Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1) is a new member of the Cupin superfamily: A possible multifunctional protein acting as an invasion suppressor down-regulated in tumors

Takamasa Uekita¹, §, Isamu Gotoh¹, Takeshi Kinoshita¹, §, Yoshifumi Itoh¹, §, Hiroshi Sato², Takayuki Shiomi³, Yasunori Okada³, and Motoharu Seiki¹,*

¹Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, 108-8639, and ²Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, 920-0934, ³Department of Pathology, School of Medicine, Keio University, Shinjuku-ku, Tokyo, 160-0016, Japan

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§Present address: T. Uekita; Growth Factor Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, 104-0045. T. Kinoshita; Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, Kanazawa, 920-0934, Japan. Y. Itoh; Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College, 1 Aspenlea Road, Hammersmith, London W6 8LH, UK

*Address correspondence to:
Motoharu Seiki, Ph.D.
Professor
Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo
4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, JAPAN
TEL: +81-3-5449-5255, FAX: +81-3-5449-5414
E-mail: mseiki@ims.u-tokyo.ac.jp

FOOTNOTES
Abbreviations:
ECM, extracellular matrix;
G3PDH, glyceraldehyde-3-phosphate dehydrogenase;
GFP, green fluorescent protein;
MMP, matrix metalloproteinase;
MT-MMP, membrane-type MMP;
PCR, polymerase chain reaction;
RT-PCR, reverse transcript PCR;
TIMP, tissue inhibitor of metalloproteinase.
ABSTRACT
Membrane-type 1 matrix metalloproteinase (MT1-MMP/MMP-14) is an enzyme that promotes tumor cell invasion in tissues. Although the proteolytic activity of MT1-MMP is indispensable for invasion, it is also regulated by functions of the cytoplasmic tail. In this study, we obtained a new human gene whose product binds to the tail sequence in yeast. The product, MTCBP-1, is a 19 kDa protein which belongs to the newly proposed Cupin superfamily composed of proteins with diverse functions. MTCBP-1 expressed in cells formed a complex with MT1-MMP and co-localized at the membrane. It was also detected in both the cytoplasm and nucleus where MT1-MMP does not exist. In human tumor cell lines, MTCBP-1 expression was significantly low compared to non-transformed fibroblasts and enforced expression of MTCBP-1 inhibited the activity of MT1-MMP in promoting cell migration and invasion. MTCBP-1 showed significant homology to the bacterial aci-reductant dioxygenase (ARD), which is an enzyme in methionine metabolism. The C-terminal part of MTCBP-1 is identical to Sip-L, which is reported to be important for HCV replication. Thus, MTCBP-1 may have multiple functions other than the regulation of MT1-MMP, which presumably depend on the subcellular compartment.
INTRODUCTION

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a member of the matrix metalloproteinase (MMP) family that collectively degrades most components of the extracellular matrix (ECM) (1). By anchoring to the plasma membrane through a transmembrane domain, MT1-MMP acts in the pericellular space on the cell surface. This property is particularly suitable for the degradation of ECM required for cellular functions such as migration, invasion, proliferation, and the regulation of cell morphology (2,3). Since MT1-MMP is frequently expressed in malignant tumors, it is believed to play a major role in tumor invasion by degrading the ECM in the direction of cell migration and by processing of cell surface molecules (2-4).

As a member of the MMP family, MT1-MMP has a propeptide, a catalytic domain, hinge, and a hemopexin-like (HPX) domain starting from the N-terminus and this extracellular portion is linked to the membrane through the transmembrane domain, which follows a short cytoplasmic tail composed of 20 amino acids (1,2,5). As an invasion-promoting enzyme, the activity, localization, and turnover of MT1-MMP are tightly regulated during cell locomotion (3). For example, MT1-MMP localizes at the leading edge of migrating cells and this localization is regulated through interaction between CD44 and the HPX domain (6). CD44 is a cell adhesion molecule that acts as a receptor for hyaluronan (HA) and mediates flexible adhesion to the provisional HA-rich matrix in the dynamic ruffling membrane area. Such adhesion is expected to be followed by a firmer adhesion through integrins to generate force for migration (3). At the migration edge, MT1-MMP forms oligomers through the HPX domain with possible participation of the cytoplasmic tail, the hinge region, and ECM that binds to the molecule (7-10). Activation of proMMP-2 by
MT1-MMP is expected to be carried out efficiently within the oligomeric complex at the leading edge (7). MT1-MMP exposed on the cell surface is regulated negatively by TIMPs (5), auto-degradation (11), and internalization (12,13). Internalization of MT1-MMP depends on the cytoplasmic tail and a LLY motif in the region was found to act as a binding site for AP-2 complex that mediates incorporation of target proteins into clathrin-coated pits (12). Deletion of the cytoplasmic tail inhibits not only the internalization of MT1-MMP but also the invasion-promoting activity against the reconstituted basement membrane (Matrigel) mediated by MT1-MMP (12,14). Thus, the proteolytic activity of MT1-MMP alone is not enough for the invasion-promoting activity and it has to be regulated further by the function of the cytoplasmic tail.

Although the cytoplasmic tail is short, containing 20 amino acids, it is reported to affect functions of MT1-MMP such as the formation of oligomers (8,9), localization of the enzyme to the proteolytically active protrusions (invadopodia) (15), internalization (12,13), and cell migration and invasion (12,14). However, it has not been elucidated how the cytoplasmic tail affects these functions except for the mechanism of internalization. To obtain clues about the mechanism by which the cytoplasmic tail regulates MT1-MMP, we attempted to isolate genes whose products interact with this portion using the yeast two-hybrid screening system. Using a cDNA library established from human fibroblast WI-38 cells, we isolated a new gene, whose product shows homology to members of the Cupin superfamily, a new family composed of proteins with diverse functions and which has a conserved 3D structure (16). We named this protein MT1-MMP cytoplasmic tail binding protein-1 (MTCBP-1). MTCBP-1 is expressed as a 19 kDa protein and is co-localized with MT1-MMP at the adherent membrane edge. It was also found in the cytoplasm and nucleus. The formation of a
complex between MTCBP-1 and MT1-MMP was confirmed by co-immunoprecipitation. Characteristics of MTCBP-1 and its possible role as an invasion-suppressor are discussed.

