Evidence of MTCBP-1 Interaction With the Cytoplasmic Domain of MT1-MMP: Implications in the Autophagy Cell Index of High-Grade Glioblastoma

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Progression of astrocytic tumors is, in part, related to their dysregulated autophagy capacity. Recent evidence indicates that upstream autophagy signaling events can be triggered by MT1-MMP, a membrane-bound matrix metalloproteinase that contributes to the invasive phenotype of brain cancer cells. The signaling functions of MT1-MMP require its intracellular domain, and recent identification of MTCBP-1, a cytoplasmic 19 kDa protein involved in the inhibition of MT1-MMP-mediated cell migration, suggests that modulation of MT1-MMP cytoplasmic domain-mediated signaling may affect other carcinogenic processes. Using qPCR and screening of cDNA generated from brain tumor tissues of grades I, II, III, and IV, MT1-MMP gene expression was found to correlate with increased grade of tumors. Inversely, MTCBP-1 expression decreased with increasing grade of brain tumor. Confocal microscopy and fluorescence resonance energy transfer (FRET) analysis revealed that overexpressing a cytoplasmic-deleted MT1-MMP recombinant protein mutant prevented MTCBP-1 recruitment to the intracellular leaf of plasma membrane in U87 glioblastoma cells. The interaction between MTCBP-1 and the 20 amino acids peptide representing the MT1-MMP cytoplasmic domain was confirmed by surface plasmon resonance. Overexpression of a full-length Wt-MT1-MMP triggered acidic autophagy vesicle formation and autophagic puncta formation for green fluorescent microtubule-associated protein 1 light chain 3 (GFP-LC3). Autophagic vesicles and GFP-LC3 puncta formation were abrogated in the presence of MTCBP-1. Our data elucidate a new role for MTCBP-1 regulating the intracellular function of MT1-MMP-mediated autophagy. The inverse correlation between MTCBP-1 and MT1-MMP expression with brain tumor grades could also contribute to the decreased autophagic index observed in high-grade tumors. © 2015 Wiley Periodicals, Inc.

Key words: Brain tumor; matrix metalloproteinase; chemoresistance

INTRODUCTION

Astrocytic tumors are the most common primary brain tumor type in humans. The World Health Organization (WHO) classifies pilocytic astrocytoma multiforme as grade I, diffuse astrocytoma as grade II, anaplastic astrocytoma as grade III, and glioblastoma multiforme as grade IV [1]. Progression from a low (I and II)- to a high (III and IV)-grade tumor has been associated with various molecular alterations [2]. Among these alterations, defective autophagy, shown via reduced expression of the Beclin-1 and LC3B-II autophagy biomarkers, has been suggested to correlate with reduced survival times of patients with astrocytic tumors [3]. On the other hand, induction of autophagy processes by pro-autophagic drugs is also becoming an emerging concept to trigger cell death in high-grade gliomas and to exploit caspase-independent programmed cell death pathways for the development of novel brain tumor therapies [4]. Although high-grade gliomas are characterized with reduced expression of autophagy-related proteins when compared to low-grade gliomas, it is still unclear whether dysregulation of autophagy in advanced brain cancer would promote survival or death upon various therapeutic settings.

A low autophagy index in tumorigenesis has been inferred by recent studies where autophagic capacity was decreased during the progression of many tumors. Supporting this, autophagy could be induced in those tumors by numerous anti-tumor agents,

Abbreviations: CNS, central nervous system; ER, Endoplasmic reticulum; FRET, Fluorescence resonance energy transfer; GFP-LC3Green, fluorescent microtubule-associated; MT1-MMP, Membrane type-1 matrix metalloproteinase; MTCBP-1Membrane-type, 1 matrix; SPR, Surface plasmon resonance; TIMP-2, Tissue inhibitor of metalloproteinase-2; WHO, World health organization.

Conflict of interest: The authors declare that they have no competing interests.

