellular protein was used. To detect MetAP-1 and MetAP-2, we used a MetAP-1 polyclonal antibody (Mediomics) and the MetAP-2 polyclonal antibody CM33 (Zymed), respectively. To detect β-actin, the monoclonal anti-β-actin antibody AC-15 (Sigma) was used.

Cloning of the Human MetAP-2 Promoter

A 2 kilo base (kb) fragment of the human MetAP-2 promoter, and a smaller 0.5 kb fragment located immediately 5′ to the MetAP-2 ORF, were amplified with the polymerase chain reaction (PCR) from a human genomic DNA library and cloned into the pTAL-Luc expression vector (BD Bioscience, Palo Alto, CA) to generate pTAL-MetAP-2-2.0-Luc and pTAL-MetAP-2-0.5-Luc, respectively, using standard molecular biology techniques. The following oligonucleotide primers were used: 2.0 kb 5′-CGACGCGTGATCTCAGCCTAATTGCTACT-3′; 0.5 kb-5′ CGACG CGGAGCTGTGATTGTGA-ATGTCAACG-3′. The common 3′ oligonucleotide primer was: 5′-GCGGGATCCGTTGCCC-GAGAGAGCGCGAGGGAAT-3′. Sequence alignments found the human *MetAP-2* gene to be located on chromosome 12 (GenBank™ Accession Number NT-024394).

Luciferase Assay

HEK293T cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained in culture in RPMI medium supplemented with fetal bovine serum (10% v/v), penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively) and L-glutamine (2 mM). HEK293T cells (10,000 cells/well) were plated in 96-well culture dishes and maintained at 37°C in 5% CO_2 overnight. Transfections were performed using 0.2 μg of plasmid DNA/well with fugene according to the manufacturer's instructions (Roche). Twenty-four hours after transfection, fresh medium containing either 100 nM or 1 μM of PPI-2458 was added for 48 h. The cells were lysed and luciferase activity was measured according to the manufacturer's instructions (Tropix PE Biosystems, Bedford, MA).

MetAP-2 Assay

The MetAP-2 assay was performed as previously described [Bernier et al., 2004]. Briefly, 10–20 μg of cellular protein was incubated with a biotinylated analog of PPI-2458. This analog covalently binds to the catalytic site of MetAP-2, which enables determination of the amount of total cellular MetAP-2, or the amount of free MetAP-2, which had not been derived by prior treatment with PPI-2458 or fumagillin. The biotinylated MetAP-2-inhibitor complex was captured on a plate with immobilized streptavidin (Pierce), and detected with the MetAP-2 antibody CM33 (0.5 μg/ml), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. The amount of free MetAP-2 was determined by measuring the absorption at 450 nm using a Labsystems Multiskan plate spectrophotometer. Human recombinant MetAP-2 (Mediomics), pre-bound to the biotinylated PPI-2458 analog, was used to generate the standard curve.

siRNA Transfection and Proliferation Assays

HUVEC were purchased from Cell Applications, Inc. (San Diego, CA), A549 human lung carcinoma cells from ATCC. HUVEC were cultured in HUVEC growth medium (Cell Applications, Inc.), A549 cells in RPMI medium supplemented with fetal bovine serum (10% v/v), penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively), and L-glutamine (2 mM). Optimized conditions for the transfection of siRNA duplexes into HUVEC and A549 cells were determined in preliminary experiments. HUVEC (15,000 cells/well), routinely used between passages 3–5, and A549 cells (12,500 cells/well) were seeded in 24-well plates (in triplicate). After 24 h, the cells reached 40%–60% confluency and were transfected with 100 nM of siRNA duplexes using TransIT-TKO (HUVEC) or oligofectamine (A549 cells). After 24 h, fresh medium containing either no inhibitor (vehicle),

or different concentrations of PPI-2458 or fumagillin, was added for another 72 h. Cell growth was determined by adding 3 μCi/well [^{3}H]-thymidine for the final 24 h of incubation. The amount of incorporated [^{3}H]-thymidine was determined by liquid scintillation counting.

Cell Viability and Measurement of Apoptosis

Cell viability was examined by Trypan Blue exclusion (Sigma). Apoptosis was analyzed by labeling cells with the Annexin V-FITC apoptosis detection kit (R & D Systems), which recognizes phosphatidylserine exposure on the outer leaflet of the plasma membrane. The percentage of Annexin V-positive cells was determined by flow cytometry (LSRII, BD Bioscience).

RESULTS

In Vivo Inhibition of *Saccharomyces cerevisiae* MAP2 by PPI-2458

Yeast strains genetically deficient in either *map1* or *map2 (map1* and *map2* null strains), or both *map* genes (*map1map2* double-null strain) have been used to determine the contribution of each *MAP* to yeast growth. Previous reports have shown that several members of the fumagillin class inhibitors of MetAP-2 function, such as fumagillin, ovalicin, and TNP-470, selectively inhibited the growth of the *S.c. map1* null strain, which is dependent on functional MAP2 for viability [Griffith et al., 1997; Sin et al., 1997]. We investigated whether PPI-2458, a novel structural analog of fumagillin, would similarly inhibit the growth of this strain. The results show that the growth of the *map1* null strain was inhibited by PPI-2458 in a dose-dependent fashion (Fig. 1), while the growth of the wild-type and *map2* null strains were not affected by this inhibitor. These results demonstrate that PPI-2458 selectively inhibits MAP2 function and confirmed that MAP2 function is essential for growth of the yeast *map1* null strain.