MATERIALS AND METHODS

Yeast two-hybrid analysis

A LexA-based yeast two-hybrid screening was performed as described in the instructions for the MATCHMAKER two-hybrid kit (Clontech, CA, USA) using the cytoplasmic tail sequence of MT1-MMP as bait and a galactose inducible prey-fusion library. A DNA fragment encoding the intracellular sequence of human MT1-MMP (R^{563}-V^{582}) was amplified by polymerase chain reaction (PCR) using primers (5'-ggaattcagacgccatgggacccccagg-3' and 5'-gagctcgcctcagaccttgtccagcagggaac-3') and ligated to the LexA-encoding sequence for expression as a fusion protein. The fragment was subcloned into the yeast expression vector pEG202. The basal transcription activation activity of the bait plasmid pLexA-MT1-MMP-tail was negligible. The MATCHMAKER cDNA library used for screening was generated from poly(A)+ RNA isolated from a human lung fibroblast cell line, WI-38 (Clontech). Potential interactors were screened by auxotrophic selection on plates supplemented with galactose or glucose, but lacking histidine, leucine, tryptophan and uracil (Gal/-HLTU or Glu/-HLTU), and for the ability to metabolize X-gal on Gal/X-gal/-HTU or Glu/X-gal/-HTU plates. Positive colonies that grew on Gal/-HLTU plates and appeared blue on Gal/X-gal/-HLTU plates were collected.

Northern blot analysis
Northern blot analysis was performed using The Human Multiple Tissue Northern (MTNTM) blot membrane (Clontech). Fragments of the cDNAs for human MT1-MMP, MTCBP-1 and G3PDH were labeled with $[\alpha^{-32}P]dCTP$ (3000 Ci / mmol, Amersham Bioscience, Inc.) and used as probes.

**Reverse-transcription polymerase chain reaction (RT-PCR)**

First-strand cDNA was synthesized from 3 µg of total RNA using 0.3 µg of each random primer (Gibco BRL, Gaithersburg, MD, USA) and 200 U of Superscript II RNase H- reverse transcriptase (RTase) (Gibco BRL). After removal of the random primers, 1 µl of the RT product was used as a template for PCR (25 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min). Primer sequences are indicated in the figure legends. G3PDH mRNA was amplified similarly as an internal control.

**Cell culture and transfection**

Human fetus lung normal diploid fibroblasts (WI-38 and TIG-20), human fibrosarcoma (HT1080), and green monkey kidney (COS-1) cells were cultured at 37 °C in DMEM (Sigma Chemical Co., MO, USA). Human fibrosarcoma (SW684) cells were cultured at 37 °C in Leibovitz's L-15 medium (Sigma). All media were supplemented with 10% fetal bovine serum. Cells were seeded in 6-well plates at 1.0 - 1.5 x 10^5 cells/well and transfection was carried out after 16 h using FuGENE6™ (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's instructions.

**Immunoprecipitation**
Transfected cells (1.5 x 10^5 cells) cultured in 6-well dishes were lysed in a lysis buffer (1% Brij-99, 50mM Tris-HCl pH7.6, 150mM NaCl, 1% deoxycholic acid, and 0.1% SDS) in the presence of a protease inhibitor cocktail (Roche). The cell lysate was clarified by centrifugation at 15,000 rpm for 15 min, and the supernatant was incubated with anti-FLAG M2 antibody-conjugated agarose beads (Sigma) for 2 h at 4°C. After the beads were collected and washed three times with lysis buffer, precipitates were eluted using a FLAG peptide and then analyzed by Western blotting using the same antibody.

**Subcellular fractionation**

After washing, cells in culture dish were collected with a cell scraper on ice and suspended in 1 ml of 10 mM Tris-buffer (pH7.4) containing 250 mM sucrose and proteinase inhibitor cocktail (Roche). Then, the cells were homogenized using a Dounce homogenizer (20 strokes) and centrifuged in a microtube at 1,500 rpm for 10 min at 4°C to remove nucleus and undisrupted cells. The supernatant fraction was collected and centrifuged further at 65,000 rpm for 1 hour at 4°C using a Beckman rotor (NTV-90). Plasma membrane fraction is recovered in the pellet and cytoplasmic proteins are in the supernatant.

**Western blot analysis**

Human tissue samples (Protein Medley™) (Clontech) and cells in culture were lysed in a SDS sample buffer containing 2-mercaptoethanol. Proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Buckinghamshire, UK). After the blocking of the membrane with 10% fat-free dry milk in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl), the membrane was probed with antibodies
for detection. The membrane was further probed with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma) to visualize the reacted antibody.

**Purification of recombinant MTCBP-1 and preparation of a polyclonal antibody**

Recombinant human MTCBP-1 was expressed in the *E. coli* strain BL21 (DE3) pLysS (Stratagene, CA) by transfecting MTCBP-1/pRSET B Vector plasmid (Invitrogen, CA), with which expression of the gene can be induced by adding 0.4 mM IPTG to the culture medium. Cells were collected and sonicated in a TNC buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl$_2$, and 0.02% NaN$_3$) containing 2 mM PMSF. Supernatant was collected, and the 6xHis-tagged protein was purified on a chelating sepharose column. The bound protein was eluted with TNC buffer containing 500mM imidazol and separated further with a gel filtration column of Sephacryl S-200 (Amersham Bioscience Inc.). To remove the His-tag, EnterokinaseMax$^{TM}$ (Invitrogen) was used under the reaction conditions recommended by the manufacturer.