Authors' contributions: J.P., S.B., and B.A. designed this study. J.P. performed all the experiments, except the SPR analysis which were performed by M.I. J.P. and S.B. performed and carried out the FRET data analysis. All authors have contributed to data analysis, discussions, and interpretations of the results. All authors have read and approved the final manuscript.

Grant sponsor: NSERC; Grant number: #288249

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Received 24 September 2014; Accepted 17 November 2014
DOI 10.1002/mc.22264
Published online 15 January 2015 in Wiley Online Library (wileyonlinelibrary.com).

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ultimately leading to apoptotic cell death [5–7]. The intrinsic molecular mechanisms involved remain, however, largely unknown. In fact, very few upstream signaling events have been shown to link apoptotic (type I) to autophagic (type II) cell death in high-grade gliomas. An interesting link between type I and type II cell death was, however, recently found to involve the CCAAT/enhancer binding protein (EBP) homologous transcription factor C/EBP homologous protein (CHOP)/growth arrest and DNA damage-inducible gene 153 (*GADD153*) in the regulation of apoptosis and autophagy [8]. MT1-MMP, a membrane type-1 matrix metalloproteinase, is also among the biomarkers documented to trigger both apoptotic and autophagic signaling events [9–13]. In fact, given the recent link between endoplasmic reticulum (ER) stress, apoptosis and autophagy [14,15], MT1-MMP's intracellular signaling roles become even more relevant as ER stress was also found to be induced in glioblastoma cells overexpressing MT1-MMP [16]. Finally, exploiting the altered metabolism that characterizes brain tumor cells [17,18], MT1-MMP was found to transcriptionally downregulate the expression of a microsomal glucose-6-phosphate transporter, whose elevated expression in glioblastoma controls cell survival [19], and whose downregulation in cells overexpressing MT1-MMP triggered cell death [20].

How MT1-MMP-mediated downstream signaling events are controlled and which intracellular components are involved in MT1-MMP transducing functions is currently poorly understood. MT1-MMP's intracellular domain-mediated signaling was already shown to trigger events that led to phosphorylation of signaling intermediates including signal transducer and activator of transcription 3 [21], extracellular signal-regulated kinase [22], and nuclear factor-kappa B [23], as well as inducing the expression of RhoA [24]. Whether such MT1-MMP-mediated events require intracellular binding partners to interact with the 20 amino acid intracellular domain of MT1-MMP remains unknown. In fact, several MT1-MMP cytoplasmic domain binding proteins have been identified and include the μ2 subunit of adaptor protein 2 [25], gC1qR [26], p130Cas [27], and the phosphorylated form of caveolin-1 [28]. MT1-MMP cytoplasmic tail-binding protein-1 (MTCBP-1), a new member of the Cupin superfamily, was also suggested as a possible multifunctional protein acting as an invasion suppressor and was shown to be downregulated in tumors [29]. Although preliminary evidence suggested that it bound to the MT1-MMP cytoplasmic domain, little is known regarding its capacity to affect the intracellular signaling events triggered by MT1-MMP.

In the current study, we first addressed the possible correlative interrelationship that would link MT1-MMP and MTCBP-1 expression using clinically-validated grade I to grade IV human brain tumor tissues. We next used fluorescence resonance energy transfer by acceptor photobleaching (FRET-AP) and surfaceplasmon resonance (SPR) analysis to evaluate the requirement of the MT1-MMP cytoplasmic domain for MTCBP-1 recruitment and interaction. Finally, we tested the functional impact of MTCBP-1 on MT1-MMP-mediated autophagy signaling in glioblastoma cells.

MATERIALS AND METHODS

Materials

Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Oakville, ON). Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The HyGLO^{TM} chemiluminescent HRP antibody detection reagents were from Denville Scientific Inc. (Metuchen, NJ). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against MTCBP-1 and GAPDH were purchased from Cell Signaling (Danvers, MA). The monoclonal anti-MT1-MMP catalytic domain antibody clone 3G4.2 was from EMD Millipore (Billerica, MA). The anti-Turbo-GFP antibody was from Origene (Rockville, MD). The polyclonal anti-TIMP-2 antibody was from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada.