siRNA-Mediated Downregulation of MetAP-1 and MetAP-2 in HUVEC and A549 Cells

Similar to the lower eukaryote *Saccharomyces cerevisiae*, mammalian cells express two MetAP isoforms, MetAP-1 and MetAP-2 [Keeling and Doolittle, 1986; Arfin et al., 1995; Bradshaw and Yi, 2002]. We investigated whether the growth dependence of yeast cells on functional MAP1 and MAP2 has been conserved in mammalian cells. Due to the lack of mammalian cells genetically deficient in either *MetAP* gene, we explored the ability of siRNA duplexes to generate cells, which are functionally deficient in either MetAP-1 or MetAP-2. For this purpose, we used selective siRNA duplexes targeted to the MetAP-1 mRNA and MetAP-2 mRNA, respectively. HUVEC and A549 cells were transfected with 100 nM of MetAP-1 siRNA or MetAP-2 siRNA, and the cellular level of both proteins was determined 72 h after transfection by Western blot analysis. The expression of MetAP-1 and MetAP-2 was efficiently downregulated by the respective targeting siRNA duplexes and no interference with the expression of either the non-targeted MetAP or β-actin was observed (Fig. 2). This activity was maintained for at least 96 h after transfection of either MetAP siRNA (data not shown).

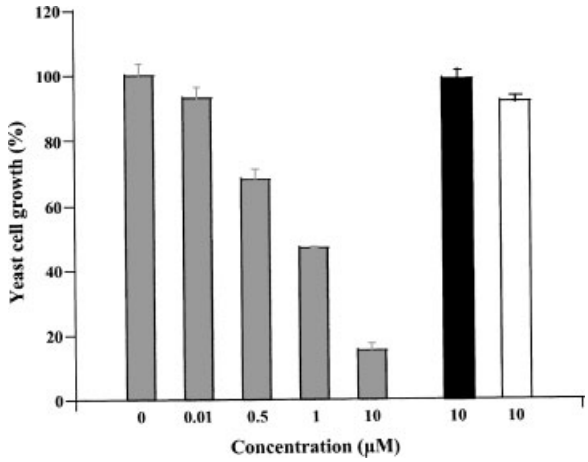

Fig. 1. Selective growth inhibition of the yeast *map1* null strain by PPI-2458. Yeast W303-1A (wild-type) (▒), YHC001 map1::HIS3 (*map1* deletion) (■), and YHC002 map2::URA3 (*map2* deletion) (□) cells were diluted to $OD_{600nm} = 0.01$, and grown for at least six generations in YPD media containing either vehicle (0.5% ethanol) or PPI-2458 at the indicated concentrations. The growth of yeast cultures containing PPI-2458 was calculated by the formula: OD_{600nm} culture (PPI-2458)/OD_{600nm} culture (vehicle) ×100%.

Differential Growth Inhibition of HUVEC and A549 Cells After MetAP-1 siRNA and MetAP-2 siRNA Treatment

After we had confirmed the ability of both MetAP siRNAs to selectively downregulate the expression of their cellular targets, we investigated the effect of this functional deficiency on the proliferation of HUVEC and A549 cells. These cell types are of specific interest due to their reported differential sensitivity to growth

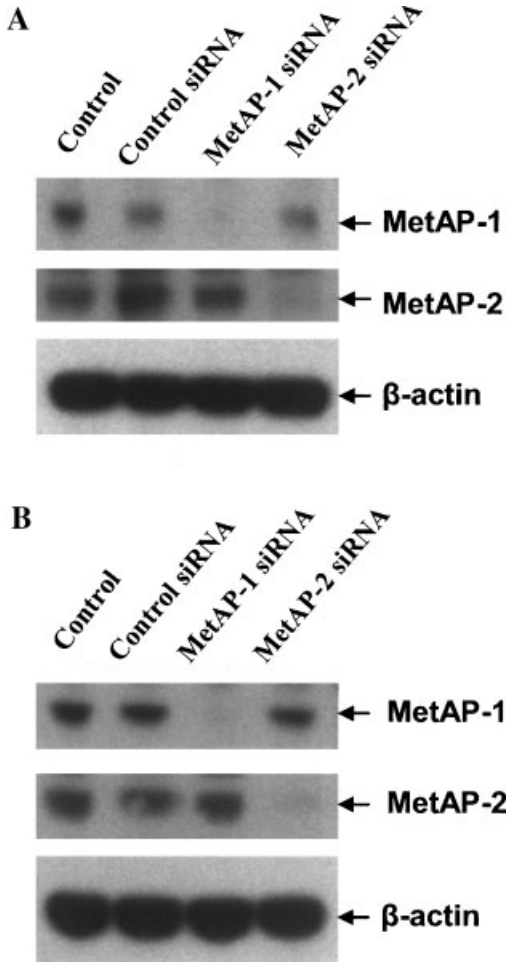

Fig. 2. siRNA-mediated downregulation of MetAP-1 and MetAP-2 in HUVEC and A549 cells. HUVEC (15,000 cells/well) (**A**) and A549 cells (12,500 cells/well) (**B**) were transfected with 100 nM of siRNA targeting duplexes against MetAP-1 (*MetAP-1 siRNA*), MetAP-2 (*MetAP-2 siRNA*), or a control duplex (*Control siRNA*) as described under Materials and Methods. Control refers to untransfected cells. After 72 h, cells were lysed and 30 μg of cellular protein was used for Western blot analysis. For MetAP-1, MetAP-2, and β-actin protein detection in cell lysates, anti-MetAP-1, anti-MetAP-2, and anti-β-actin antibodies were used.