To obtain polyclonal antibody for MTCBP-1, two female rabbits were immunized with the purified recombinant protein conjugate emulsified with an equal volume of Freund’s complete adjuvant. A week after the last injection, serum was obtained from the animals, and subjected to a 40% saturated ammonium sulfate fractionation. Antibody reactive to MTCBP-1 was further purified using an affinity column of sepharose 4B conjugated with rMTCBP-1.

**Indirect immunofluorescence staining**

Transfected HT-1080 cells were seeded on fibronectin/gelatin coated coverslips. After 24h,
the cells were washed with phosphate-buffered saline (PBS) three times, and fixed with 3% paraformaldehyde in PBS. To detect the MTCBP-1 signal, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. After blocking treated with 5% goat serum and 3% bovine serum albumin in PBS for 1 h at room temperature, cells were reacted with rabbit anti-MTCBP-1 antibody or mouse anti-FLAG M2 antibody at room temperature for 2 h. To visualize MTCBP-1 and FLAG-tagged MT1-MMP, cells were further incubated with Alexa™488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., OR, USA) and Cy3-conjugated goat anti-mouse IgG (Jackson Immuno Research laboratories, Inc., PA). Fluorescence signals were detected using a BIO-RAD MRC-1024 confocal laser microscope.

**Gelatin zymography**

Gelatin zymography was conducted with an SDS-polyacrylamide gel containing gelatin (0.8 mg/ml) as described previously (4). The samples were mixed with SDS/PAGE loading buffer without a reducing agent and subjected to electrophoretic analysis at room temperature. Enzyme activity was visualized as negative staining with Coomassie Brilliant Blue R-250.

**Phagokinetic track motility assay**

The phagokinetic track motility assay was performed as described previously (12). Colloidal gold-coated coverslips were placed in a 12-well plate and transfected cells were seeded at 3 x 10³ cells/well. After 12 h incubation at 37°C, the phagokinetic tracks were visualized under bright-field illumination using a CoolSNAP-fx monochrome CCD camera (Roper Scientific). The track area was measured using NIH Image software v1.62.
Matrigel invasion assay

The Matrigel invasion assay was performed as described previously (7) according to the manufacturer's instructions (Becton Dickinson Labware, MA). MTCBP-1 plasmid was introduced into HT-1080 cells with a plasmid for the expression of green fluorescent protein (GFP). The transfected cells were suspended in DMEM without serum and seeded onto a Matrigel-coated filter (8 µm pore) in the transwell chamber. FBS (10%) was added to the medium in the lower chambers and incubated for 12 h at 37°C. Non-invading cells remaining on the upper surface of the filter were removed and fixed with 3% paraformaldehyde. The GFP-positive cells on the lower surface of the filter were enumerated under microscope at a magnification of x400. Each assay was performed in triplicate and five microscopic fields from each of the three filters were counted.

RESULTS

Screening of human genes whose products bind MT1-MMP at the cytoplasmic tail

To isolate candidate genes whose products bind the cytoplasmic tail of MT1-MMP, the yeast two-hybrid system was used to screen a human cDNA library established from lung fibroblast cells (WI-38). The cytoplasmic tail peptide (RRHGTPRRLYCQRSLLDKV) was expressed as bait fused to the DNA binding domain of LexA protein. From 8 x 10⁷ transformants, 23 clones were obtained and confirmed to be true positives. These clones can be divided into two groups by their size (1062 and 1617 bp) with one exception. DNA sequence analysis revealed that the 1062 bp fragment matched exactly the 5' portion of the 1617 bp fragment, sharing the same 5’ end, and that both had a poly (A) tail at their 3’ end
(Fig. 1). Thus, these two cDNAs are presumably derived from transcripts of a single gene that has multiple transcriptional termination sites. Both transcripts have one open reading frame that potentially encodes a 179 amino acid polypeptide. Since the product of the gene is expected to bind to the cytoplasmic tail of MT1-MMP, it was named MT1-MMP cytoplasmic binding protein-1 (MTCBP-1). One cDNA that did not show any homology to the MTCBP-1 gene was not analyzed further in this study.

**Structure of MTCBP-1 and its homologues**

A BLAST search with MTCBP-1 of the human DNA databases yielded a gene called Sip-L (Fig. 2), the expression of which is reported to render cells susceptible to infection by human hepatitis C virus (HCV) by allowing its replication (17). Although the two genes matched completely, the reported Sip-L cDNA lacks the 5’ sequence of MTCBP-1 that includes the non-coding and part of the 5’ coding sequence. As a result, Sip-L lacks the N-terminal 63 amino acids of MTCBP-1. However, the transcripts and protein product of Sip-L have not been characterized well in the previous study (17) as discussed later.

MTCBP-1 also showed significant homology to the bacterial genes that encode aci-reductant dioxygenase (ARD) used in the salvage pathway of methionine metabolism. The homology of MTCBP-1 to the ARD of Klebsiella oxytoca (28.2 %) and other homologues in different species is summarized in Fig. 2B. MTCBP-1 homologues are composed of 178-188 amino acids. Among them, ARDs of Klebsiella pneumoniae and oxytoca, including the 3D structure, are the best characterized (18-20), though the functions of the eukaryotic homologues have yet to be studied.

ARD has a β-barrel hold that is also known as a double stranded beta helix (DSBH)
domain (20) and is characteristically conserved in members of the Cupin superfamily that was proposed recently (16). In spite of this structural conservation, the members of this family vary widely in function, and include metabolic enzymes, transcription factors, scaffold proteins etc.