Cell Culture

The human U87 glioblastoma cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in Eagle's Minimum Essential Medium containing 10% (v/v) calf serum (HyClone Laboratories, Logan, UT), 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated at 37 °C with 95% air and 5% CO_2.

Transfection Method and RNA Interference

Cells were transiently transfected with 20 nM siRNA against MT1-MMP (Hs_MMP14_6 HP validated siRNA; QIAGEN, SI03648841) or scrambled sequences (AllStar Negative Control siRNA; QIAGEN, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen, CA). MT1-MMP-specific gene knockdown was evaluated both by qRT-PCR and typically ranged over 90% of inhibition of gene expression (not shown) as well as through the functional activation of latent proMMP-2 into active MMP-2 by gelatin zymography.

TissueScan cDNA Arrays of Grades I–IV Brain Tumor Tissues

TissueScan^{TM} cancer and normal tissue cDNA arrays were purchased from OriGene (Rockville, MD), covering

43 clinical samples of the four stages of brain cancer as well as normal tissues, and were used to assess MT1-MMP and MTCBP-1 gene expression according to the manufacturer's recommendation. Tissue cDNAs in each array are synthesized from high quality total RNAs of pathologist-verified tissues, normalized and validated with ß-actin in two sequential qPCR analyses, and accompanied by clinical information for 18 WHO grade I, 11 WHO grade II, 10 WHO grade III, and two WHO grade IV brain tumors.

Gelatin Zymography

Gelatin zymography was used to assess the extent of proMMP-2 gelatinolytic activity and activation status as previously described [21]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin, a substrate that is efficiently hydrolyzed by proMMP-2 and MMP-2. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting Procedures

Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin and 0.1% sodium azide, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2,500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by chemiluminescence.

Immunofluorescent Microscopy

U87 cells were harvested on cover slips, transiently transfected with a combinaison of cDNA plasmids encoding for a full length Wt-MT1-MMP recombinant protein (Wt-MT1-MMP), a deleted-cytoplasmic domain recombinant protein (Δcyto-MT1-MMP), green fluorescent protein (GFP), GFP-LC3, GFP-mCherry-LC3, or a chimeric GFP-MTCBP-1. Media was removed and cells were fixed in 10% formalin phosphate buffer (Fisher Scientific, Ottawa, ON) for 20 min, and then blocked for 1 h in 1% BSA/PBS/NaN_3. Immunostaining was performed for 1 h with the anti-MT1-MMP antibody 1:200 in 1% BSA/PBS/NaN_3, followed by 1:200 Rhodamine Red-X donkey anti-mouse IgG (Invitrogen, ON). A solution of 10 μg/ml DAPI diluted in PBS was used to stain the nuclei. Fluorescence was then examined by microscopy.

Detection of Acidic Vesicular Organelles (AVO) and of GFL-LC3 Puncta

U87 glioblastoma cells were serum-starved in media containing the different conditions of transfections (siRNA, cDNA plasmid encoding recombinant forms of MT1-MMP) or treatments with Concanavalin-A (ConA; Sigma-Aldrich Canada, L7647). Acridine Orange (0.5 μg/ml; Sigma-Aldrich Canada, A6014) was added to each well, cells were incubated for 15 min at 37 °C in the dark. Fluorescence was then examined by flow cytometry in FL3-H upper quadrants. Transient cell transfection with pEGFP-LC3 or pEGFP-m-Cherry-LC3 (generously provided by Dr. Patrick Labonté, INRS-IAF, Qc), Wt-MT1-MMP, or Δcyto-MT1-MMP cDNA plasmids was performed using lipofectamine 2000 as previously described [16]. Fluorescence of GFP-LC3 puncta was then examined by flow cytometry in FL1-H lower right quadrants.