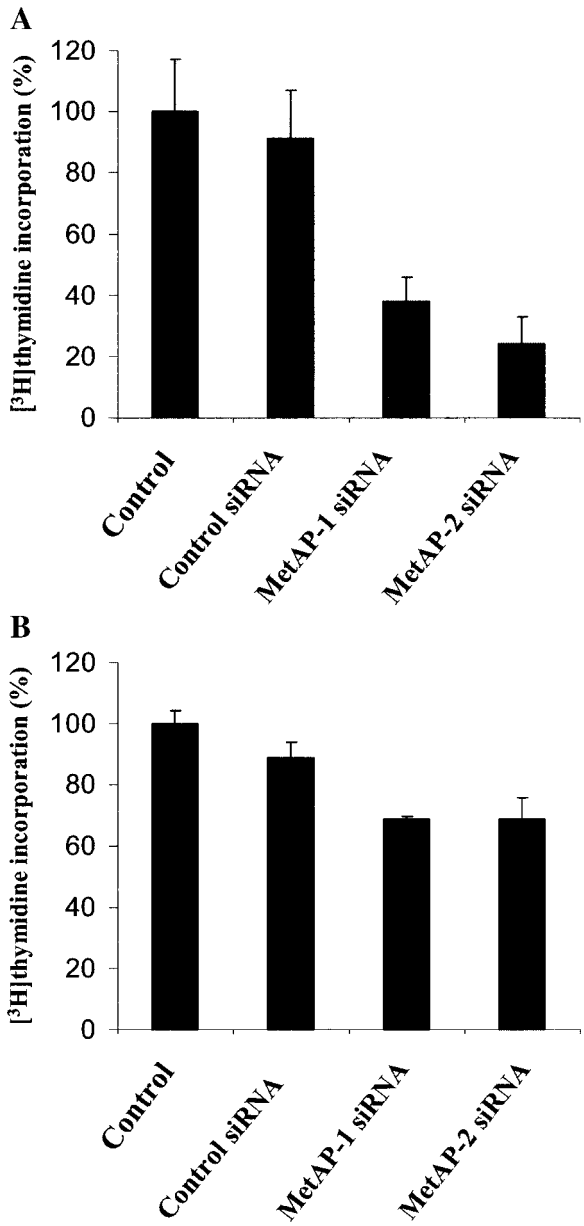

Fig. 3. Differential sensitivity to growth inhibition of HUVEC and A549 cells after MetAP-1 siRNA or MetAP-2 siRNA transfection. HUVEC (15,000 cells/well) (**A**) and A549 cells (12,500 cells/well) (**B**) were transfected with 100 nM of MetAP-1 siRNA, MetAP-2 siRNA, or control siRNA as described under Materials and Methods. Control refers to untransfected cells. The cells were grown for 96 h, and 3 μCi/well of [^{3}H]-thymidine was added for the final 24 h of incubation. Cell proliferation was determined by the amount of incorporated [^{3}H]-thymidine using liquid scintillation counting. The results represent the mean and standard deviation of triplicate wells and are representative of at least 3 independent experiments.

inhibition by the MetAP-2 enzyme inhibitor fumagillin [Towbin et al., 2003; Wang et al., 2003]. Furthermore, the results of fumagillin-induced growth inhibition in A549 cells vary significantly [Towbin et al., 2003; Wang et al., 2003]. We transfected HUVEC and A549 cells with 100 nM of either MetAP-1 siRNA or MetAP-2 siRNA, and determined the growth inhibitory activity of both MetAP siRNAs after 96 h by [^{3}H]-thymidine incorporation. Our results show that MetAP-1 siRNA and MetAP-2 siRNA significantly inhibited HUVEC proliferation (70%–80%) (Fig. 3A), while the proliferation of A549 cells was much less affected (20%–30%) (Fig. 3B). The efficient growth inhibition of HUVEC by MetAP-2 siRNA was reflective of the growth inhibition of HUVEC

observed after treatment with fumagillin class inhibitors of MetAP-2 function, such as PPI-2458, TNP-470, and fumagillin [Abe et al., 1994; Kusaka et al., 1994; Turk et al., 1999; Wang et al., 2000; Bernier et al., 2004]. The markedly lower degree of A549 cell growth inhibition by either MetAP siRNA suggests important cell-type specific differences in the dependence upon MetAP-1 and MetAP-2 function for cell proliferation. No growth inhibition was observed in either cell type after transfection of the control siRNA.

Quantitative Measurement of the MetAP-2 siRNA-Mediated Downregulation of MetAP-2 in HUVEC and A549 Cells

To determine if the MetAP-2 siRNA-mediated downregulation of MetAP-2 is correlated with the growth inhibitory activity of HUVEC and A549 cells, and to measure the time-dependent decrease in the amount of MetAP-2 after MetAP-2 siRNA transfection, we used a recently developed quantitative MetAP-2 assay [Bernier et al., 2004]. HUVEC and A549 cells were transfected with 100 nM of MetAP-2 siRNA, and the amount of free MetAP-2 was measured after 4, 24, or 96 h. The results demonstrated a time-dependent downregulation of MetAP-2 in both cell types, with similar levels of down-regulation achieved at each time point (Fig. 4). In comparison, after treatment of HUVEC and A549 cells with either 100 nM of PPI-2458 or fumagillin, rapid and complete inhibition of MetAP-2 function was already achieved after 4 h, and this degree of inhibition was maintained for at least 96 h (Fig. 4). The transfection of MetAP-1 siRNA only marginally affected the level of free MetAP-2 after 4 and 24 h, when compared to vehicle-treated cells. Over time the free MetAP-2 did not increase in response to MetAP-1 siRNA. This apparent decrease is due to an increase in vehicle-treated cells at 96 h (Fig. 4).