**Interaction between MTCBP-1 and MT1-MMP in cells**

Recombinant MTCBP-1 protein (rMTCBP-1) was expressed in *E.coli* and purified as described in the Materials and Methods section. Rabbit polyclonal antibody was also prepared using rMTCBP-1 as an immunogen. To examine whether MTCBP-1 binds to the cytoplasmic tail of MT1-MMP directly, a cytoplasmic tail peptide with 6xHis-tag (CP-WT) and a control peptide with a randomized sequence (CP-RD) were prepared. The peptides were immobilized on a nitrocellulose membrane filter and then blotted with rMTCBP-1. The protein bound to the filter was visualized using anti-MTCBP-1 antibody conjugated with alkaline phosphatase (Fig. 3A). MTCBP-1 was bound to CP-WT but not CP-RD. The binding to CP-WT was specific, because it was blocked almost completely with an excess amount of the CP-WT peptide but not with CP-RD.

To confirm the binding within cells, either a full-length MT1-MMP having a FLAG-tag immediately downstream of the furin-site (MT1F) or its mutant lacking the cytoplasmic tail (dCPF) was expressed together with MTCBP-1 in human fibrosarcoma HT1080 cells. Transfected cells were lysed and MT1F was immunoprecipitated using anti-FLAG antibody. The precipitates were eluted from the antibody using FLAG peptide and the eluate was analyzed further by Western blotting using anti-MTCBP-1 antibody (Fig. 3B). Expression of each protein in the cells was confirmed by Western blotting (Fig. 3B,
total lysate). Although MTCBP-1 itself was not precipitated by the anti-FLAG antibody, it was precipitated with MT1F when both were co-expressed. The mutant MT1F lacking the cytoplasmic tail (dCPF) failed to precipitate MTCBP-1. Thus, MTCBP-1 binds to the cytoplasmic tail of MT1-MMP in the cells.

MT1-MMP recruits MTCBP-1 to the plasma membrane fraction

If MTCBP-1 bound MT1-MMP, the proteins would co-localize in the cells. MT1-F and MTCBP-1 were co-expressed in HT1080 cells and localization of the products was examined by immunostaining (Fig. 3C). MT1F was detected on the cell surface especially at the ruffling edge when the cells were examined without permeabilization (no treatment with Triton X-100). Under the same conditions, MTCBP-1 was not detected as it is a cytoplasmic protein. After the cells were treated with Triton X-100, co-localization of MTCBP-1 and MT1F was detected at the periphery of the cells (Fig. 3C). Some MT1F signals within the cells presumably represent the translation products in vesicles and the Golgi apparatus. Although MTCBP-1 was also detected in the cytoplasm, its localization did not coincide with that of MT1F. Some MTCBP-1 was detected in the nucleus as well, indicating the possibility that MTCBP-1 shuttles between the three compartments, membrane, cytoplasm, and nucleus.

To confirm the interaction of both proteins further, plasma membrane-enriched fraction was prepared and examined by Western blotting (Fig. 3D). Accumulation of MTCBP-1 in the membrane fraction was not much when it is expressed without MT1F. However, co-expression increased the amount of MTCBP-1 in the membrane fraction significantly. This accumulation was dependent on the cytoplasmic tail of MT1F, because
co-expression of dCPF failed to increase this amount. Appropriate fractionation was monitored using marker proteins such as transferring receptor (TfnR) for membrane proteins and actin for cytoplasmic proteins. Expression of MT1F and dCPF did not affect the expression levels of MTCBP-1 and the fractionation (Fig. 3D). Thus, the results strongly suggest that MT1-MMP forms a complex with MTCBP-1 within the cells.

**Expression and tissue distribution of MTCBP-1**

The expression of MTCBP-1 mRNA in different human tissues was examined by Northern blotting and compared to that of MT1-MMP (Fig. 4A). Three MTCBP-1 transcripts (1.2, 1.5 and 2.0 Kb) were detected in tissue samples. The three presumably represent transcripts terminating at different sites. Corresponding to the three transcripts, three potential poly(A) signals can be seen in the 3’ non-coding region of the 1.6 kb cDNA sequence as indicated in Fig. 1. The expression levels were higher in heart, and lower in brain and placenta (Fig. 4A). On the other hand, MT1-MMP mRNA was predominantly expressed in lung and placenta where MTCBP-1 expression is low. Thus, MT1-MMP may not require MTCBP-1 to function, at least in these tissues.

The antibody against MTCBP-1 detected a 19 kDa protein in the COS-1 cells transfected with the expression plasmid for MTCBP-1 but not in the mock-transfected cells (Fig. 4B). A band similar in size was also detected in the tissue extract from human lung and liver.

**Inverse correlation of MTCBP-1 and MT1-MMP expression in the transformed and non-transformed human cell lines**
MT1-MMP is frequently expressed in human tumors and tumor-derived cell lines. Thus, expression of MTCBP-1 and MT1-MMP was examined further using cell lines to confirm the relationship between the two (Fig. 5A and B). As tumor cell lines, four gastric carcinoma (MKN-7, MKN-28, NUGU-3 and NUGC-4) and two fibrosarcoma (HT1080 and SW684) cell lines were analyzed by RT-PCR using specific primers (Fig. 5A). Non-transformed fibroblasts WI-38 and TIG-20 were also examined. *MT1-MMP* mRNA was detected in the tumor cell lines as reported at higher levels than in the non-transformed cells (Fig. 5B). In contrast, although *MTCBP-1* mRNA was detected in all the cells, the expression was weak in the transformed cells compared to the non-transformed cells. Although we analyzed more than 20 different types of human tumor cell lines, none of them expressed a level of *MTCBP-1* mRNA comparable to that in the non-transformed fibroblasts (data not shown). Amount of the transcripts measured by real-time PCR after reverse-transcription revealed that the expression of *MTCBP-1* mRNA in the fibrosarcomas was less than 5-10% of that in the non-transformed fibroblasts (data not shown). Expression of the protein products was also confirmed by Western blotting (Fig. 5C). Thus, the expression of *MTCBP-1* appears to be down-regulated in the tumor-derived cell lines compared to the non-transformed cell lines and inversely correlated with that of *MT1-MMP*. These results raise the possibility that MTCBP-1 is a negative regulator of MT1-MMP function.