FRET Acceptor Bleaching Microscopy

Acceptor bleaching experiments were performed using a Nikon Eclipse Ti confocal microscope. Fluorescence images of the fixed cells were taken as single optical sections using a 63X Planapochromat (NA = 1.4) oil immersion objective focused to the middle of the cell along the Z axis. The pinhole size was adjusted to 1.5 Airy units. The cells overexpressing GFP fusion proteins (GFP alone or GFP-MTCBP-1) were imaged using a 488 nm laser line excitation and a 530DF30 filter for emission. Alexa Fluor 568-coupled secondary antibody was used to assess the overexpressed cell surface-localized recombinant Wt-MT1-MMP or Δcyto-MT1-MMP proteins using a 561 nm laser line excitation and a LongPass (LP570) filter for emission. Images were analyzed using NIS-Elements 4.0 C software from Nikon. Bleaching experiments were performed as follows: A region of interest (ROI) was chosen at the cell membrane (Yellow ellipse), inside the cell (Blue rectangle) and outside the cell (Background). Before starting the repetitive bleaching procedure, three prebleaching images were recorded in both channels. Afterwards, the power of the 561 nm laser line was set to 5% and 100% for the imaging and bleaching illuminations, respectively. Five consecutive cycles of one second of bleaching in the region of interest and 1 s of sequential imaging acquisitions of the green and red channels were performed. These FRET acceptor bleaching experiments were performed on 10 cells from each condition and for three different regions of interest, for a total of 30 FRET analysis for each condition of co-transfections.

Surface Plasmon Resonance (SPR) Analysis

SPR analyses were performed using a Biacore T200 instrument (GE Healthcare). MTCBP-1 recombinant

protein (Fitzgerald industries international, Acton, MA) was immobilized on a carboxymethylated dextran CM5 sensor chip (GE Healthcare, Baie D'Urfé Quebec) using an amine-coupling strategy. Briefly, the sensor chip surface was activated with a 1:1 mixture of N-hydroxysuccinimide and 3-(N,Ndimethylamino)-propyl-N-ethylcarbondiimide. MTCBP-1 solution (20 μg/mL) was injected at a flow rate of 10 μL/min using HBS-N running buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) to reach a level of immobilization of 400 RU. Surfaces (protein and reference) were blocked by the injection of an ethanolamine solution. Binding kinetics of a peptide encompassing the entire intracellular cytoplasmic domain of MT1-MMP (RRHGTPRRLLYCQRSLLDKV) and of a relative scrambled peptide (YHLSPQPRKEKRVYYKRTAR) over the MTCBP-1 sensor chip was evaluated in HBS-N buffer at increased concentrations (6.25 to 100 μM) at a flow rate of 20 μL/min. The sensor chip was regenerated by injecting 20 μL of a 10 mM glycine solution, pH 3. Binding sensorgrams were obtained by subtraction of the reference flow cell. Experiments were performed in duplicate and data analysis was performed using the BIAevaluation software package (GE Healthcare).

Statistical Data Analysis

Fret data are representative of 30 FRET analysis on 10 cells from each condition. For all the other experiments, three independent experiments were performed and statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk identifies such significance in the respective figures.

RESULTS

High-grade Brain Tumors Express Low MTCBP-1 and High MT1-MMP

The classification of brain tumors is based on the premise that each type of tumor results from the abnormal growth of a specific cell type. To the extent that the behavior of a tumor correlates with basic cell type, tumor classification often dictates the choice of therapy and predicts prognosis. The WHO grading system classifies grade I brain tumors as benign and slow growing, while grade IV are the most malignant [1]. In order to evaluate the extent of MT1-MMP and MTCBP-1 expression in all four brain tumor grades, we used a cDNA tissue array to assess the transcript levels of each of these biomarkers. We found that MTCBP-1 was highly expressed in low invasive grade I brain tumor tissues and tended to decrease in highly invasive high-grade tumors (Figure 1A, right panel). Inversely, MT1-MMP expression increased with the tumor grade (Figure 1A, left panel) in accordance with previous observations reporting that increased MT1-MMP expression predicts poor survival in human glioma [30]. A scheme of the molecular signature characterizing the current hypothesized models of low MT1-MMP/MTCBP-1 expression ratio in low-grade brain tumors versus high MT1-MMP/MTCBP-1 expression ratio is depicted (Figure 1B). Whether overexpressing MTCBP-1 in U87 cells derived from high-grade glioblastoma translates into colocalization with MT1-MMP at the plasma membrane, and how this affects MT1-MMP-mediated autophagy was next investigated.