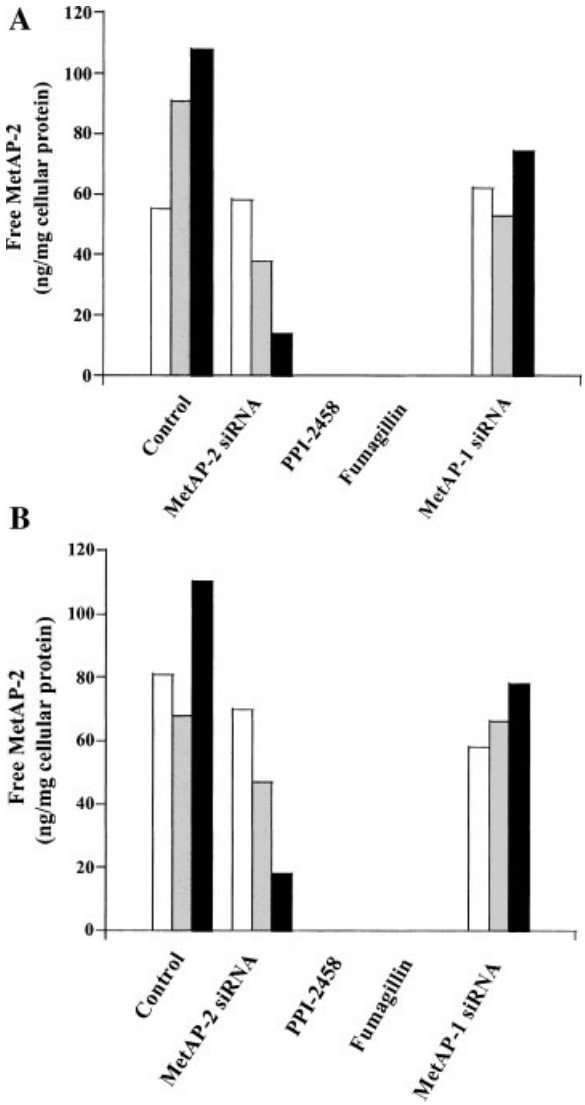

Fig. 4. Time-dependent decrease in free MetAP-2 levels after MetAP-2 siRNA transfection. HUVEC (15,000 cells/well) (**A**) and A549 cells (12,500 cells/well) (**B**) were transfected with 100 nM of MetAP-1 siRNA, or MetAP-2 siRNA as described under Materials and Methods, and cultured for 4, 24, and 96 h. Alternatively, HUVEC (A) and A549 cells (B) were treated for 4 (□), 24 (▒), and 96 h (■) with 100 nM PPI-2458, 100 nM fumagillin or vehicle (control). At each time point, cells were processed as described under Materials and Methods, and the amount of free MetAP-2 (ng/mg cellular protein) was calculated from a standard curve which was generated with human recombinant MetAP-2, pre-bound to a biotinylated analog of PPI-2458.

MetAP-2 Is the Critical Cellular Target for Cell Growth Which Is Inhibited by MetAP-2 siRNA and Inhibitors of MetAP-2 Function

MetAP-2 is generally considered to be the common cellular target of fumagillin class molecules, and potent anti-angiogenic and anti-proliferative activity has been associated with this class of compounds [Sin et al., 1997; Griffith et al., 1998; Turk et al., 1999]. Since a recent report suggested that MetAP-2 is not the critical cellular target for this activity and that the anti-proliferative activity is instead due to alternative cellular target(s) [Kim et al., 2004], we tested this hypothesis in proliferation assays designed to discriminate between MetAP-2 and alternative cellular targets. First, HUVEC or

A549 cells were transfected with 100 nM MetAP-2 siRNA and incubated for 96 h. At the same time, parallel plates of both cell types were treated with increasing amount of PPI-2458 or fumagillin for 72 h. In addition, HUVEC or A549 cells were transfected with 100 nM MetAP-2 siRNA and increasing amounts of either PPI-2458 or fumagillin were added 24 h after transfection, and these cells were incubated for another 72 h. The maximum level of growth inhibition observed after treatment of HUVEC and A549 cells with either single MetAP-2 targeting approach was similar to the level observed after combined treatment, which suggests that MetAP-2 is the critical molecular target involved in cell growth (Fig. 5A,B).