*MTCBP-1 suppresses cell migration mediated by MT1-MMP*

One well-known function of MT1-MMP is to activate proMMP-2 (2). The activation requires at least two MT1-MMP molecules, which act as a receptor for binding proMMP-2 using TIMP-2 as an adaptor, and an activator that is free of TIMP-2 and attacks...
the proMMP-2 bound to the MT1-MMP/TIMP-2 complex. The oligomerization of MT1-MMP accompanies the enhanced activation of proMMP-2 presumably by maintaining a distance appropriate for the reaction (7). Although the HPX plays the primary role in the oligomerization directly and indirectly (7,8,10), the cytoplasmic tail is also reported to contribute the process (8,9). Thus, we examined the effect of MTCBP-1 on the activation of proMMP-2 by MT1-MMP. Expression of MT1F in COS-1 cells induced activation of the exogenously added proMMP-2 and this effect was inhibited by TIMP-2 but not TIMP-1 (Fig. 6A). However, co-expression of MTCBP-1 did not affect the activation rate at all. Similarly, expression of MTCBP-1 did not affect the rate of internalization of MT1-MMP (data not shown).

MT1-MMP is also known to promote cell migration and invasion requiring its proteolytic activity. Cell surface targets may be cell adhesion molecules such as CD44, integrin αv chain, tissue transglutaminase, and laminin-5 etc. (3). We reported previously that the cytoplasmic tail plays some roles in the migration- and invasion-promoting activity of MT1-MMP as well (12). Thus, we examined the possibility that expression of MTCBP-1 affects the activity of MT1-MMP to promote cell migration. We used COS-1 cells that do not express endogenous MT1-MMP and are known to migrate upon expression of MT1-MMP (12). Transient expression of MT1-MMP promoted cell migration and the effect required the cytoplasmic tail, because on deletion of the tail (dCPF) the migration was not promoted as reported previously (12) (Fig. 6B). Expression of MTCBP-1 itself had no effect on cell migration. However, MTCBP-1 significantly suppressed the cell migration promoted by MT1-MMP. The effect of MTCBP-1 was specific to the migration promoted by MT1-MMP,
because MTCBP-1 did not show such a suppressive effect on the TPA-induced cell migration. This is consistent with the evidence that MTCBP-1 had no effect on the basal migration activity of the COS-1 cells.

**MTCBP-1 reduces the invasion-promoting activity mediated by MT1-MMP**

To examine the effect of MTCBP-1 on invasion, we used HT1080 cells that aggressively invade a reconstituted basement membrane (Matrigel) using endogenous MT1-MMP and express low levels of MTCBP-1 (Fig. 7). In the previous study, we demonstrated that specific down regulation of MT1-MMP by RNAi abrogated the activity to invade Matrigel (21). As expectedly from this result, invasion by the cells strictly requires MMP activity, as the synthetic MMP inhibitor BB94 or natural inhibitor TIMP-2 inhibited it efficiently (Fig. 7). TIMP-1 also inhibited the invasion, though it was not comparable to that of BB94 or TIMP-2. Expression of MTCBP-1 significantly reduced the invasion (Fig. 7). Although the inhibition by MTCBP-1 was greater than by TIMP-1, it was less than that by BB94 or TIMP-2.

**DISCUSSION**

**Identification of MTCBP-1 and its homologues**

MTCBP-1 was identified as a protein binding to the cytoplasmic tail of MT1-MMP. From the database search, part of *MTCBP-1* was found to be identical to *Sip-L*, which is reported to confer susceptibility to HCV infection, though the mechanism has not been clarified yet (17). However, the tissue distribution pattern of MTCBP-1 transcripts is clearly different from that of *Sip-L* studied by RT-PCR. The transcripts of *Sip-L* may not be detected
appropriately in the previous study. It is not clear at this moment whether Sip-L gene is merely a cloning artifact or if there is a specific regulation to express short forms of MTCBP-1 such as Sip-L.

MTCBP-1 also showed homology to the products of prokaryotic and eukaryotic genes listed in Fig. 2. Among them, ARDs of *Klebsiella pneumonia* and *oxytoca* are the best characterized ones as a dioxygenase acting in the salvage pathway of methionine metabolism (22,23). The ARD converts aci-reductone, an intermediate in the salvage pathway of methionine metabolism, into two different metabolites depending on the metal ions available (Ni$^{2+}$ and Fe$^{2+}$) (18). Since ARD activity is conserved in a wide array of organisms, it is possible that MTCBP-1 acts as a human ARD.

MTCBP-1 homologues in different organisms share an evolutionally conserved domain called DSBH or β-barrel hold that unites these proteins as members of the Cupin superfamily (16). A characteristic feature of this family is that the members have acquired diverse functions during evolution while retaining the DSBH scaffold. For example, they function as isomerases, epoxidases, dioxygenases, decarboxylases, transcription factors, and centromeric proteins (16). Thus, it is plausible that MTCBP-1 has acquired an ability to regulate MT1-MMP in addition to the ARD activity during the process of evolution.