FRET- and SPR-based Evidence of MTCBP-1 Interaction With MT1-MMP's Cytoplasmic Domain in U87 Glioblastoma Cells

In order to accurately determine MTCBP-1's capacity to interact with the intracellular domain of MT1-MMP, the protein expression of a recombinant form of MTCBP-1 was first validated in transiently-transfected U87 glioblastoma cells with various amounts of a cDNA plasmid encoding MTCBP-1 fused to green fluorescent protein (GFP). When cell lysates were electrophoresed and immunoblotting performed, we found that immunoreactive material increased dose-dependently with maximal expression reached at 1 μg of plasmid DNA, and that the protein could be detected using either an anti-MTCBP-1 antibody or an anti-turbo-GFP antibody (Figure S1A, in the supplemental data section). Fluorescence microscopy also enabled us to confirm the expression of the recombinant GFP-MTCBP-1 protein (Figure S1B, in the supplemental data section). Using confocal microscopy and fluorescence resonance energy transfer (FRET) analysis, we next looked for evidence of interaction between MTCBP-1 and MT1-MMP. We performed transient co-transfections with combinations of cDNA plasmids encoding Wt-MT1-MMP with GFP or with GFP-MTCBP-1, as well as co-transfections of cDNA plasmids encoding a cytoplasmic-deleted form of MT1-MMP (Δcyto-MT1-MMP) also with GFP or GFP-MTCBP-1 in U87 glioblastoma cells. As expected, the co-expression of GFP alone with either Wt-MT1-MMP or Δcyto-MT1-MMP, the latter being demonstrated by increased red labeling at the cell surface, did not show any significant changes in green fluorescence (Figure 2A and C). Interestingly, only co-expression of GFP-MTCBP-1 with Wt-MT1-MMP showed an increased green fluorescence after acceptor bleaching, suggesting that MTCBP-1 is recruited and interacts with the cytoplasmic domain of recombinant MT1-MMP (Figure 2B and D). To confirm FRET in the region of interest (ROI) only, measurements in the control region (blue rectangles) and in the ROI (yellow ellipse) were taken. None of the co-transfections revealed any significant changes in the green or red emission fluorescence in the control region, confirming that there are no diffused FRET signals outside the ROI (Figure 2E and F). FRET between GFP-MTCBP-1 and Wt-MT1-MMP (Figure 2H, open circles) is confirmed by the increase in 488 nm green fluorescence, while the other co-transfections didn't