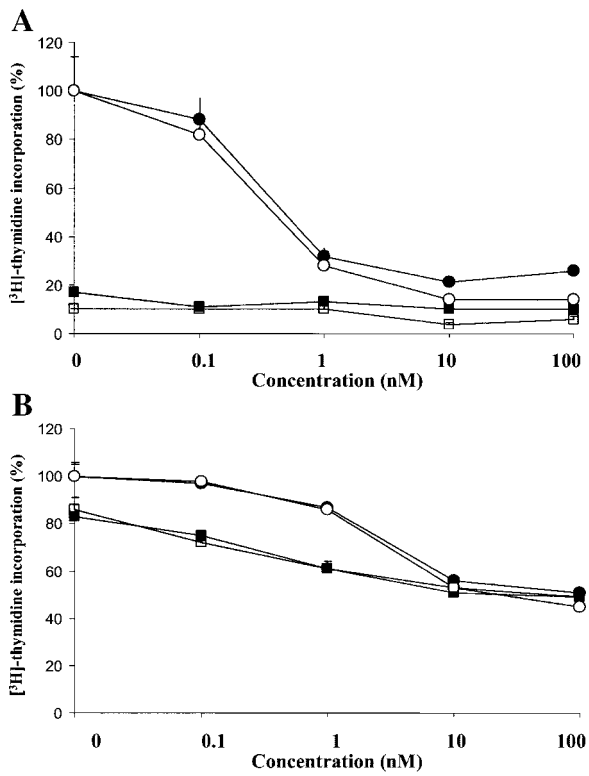

Fig. 5. Growth inhibition of cells under the control of MetAP-2. HUVEC (15,000 cells/well) (**A**) and A549 cells (12,500 cells/well) (**B**) were transfected with 100 nM of MetAP-2 siRNA (squares) as described under Materials and Methods. Control cells on parallel plates were treated with vehicle (control) (circles). After 24 h, fresh medium containing increasing concentrations of PPI-2458 (●/■) or fumagillin (○/□) was added for another 72 h. Three μCi/well [^{3}H]-thymidine was added for the final 24 h of incubation. Cell proliferation was determined by the amount of incorporated [^{3}H]-thymidine, using liquid scintillation counting. The results represent the mean and standard deviation of triplicate wells and are representative of at least three independent experiments.

The Increase in MetAP-2 Protein Levels After Treatment With PPI-2458 and Fumagillin Is Transcriptionally Controlled

Previous reports have shown a dose-dependent increase in MetAP-2 protein levels in HUVEC and other cell types after exposure to PPI-2458 and fumagillin, most likely as a compensatory response to stress signals due to aberrant protein processing [Wang et al., 2000; Bernier et al., 2004]. The level of cellular MetAP-2, however, did not affect the growth inhibitory activity of either inhibitor [Bernier et al., 2004]. We tested two independent strategies to investigate whether this observed increase in cellular MetAP-2 protein is controlled at the transcriptional level, or due to changes in the rate of MetAP-2 turnover. First, HUVEC were transfected with 100 nM MetAP-2 siRNA 24 h prior to exposure to PPI-2458 or fumagillin. Second, we measured the expression of the luciferase reporter gene driven by the human MetAP-2 promoter after the exposure of transiently transfected HEK293T cells to PPI-2458. Results demonstrate that no MetAP-2 protein was detected in HUVEC after treatment for 24 h with 100 nM PPI-2458 or fumagillin when these cells were transfected with MetAP-2 siRNA prior to exposure to PPI-2458 or fumagillin (Fig. 6A). Prior transfection of MetAP-1 siRNA or control siRNA, however, did not affect the MetAP-2 inhibitor-induced increase of MetAP-2 protein (Fig. 6A). Similar results were observed in A549 cells (data not shown). One likely explanation for the observed increase in MetAP-2 protein levels after exposure to these MetAP-2 enzyme inhibitors is transcriptional regulation of this gene. Moreover, the lack of detectable MetAP-2 protein after MetAP-2 siRNA transfection indicates that the regular MetAP-2 turnover continues in the absence of de novo MetAP-2 synthesis. In addition, we cloned a 2.0 and 0.5 kb promoter fragment located immediately 5′ upstream of the human MetAP-2 ORF into the pTAL-Luc expression vector. The exposure of transiently transfected HEK293T cells for 48 h with either 100 nM or 1 μM PPI-2458 induced a 2–3 fold increase in the expression of the reporter gene (Fig. 6B). Taken together, these results are consistent with a transcriptionally controlled mechanism to increase the cellular level of MetAP-2 protein after exposure to fumagillin class MetAP-2 inhibitors and are further consistent with previous reports

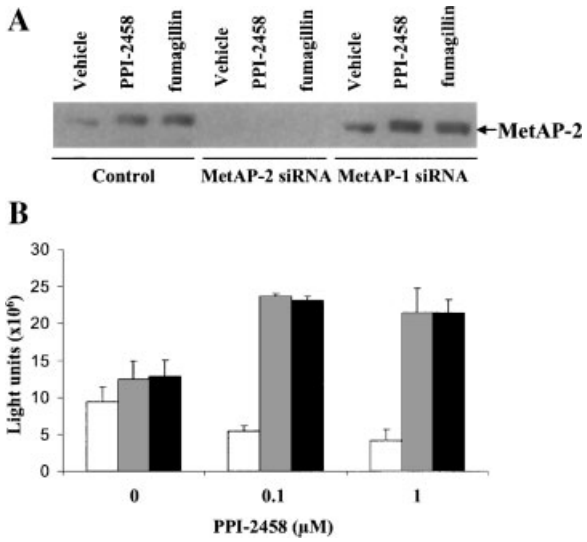

Fig. 6. Transcription control of cellular MetAP-2 protein levels by PPI-2458 and fumagillin. **A**: HUVEC (15,000 cells/well) were transfected with 100 nM MetAP-1 siRNA or 100 nM MetAP-2 siRNA, as described under Materials and Methods. Control refers to untransfected cells. After 24 h, the cells were treated with either no inhibitor (vehicle), or 100 nM PPI-2458 or 100 nM fumagillin for another 48 h. After 72 h, the cells were lysed and 30 μg of cellular protein was used for Western blot analysis. For MetAP-2 detection in cell lysates, anti-MetAP-2 antibody CM33 was used. **B**: HEK293T cells (10,000 cells/well) were transfected with either 0.2 μg of pTAL-Luc (□), 0.2 μg of pTAL-MetAP-2-0.5-Luc (▩), or 0.2 μg of pTAL-MetAP-2-2.0-Luc (■) as described under Materials and Methods. After 24 h, fresh medium was added containing either no inhibitor (vehicle), or 100 nM or 1 μM PPI-2458 for another 48 h. The cells were lysed, and luciferase activity was measured according to the manufacturer's instructions. Results represent the mean and standard deviation of triplicate wells.

that MetAP-2 is regulated at the transcriptional level under certain physiological conditions [Gupta et al., 1995, 1997].