**MTCBP-1 down-regulates the cell migration and invasion promoted by MT1-MMP**

MTCBP-1 co-localizes with MT1-MMP at the ruffling membrane by forming a complex and recruitment of MTCBP-1 to the membrane fraction was dependent on the cytoplasmic tail of MT1-MMP (Fig. 3D). Expression of MT1-MMP is known to promote cell migration and invasion (2,3). Co-expression of MTCBP-1 inhibited the cell migration
promoted by MT1-MMP while it showed no effect on the TPA-induced migration. Thus, the effect of MTCBP-1 on cell migration is specific to the one promoted by MT1-MMP. MTCBP-1 also inhibited the invasion-promoting activity of MT1-MMP against Matrigel. The invasion and migration-promoting activity of MT1-MMP is mediated through proteolysis on the cell surface, because the activity was inhibited by BB94 (Fig. 6 and 7). On the other hand, MTCBP-1 did not affect proMMP-2 activation by MT1-MMP. In addition, the cytoplasmic tail of MT1-MMP has no effect on degradation of and invasion into type I collagen gel (24,25). Thus, we speculate that the effect of the cytoplasmic tail on the proteolytic activity of MT1-MMP differs depending on its substrates. The difference may be explained by the mode of interactions of MT1-MMP with substrates. Namely, MT1-MMP binds type I collagen at the HPX domain (10) and does not require the cytoplasmic tail to degrade it (24). However, some substrates in the Matrigel may bind MT1-MMP indirectly through the membrane proteins that bind the cytoplasmic tail of MT1-MMP within the cells and bind substrates outside. In this case, deletion of the tail would abolish the activity to degrade the substrates in the Matrigel and inhibit the invasion. Binding of MTCBP-1 to the cytoplasmic tail may compete this sort of interaction, and eventually reduce the invasion-promoting activity of MT1-MMP.

All the results presented support the idea that MTCBP-1 is a negative regulator of MT1-MMP through its cytoplasmic tail. Interestingly, the expression levels of MTCBP-1 in all the tumor cell lines examined was quite low compared to the non-transformed fibroblasts. Thus, down-regulation of MTCBP-1 appears to associate with tumor progression.

**Subcellular localization and possible physiological functions**
MTCBP-1 appears to shuttle between the three subcellular compartments, membrane, cytoplasm, and nucleus. The best characterized MTCBP-1 homologue is the ARD of *Klebsiella pneumoniae* (18). It is an enzyme in the salvage pathway of the methionine metabolism. As we already discussed, MTCBP-1 may be a human homologue of the bacterial ARD. Because the reactions of the metabolic pathway are thought to be carried out in the cytoplasm, the ARD activity of MTCBP-1 may be active when it is in the cytoplasmic fraction. In relation to the nuclear localization of MTCBP-1, the product of the yeast homologue (YMR009w) is listed as a possible interactor with a splicing factor, SNP-1, in the comprehensive yeast protein-protein interaction database constructed by the two-hybrid methodology (26). Therefore, MTCBP-1 may regulate gene expression as a component of the splicing machinery in the nucleus. Thus, MTCBP-1 is a possible multifunctional protein carrying out different functions depending on its localization. One characteristic feature of Cupin superfamily is the diverse functions of the members while they have a conserved 3D structure (16). The DSBH domain conserved in the family appears flexible and able to accept such diverse functions that were necessary during evolution. Thus, it is not be surprising that MTCBP-1 has acquired multiple functions by having the DSBH domain.

In conclusion, we identified MTCBP-1 as a protein binding to the cytoplasmic tail of MT1-MMP. MTCBP-1 has a DSBH domain that is conserved in a recently identified Cupin superfamily composed of proteins with diverse functions. The expression levels of MTCBP-1 in tumor cell lines are low compared to the non-transformed fibroblasts and enforced expression of MTCBP-1 suppressed the invasion and migration-promoting activity of MT1-MMP. Since MTCBP-1 disrupts the essential role of the cytoplasmic tail during cell
migration and invasion, it may be a useful tool to analyze the regulation of MT1-MMP through the cytoplasmic tail.

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FIGURE LEGENDS

Figure 1. Structure of the cDNA and deduced amino acid sequence of human MTCBP-1 (DDBJ/EMBL/GenBank Accession number: AB158319). The nucleotide sequence of the longer cDNA fragment is presented. The open reading frame (ORF) that starts from the first methionine codon (537 bp) and encodes the 179-amino acid polypeptide presented. Three hypothetical poly(A) signals in the 3’ non-coding region are indicated in bold.

Figure 2. Alignment of the amino acid sequence of human MTCBP-1 with its homologues in the DNA database. A, Genes in the DNA database, whose products show significant homology to MTCBP-1, are listed and their amino acid sequences are aligned with MTCBP-1. Identical amino acids were surrounded with black boxes and homologous ones with gray boxes. B, The degree of homology in amino acid sequence (%) to MTCBP-1 or DSBH domain of ARD is listed for each gene product.