The Functional MetAP-1-MetAP-2 "Double-knockout" Significantly Inhibits the Growth of HUVEC and A549 Cells

Our findings that HUVEC and A549 cells possess significantly different sensitivity to growth inhibition in response to the inhibition of either MetAP-1 or MetAP-2, and that MetAP-2 is the critical molecular target of PPI-2458 and fumagillin to induce growth inhibition, prompted us to investigate whether a functional double-knockout of MetAP-1 and MetAP-2 would result in complete growth inhibition, similar to that observed in the yeast *map1map2* double-null strain. In order to generate HUVEC and A549 cells functionally double-deficient in MetAP-1 and MetAP-2, we first transfected these cells with 100 nM MetAP-1 siRNA. Secondly, increasing concentrations of either PPI-2458 or fumagillin were added 24 h after MetAP-1 siRNA transfection and the cells were incubated for another 72 h. HUVEC proliferation was already significantly inhibited after the transfection of the MetAP-1 siRNA, but the addition of either PPI-2458 or fumagillin resulted in complete growth inhibition at concentrations of $\geq$1 nM of either inhibitor (Fig. 7). The effect of the functional "double-knockout" was even more apparent in A549 cells. While the transfection of the MetAP-1 siRNA only caused minor growth inhibition, the addition of either MetAP-2 inhibitor to these cells resulted in almost complete growth inhibition ($\sim$ 90%), in a dose-dependent fashion (Fig. 7). These results suggest that MetAP-1 and MetAP-2 are essential for mammalian cell growth, and that the simultaneous loss of both critical enzyme functions cannot be compensated for by other MetAPs or MetAP unrelated cellular proteins.

MetAP-1 siRNA and MetAP-2 siRNA-Mediated Growth Inhibition Is Exerted Through a Cytostatic Mechanism

We further investigated whether the observed growth inhibition of HUVEC and A549 cells

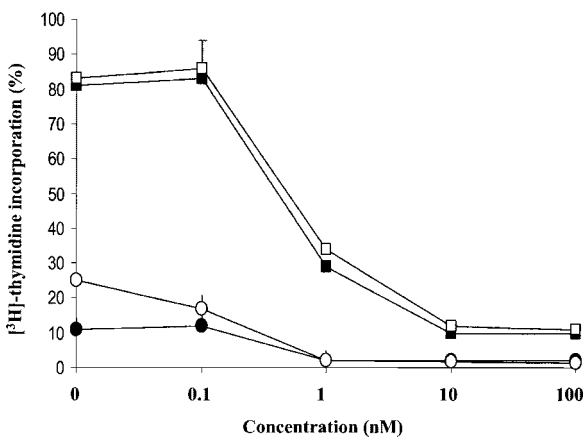

Fig. 7. A functional MetAP-1 MetAP-2 double knockout significantly inhibits growth of HUVEC and A549 cells. HUVEC (15,000 cells/well) (circles) and A549 cells (12,500 cells/well) (squares) were transfected with 100 nM of MetAP-1 siRNA as described under Materials and Methods. After 24 h, fresh medium containing increasing concentrations of PPI-2458 (●, HUVEC; ■, A549 cells) or fumagillin (○, HUVEC; □, A549 cells) was added for another 72 h. Three μCi/well [^{3}H]-thymidine was added for the final 24 h of incubation. Cell proliferation was determined by the amount of incorporated [^{3}H]-thymidine, using liquid scintillation counting. Percent [^{3}H]-thymidine incorporation is in reference to control siRNA-treated cells. The results represent the mean and standard deviation of three independent experiments.

after the transfection of MetAP-1 siRNA or MetAP-2 siRNA is exerted through a cytostatic mechanism similar to that observed after the inhibition of MetAP-2 function by PPI-2458 or fumagillin or through direct cytotoxicity. HUVEC and A549 cells were transfected with 100 nM of MetAP-1 siRNA or MetAP-2 siRNA and cultured for 4 days. The cells were then stained with either Trypan blue, a marker for cell membrane integrity, to determine cytotoxicity or Annexin V-FITC, which binds to phosphatidylserine exposed at the outer leaflet of the plasma membrane, a marker for early apoptosis. We did not detect any increase in the number of Trypan blue stained HUVEC or A549 cells in response to transfection with either siRNA (Table I), nor did we find any increase in the number of Annexin V-FITC positive cells (Table I). Thus, transfection with MetAP siRNA was not directly cytotoxic and the results are consistent with a cytostatic mechanism of growth inhibition due to MetAP inhibition. Finally, we investigated whether the complete growth inhibition of HUVEC and A549 cells observed after the simultaneous inhibition of MetAP-1 and MetAP-2 was due to cytostatic activity, or whether the "double-knockout" condition would trigger a cell death pathway. HUVEC and A549 cells were transfected with 100 nM of MetAP-1 siRNA and 100 nM of PPI-2458 was added 24 h later for another 72 h. Furthermore, cells were transfected with 100 nM of MetAP-2 siRNA, and similarly treated with PPI-2458. Our results closely resembled those observed after transfection with either MetAP-1 siRNA or MetAP-2 siRNA, or after treatment with pharmacological inhibitors of MetAP-2 function, which supports a cytostatic mechanism of growth inhibition by MetAP-1 and MetAP-2 (Table I).

DISCUSSION

Genetic studies in yeast have shown that *map1* and *map2* null strains are viable, albeit a slow-growth phenotype, while the *map1map2* double null strain is lethal [Li and Chang, 1995a]. These studies provide evidence that yeast growth is dependent on the activity of at least one MAP. It further demonstrates that despite their high degree of sequence diversity, sufficient overlapping functions have been conserved to rescue the reciprocal *map* null strain. Consistent with previous reports on fumagillin class MetAP-2 inhibitors, PPI-2458 selectively inhibited the growth of the *map1* null strain, while growth of the wild-type and *map2* null strains was not affected. Database searches of the yeast genome have not yet uncovered additional *MAPs*, however, even if present, this putative MAP activity is apparently unable to compensate for the functional loss of *MAP1* and *MAP2*. Moreover, no salvage pathways independent of *MAP1* and *MAP2* have evolved to rescue growth. Therefore, in the lower eukaryote *S.c.* functional *MAP1* and *MAP2* genes are essential for growth.

In the current studies we asked the question whether this mechanism of MetAP-1 and MetAP-2 dependent cell growth control is conserved in mammalian cells, despite a more complex cellular organization and signaling networks. In contrast to yeast, no mammalian

TABLE I. Cell Growth Inhibition is Exerted through a Cytostatic Mechanism

	Trypan blue stained cells (%)		Annexin V-positive cells (%)	
Treatment	HUVEC	A549 cells	HUVEC	A549 cells
Control	4	2	7	5
MetAP-1 siRNA	2	1	5	2
MetAP-2 siRNA	2	1	5	2
Control + PPI-2458	3	1	6	2
MetAP-1 siRNA + PPI-2458	2	1	5	6
MetAP-2 siRNA + PPI-2458	4	1	8	2

HUVEC and A549 cells were transfected with 100 nM MetAP-1 siRNA or MetAP-2 siRNA and cultured for 96 h as described under Materials and Methods. In order to generate the MetAP-1-MetAP-2 functional double-knockout, HUVEC and A549 cells were first transfected with 100 nM of MetAP-1 siRNA. After 24 h, fresh medium containing no inhibitor (vehicle) or 100 nM of PPI-2458 was added for another 72 h. In parallel experiments, the cells were transfected with 100 nM of MetAP-2 siRNA, and similarly treated with PPI-2458. After 96 h, HUVEC and A549 cells were stained with either Trypan blue and counted with a hemacytometer to determine cytotoxicity, or stained with Annexin V-FITC and analyzed by flow cytometry (10,000 cells) to determine the number of apoptotic cells.

cells genetically deficient in either MetAP exist. Therefore, we selected two alternative strategies to target MetAP function: siRNA-mediated post-transcriptional gene silencing to trigger the degradation of endogenous MetAP mRNAs, and two irreversible pharmacological inhibitors of MetAP-2, PPI-2458, and fumagillin, to selectively inhibit the catalytic activity of MetAP-2. While the role of MetAP-1 in mammalian cell growth control has not yet been thoroughly studied, the overexpression of the human *MetAP-1* gene in a yeast *map1* null strain functionally complemented the slow growth phenotype of this strain, in a dose-dependent manner [Dummitt et al., 2002]. This ability to rescue the slow growth phenotype of the *map1* null strain suggests that MetAP-1 has in vivo MetAP activity and is involved in growth control. Here we show for the first time the direct involvement of human MetAP-1 in the process of growth control of mammalian cells. The specific downregulation of MetAP-1 protein levels using a siRNA-targeting approach inhibited HUVEC proliferation by 70%–80%, while the proliferation of A549 cells was significantly less inhibited (20%–30%). There are several hypotheses to explain this differential sensitivity. First, MetAP-1 activity in HUVEC is essential to promote proliferation and the processing of one or more potentially cell type-specific substrates in this pathway may be exclusively dependent on MetAP-1 function. This further suggests that in HUVEC, the degree of overlapping substrate specificity between MetAP-1 and MetAP-2 is not sufficient for reciprocal complementation. Second, the proliferation of A549 cells is either less dependent on the contribution of this pathway or the critical substrate(s) in this cell type which promotes cell growth is processed by MetAP-2, albeit with slightly less efficiency. A very similar pattern of HUVEC and A549 cell growth inhibition was observed after the transfection of MetAP-2 siRNA, when compared to the level of growth inhibition after the transfection of MetAP-1 siRNA. Moreover, although both the level of siRNA-mediated downregulation of MetAP-2 and the inhibition of MetAP-2 enzyme function by PPI-2458 and fumagillin in HUVEC and A549 cells were almost superimposable, A549 cells were significantly less sensitive to growth inhibition. Therefore, it appears that differences in the observed growth inhibitory response between these two cell types are cell-type specific and are determined by yet unknown proteins directly regulated by MetAP enzymatic activities.