Figure 3. Interaction between MTCBP-1 and MT1-MMP. A, Binding of rMTCBP-1 to the peptide, CP(WT), which has the sequence derived from the cytoplasmic tail of MT1-MMP. CP(RD) has the same amino acid composition but the sequence was randomized. Two peptides (10 ng) were immobilized on a nitrocellulose membrane filter. After blocking with 10% fat-free dry milk in Tris-buffered saline, the filter was blotted with rMTCBP-1 (1 µg/ml) for 1 hour at room temperature. After the membrane filter was washed with Tris-buffered saline containing 0.05% Tween 20, proteins that remained on the filter were reacted with the antibodies listed in the right and visualized with secondary antibody conjugated with alkaline
phosphatase. A 100-fold molar excess of CP(WT) or CP(RD) was used as competitor for the binding as indicated in the left. B, HT1080 cells were transiently transfected with expression plasmids for MT1F, dCPF and MTCBP-1, as indicated. Immunoprecipitation was carried out using anti-FLAG M2 mouse monoclonal antibody conjugated with agarose beads. Precipitates were specifically eluted with the FLAG peptide and then the eluate was analyzed by Western blotting using mouse monoclonal antibody (FLAG M2) for MT1-MMP or rabbit polyclonal antibody (MTCBP-1) for MTCBP-1. C, HT1080 cells were transfected with expression plasmids for MT1-F and MTCBP-1. Transfected cells were seeded on fibronectin/gelatin-coated glass coverslips and incubated for 24 h. Following fixation, the cells were treated with anti-FLAG M2 antibody and anti-MTCBP-1 antibodies for 1 h. The bound antibodies were visualized with Cy3-conjugated anti-mouse IgG (red) and Alexa488-conjugated anti-rabbit IgG (green), respectively. Scale bar, 10 µm. D, HT1080 cells were transiently transfected with expression plasmids for MT1F and dCPF together with MTCBP-1. Mock transfection was carried out with the vector plasmid. Total cell lysate (total lysate) and membrane-enriched fraction were prepared as described in the Materials and Methods. The samples were separated by electrophoreses on PAGE and analyzed by Western blotting using antibodies against FLAG sequence, MTCBP-1, TfnR, and actin as indicated.

**Figure 4.** Tissue distribution of MTCBP-1. A, Northern blot analysis of MTCBP-1 mRNA in human tissues. A membrane filter that had mRNAs extracted from different tissues and transferred after electrophoresis (Multiple Tissue Northern, Clontech) was probed with P32-labeled cDNA for MTCBP-1 or MT1-MMP, and radioactivity was detected by
autoradiography. The same membrane was rehybridized with a probe for the glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) to confirm the amount of RNA loaded. The size of the mRNA is 4.2 kb for MT1-MMP, 2.0, 1.5 and 1.2 kb for MTCBP-1, and 1.8 kb for G3PDH. B, Detection of the product of MTCBP-1. Protein samples from the indicated cells and tissues were examined by Western blot analysis. COS-1(Mock) and COS-1(MTCBP-1) are the lysates of COS-1 cells transfected with plasmids carrying empty and MTCBP-1 cDNA, respectively. Tissue homogenates (75 µg) were obtained from lung and liver. The membrane filter was probed with anti-MTCBP-1 antibody or anti-actin antibody, and visualized using secondary antibody conjugated with alkaline phosphatase.

**Figure 5.** Expression of MTCBP-1 transcript and protein in tumor cell lines and non-transformed fibroblasts obtained from normal tissues. A, The list represents the primers used to amplify reverse-transcribed transcripts for MTCBP-1 and MT1-MMP. B, Expression of MTCBP-1 and MT1-MMP was analyzed by RT-PCR. Group 1 contains gastric carcinoma cells (MKN-7, MKN-27, NUGC-3, and NUGC-4). Group 2 contains fibrosarcoma cells (HT1080 and SW684) and group 3 non-transformed fibroblasts from lung (WI-38 and TIG-20). RT-PCR products were analyzed by agarose gel electrophoresis. Plasmid carrying either cDNA was used as a control template. C, Detection of MTCBP-1 protein in tumor cell lines. Cell lysates prepared from the tumor cell lines (1 x 10^7 cells) indicated were immunoprecipitated using anti-MTCBP-1 rabbit polyclonal antibody conjugated with protein sepharose A. After the beads were collected and washed three times with lysis buffer, the immune complex was solubilized in a SDS sample buffer and subjected to Western blot analysis.
Figure 6. Effect of MTCBP-1 on the functions of MT1-MMP. A, Activation of proMMP-2. COS-1 cells (1.0 x 10^5) were transfected with the indicated expression plasmids and incubated with purified proMMP-2 in serum-free culture medium. Either TIMP-1 (1 µg/ml) or TIMP-2 (1 µg/ml) was added in the culture medium as indicated. After 18 h, gelatinolytic activity in the culture medium was analyzed by gelatin zymography. Transfected cells were subjected to Western blot analysis using the anti-FLAG M2 monoclonal antibody (MT1F) and anti-MTCBP-1 polyclonal antibody (MTCBP-1). To confirm the amount of protein loaded, anti-Actin monoclonal antibody was used. B, Migration track assay. COS-1 cells (1.0 x 10^5) were transfected with the expression plasmids for MT1F and MTCBP-1. Cells were treated with TPA (TPA+) or left untreated (TPA-). The migration area was visualized under bright-field illumination and analyzed using NIH Image software v1.62. The average of 40 cells ± SEM is shown.

Figure 7. Effect of MTCBP-1 on Matrigel invasion. HT1080 cells (1.5 x 10^5) were transfected with an expression plasmid for GFP as a transfection marker either alone or together with that for MTCBP-1. The cells (1.0 x 10^4) were subjected to the Matrigel invasion assay as described in the Materials and Methods. Either BB94 (10 µM), TIMP-1 (1 µg/ml) or TIMP-2 (1 µg/ml) was added to both upper and lower chambers as indicated in the figure. After 24 h of incubation, the GFP-positive cells on the lower surface of the filters were counted under a fluorescence microscope. Each assay was performed in triplicate and the average for four fields is shown (mean ± SD). *, P<0.05. The cells were also subjected for Western blot (WB) analysis using anti-MTCBP-1 and anti-actin antibodies as indicated.
Uekita et al. Figure. 1

CTGGTTCTGAACACGCACCCGCACCTGGCT

ATGGTGAGGCGCTGGTATATGGAGAGCCCGCCGCCGGCCAGCCGGCGGCCGGCACCCGCCAGCCGCGCCGGC
M V Q A W Y M D D A P G D P R P Q P H R P

GACCCGGCCGCCAGTGGGCGCTGACGTGGCGGCTGCGGTTCTACTGGAG
D P G R P V G E Q L R R L G V L Y W K

CTGGAATGGTACCCAAATATGAGATGTCATCCAGTTAAAGGATAGTGACAGAGAG
L D A K Y E N D P E L K I R R N E N

TACTCCCTGATGGCACTATACATATAGCAGGAAAGTTTACTACAAATATGAGGAAAG
Y S W M D I I I I T I C D K L P N Y E K

ATTAAGATGGTCTACGGAGGACTTGGAGCTGGATCCCTACATTTGGAT
I K M P Y E E H L H L D E I R Y I L D

GGCAATGCTGCTGACTGTAATGGAGCAGCTCCGCTCCTGGAGAGAG
G S G Y F D V R D K E D Q W I R I F M E

AAGGGAGACATGGTACGCCTCCCGGGGATCTATCCACCGTACCTGGAT
K G D M V T L P A G I Y H R F T V D E K

AACTACACGGACACCTGCGCTTTGGAGGAGACCCGTCGACCGGAGGC
N Y T K A M R L F V G P V W T A Y N R

CCCGCTGGACATTGTTAGAAGCGCCGCCGGGGAGCTGGAAATTCTTGGGCAAGAGCAGCCGCTAG
P A D H F E A R G Q Y V K F L A Q T A *

Nuc A.A
33
93 20
153 40
213 60
273 80
333 100
393 120
453 140
513 160
573 179
633
693
753
813
873
933
993
1053
1113
1173
1233
1293
1353
1413
1473
1533
1593
1617
Uekita et al. Figure 3

**A**

MT1-CP Peptide
CP(WT): 6xHis-RRHGTPRLLCYCQRSSLKDV
CP(RD): 6xHis-KTLRYSVLVRQCRGLRPLR

|          | CP(WT) | CP(RD) |
|----------|--------|--------|
| rMTCBP-1 |        |        |
| rMTCBP-1 + CP(WT) |        |        |
| rMTCBP-1 + CP(RD) |        |        |

**B**

IP (FLAG)
(kDa) 63 19
Total lysate
(kDa) 63 19 43

**C**

|          | MTCBP-1 | Merge | MT1F |
|----------|---------|-------|------|
| Triton X-100 (-) |        |       |      |
| Triton X-100 (+) |        |       |      |

**D**

|          | membrane | total lysate |
|----------|-----------|--------------|
| (kDa)    | Mock      | MT1F         | dCPF |
| 63       | anti-FLAG |              |      |
| 19       | anti-MTCBP-1 |            |      |
| 95       | anti-TfnR  |              |      |
| 43       | anti-Actin |              |      |
### A

| Gene   | Primer Sequence                                      | RT-PCR Product (bp) |
|--------|------------------------------------------------------|---------------------|
| MT1-MMP | 5’-GCTTGCAAGTAACAGGCAA-3’<br>5’-TGGATGGACACGGGAATT-3’ | 743                 |
| MTCBP-1 | 5’-ATGTTGAGGCTGCTTATATG-3’<br>5’-GGCCGTCCTGAGCAAAATTTC-3’ | 537                 |
| G3PDH  | 5’-AAGGCTGAGAAGGGGAGCTTGATCATAAT-3’<br>5’-TTCCCGTCTAGGTGATGACCTTGGC-3’ | 500                 |

### B

|          | 1                | 2 | 3 | 4   | 5  | 6  | 7 | 8 | 9 | 10 | 11 |
|----------|------------------|---|---|-----|----|----|---|---|---|----|----|
|          | MKN-7            |   |   |     |    |    |   |   |   |    |    |
|          | MKN-28           |   |   |     |    |    |   |   |   |    |    |
|          | NUGC-3           |   |   |     |    |    |   |   |   |    |    |
|          | NUGC-4           |   |   |     |    |    |   |   |   |    |    |
|          | MTK1080          |   |   |     |    |    |   |   |   |    |    |
|          | SW684            |   |   |     |    |    |   |   |   |    |    |
|          | WI-38            |   |   |     |    |    |   |   |   |    |    |
|          | TIG-20           |   |   |     |    |    |   |   |   |    |    |
|          | Control          |   |   |     |    |    |   |   |   |    |    |
| MT1-MMP  | ![Image](image1)  |   |   |     |    |    |   |   |   |    |    |
| MTCBP-1  | ![Image](image2)  |   |   |     |    |    |   |   |   |    |    |
| G3PDH    | ![Image](image3)  |   |   |     |    |    |   |   |   |    |    |

### C

|          | Control | WI-38 | TIG-20 | HT1080 | SW684 |
|----------|---------|-------|--------|--------|--------|
| anti-MTCBP-1 | ![Image](image4) |       |       |        |        |
| anti-Actin  | ![Image](image5)  |       |       |        |        |

(kDa)
A

MMP-2
pro
act
72
65

MT1-MMP
63

MTCBP-1
19

Actin
43

B

Migration Area (x10³ Pixels)

TPA(-)

Mock
dCPF
MT1F
MTCBP-1
MT1F + MTCBP-1

TPA(+)

Mock
MTCBP-1

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Uekita et al. Figure 7

![Bar graph showing the number of cells per field for different treatments: Mock, MTCBP-1, BB94, TMP-1, and TMP-2. The graph includes error bars indicating variability. A Western Blot (WB) shows bands for anti-MTCBP-1 and anti-Actin antibodies, with molecular weights indicated (19 kDa and 43 kDa).]
Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1) is a new member of the Cupin superfamily: A possible multifunctional protein acting as an invasion suppressor down-regulated in tumors
Takamasa Uekita, Isamu Gotoh, Takeshi Kinoshita, Yoshifumi Itoh, Hiroshi Sato, Takayuki Shiomi, Yasonori Okada and Motoharu Seiki

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