Using a recently developed quantitative MetAP-2 assay, we have previously shown that the growth inhibition of HUVEC by PPI-2458 is directly proportional to the amount of MetAP-2 enzyme inhibition, and have suggested that this inhibition of MetAP-2 function is the first critical step in the growth inhibition of PPI-2458 sensitive HUVEC [Bernier et al., 2004]. The MetAP-2 assay has the ability to measure the amount of total cellular MetAP-2, or the amount of free MetAP-2 that has not been inactivated by prior treatment of cells with MetAP-2 enzyme inhibitors such as PPI-2458 or fumagillin [Bernier et al., 2004]. Such an assay has not yet been reported for MetAP-1. Although the MetAP-2 siRNA-mediated downregulation of MetAP-2 was less efficient than the treatment with MetAP-2 enzyme inhibitors and required a prolonged period of time after transfection, it appears that this remaining amount of free MetAP-2 in HUVEC is below the necessary cellular threshold to support proliferation. This would account for the comparable degree of growth inhibition by either MetAP-2 targeting approach in HUVEC. All of these results suggest an important role of MetAP-2 in the regulation of mammalian cell growth and are further supported by several previously published observations. First, a semi-quantitative relationship between the cellular level of MetAP-2 protein and the concentrations of the MetAP-2 enzyme inhibitors A-357300 (a bestatin-type inhibitor) and fumagillin in the inhibition of cell growth has been reported [Wang et al., 2003]. Furthermore, growth inhibition of mesothelioma and rat hepatocarcinoma cells was observed after the transfection of MetAP-2 specific antisense nucleotides, a mechanistically related approach to *siRNA* gene silencing [Datta and Datta, 1999; Catalano et al., 2001]. Finally, the overexpression of the human *MetAP-2* gene in yeast complemented the *map2* deficiency and was able to rescue the lethal phenotype of a yeast *map1-map2* double-null strain [Brdlik and Crews, 2004].

The fungal metabolite fumagillin and several related molecules such as PPI-2458 are irreversible inhibitors of MetAP-2 as a result of covalent modification of the His-231 residue in the catalytic site of the enzyme [Griffith et al., 1997, 1998; Sin et al., 1997; Turk et al., 1999], and the crystal structure of human MetAP-2

complexed with fumagillin has also been reported [Liu et al., 1998; Lowther and Matthews, 2000]. A recent report, however, challenged the notion that MetAP-2 is directly involved in the control of cell growth and suggested that the growth inhibitory activity of fumagillin is due to the inhibition of an alternative cellular target(s) [Kim et al., 2004]. If this hypothesis were correct, then the growth inhibition observed after simultaneous treatment of cells with MetAP-2 siRNA plus PPI-2458 or fumagillin should only reflect the inhibitory activity of the yet unknown target(s) which is inhibited by PPI-2458 or fumagillin. We reasoned, however, that if MetAP-2 is the critical target for cell growth blocked by MetAP-2 siRNA or pharmacological inhibitors of MetAP-2 enzyme function, then no additive effect on maximal growth inhibition should be observed after simultaneous treatment of cells with both MetAP-2 targeting approaches. Our results provide evidence to confirm the hypothesis that MetAP-2 is a critical molecular regulator of cell proliferation. First, we have demonstrated that the combined treatment with MetAP-2 siRNA and PPI-2458 or fumagillin is not additive beyond the level of maximal growth inhibition achieved by either MetAP-2 targeting approach. Second, the direct measurement of the amount of free MetAP-2 showed that the treatment of HUVEC and A549 cells with PPI-2458 or fumagillin decreased the amount of free MetAP-2 far more rapidly and quantitatively than after transfection of MetAP-2 siRNA. There is at least one possible explanation for the previously reported observation that MetAP-2 is not involved in cell growth [Kim et al., 2004]. While the MetAP-2 siRNA used in our studies downregulated MetAP-2 protein expression levels below detectable levels by Western blot analysis, the downregulation with the previously studied MetAP-2 siRNA was less efficient and immunoreactive MetAP-2 was still detectable with Western blot analysis [Kim et al., 2004]. Therefore, it is conceivable that under these experimental conditions, the amount of active MetAP-2 was still above the necessary cellular threshold to support cell proliferation.

Finally, we rationalized that if the enzymatic activity of MetAP-1 and MetAP-2 is essential to regulate cell growth, then the simultaneous treatment of HUVEC or A549 cells with MetAP-1 siRNA and pharmacological inhibition of MetAP-2 with PPI-2458 or fumagillin should functionally mimic the yeast *map1map2* double-null phenotype and should therefore result in complete growth inhibition. Our results support this hypothesis and demonstrate that despite their markedly different cell type-specific sensitivity to growth inhibition by single MetAP targeting approaches, the simultaneous inhibition of both MetAP-1 and MetAP-2 was additive and completely inhibited HUVEC growth, and the growth of A549 cells by ~90%. These results make it unlikely that there is an essential role in control of cell growth for the recently cloned *MAP1D* gene, a putative MetAP postulated to be part of the dedicated machinery for N-terminal excision in the mitochondria of mammalian cells [Serero et al., 2003]. It appears likely that, if MAP1D and its *Drosophila* and Arabidopsis homologs are authentic MetAPs, they must serve as yet unidentified specialized functions limited to their respective cellular compartments [Giglione et al., 2000]. Moreover, no other cellular protein appears to be able to functionally compensate for the complete or partial loss of MetAP-1 and MetAP-2 activity.

In summary, we conclude that both MetAP-1 and MetAP-2 are essential components of the cell growth machinery and suggest a broad phylogenetic conservation from yeast to man of a mechanism of growth control regulated by these two enzymes. Because of a central role in the regulation of cell proliferation, MetAP-1 and MetAP-2 are promising drug targets for the development of novel therapeutics.

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