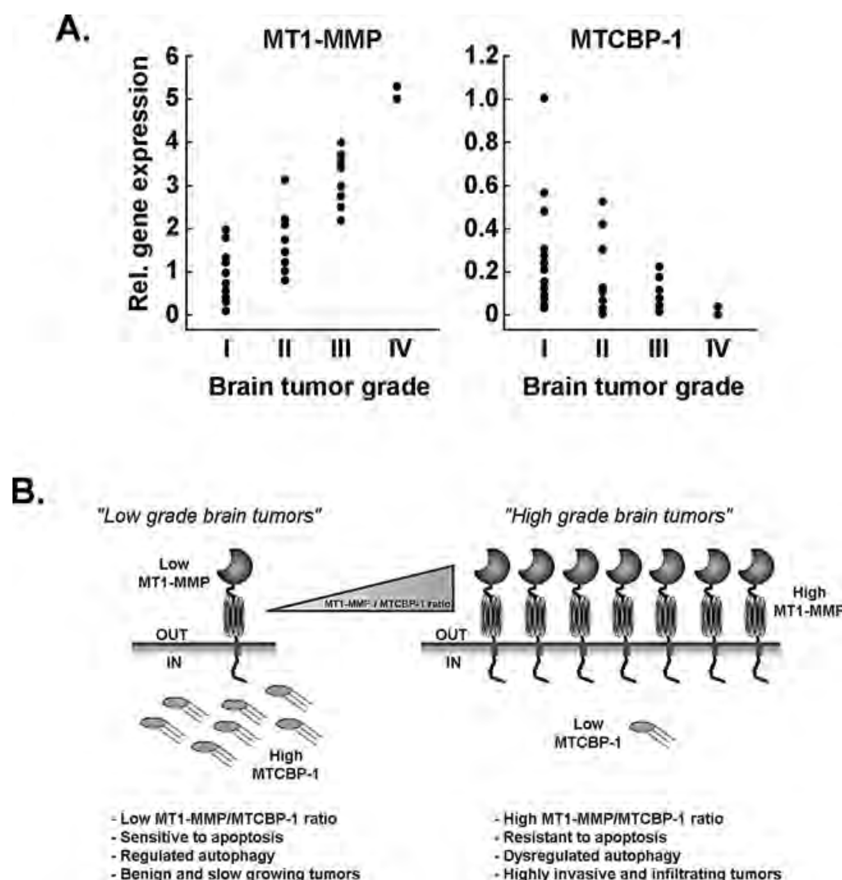

Figure 1. MT1-MMP and MTCBP-1 gene expression profiling in grades I–IV brain tumor tissues. A) TissueScan^{TM} cancer and normal tissue cDNA arrays from 43 clinical samples covering four stages of brain cancer were used to compare MT1-MMP and MTCBP-1 gene expression. Tissue cDNAs in each array were synthesized from high quality total RNAs of pathologist-verified tissues, normalized and validated with ß-actin and provided with clinical information for 18 WHO grade I, 11 WHO grade II, 10 WHO grade III, and two WHO grade IV brain tumors. B) Scheme summarizing the hypothesis that MT1-MMP-to-MTCBP-1 ratio may be indicative of high-grade tumors resistance to cell death. Low-grade astrocytic tumors are benign and slow growing tumors characterized by regulated autophagy, sensitivity to apoptosis and a low MT1-MMP/MTCBP-1 ratio. Transition to highly invasive and infiltrating high-grade tumors is characterized by dysregulated autophagy, resistance to apoptosis, and high MT1-MMP/MTCBP-1 ratios. We hypothesize new functions of MTCBP-1 in the regulation of MT1-MMP-mediated autophagy signaling, and suggest that the MT1-MMP-to-MTCBP-1 ratio may contribute to the acquisition of a cell death resistant phenotype in high-grade brain tumors.

show any changes in any fluorescence filter (Figure 2G and H). Finally, surface plasmon resonance (SPR) analyses were performed in order to monitor the interaction between a 20 amino acids peptide encompassing the entire MT1-MMP's cytoplasmic domain and MTCBP-1 immobilized on the sensor chip surface. Using a steady-state affinity model, we found that the peptide encoding the MT1-MMP cytoplasmic sequence interacted with MTCBP-1 with a K_D of 12.8 μM (Figure 2I, right panel). In a control experiment, injection of a scrambled 20 amino acids sequence peptide with similar physico-chemical properties (net charge at physiological pH, hydropathicity index, theoretical pI, molecular weight) resulted in a sensorgram showing no significant interaction (Figure 2I, left panel), strongly suggesting that the peptide derived form MT1-MMP's cytoplasmic domain interacted specifically but with moderate affinity with MTCBP-1. Co-immunoprecipitation experiments have also been performed using anti-MT1-MMP antibody to immunoprecipitate recombinant MTCBP-1 from MTCBP-1-transfected cells (Figure S2, in the supplemental data section).

MT1-MMP Overexpression Triggers Autophagy Events and is Abrogated By MTCBP-1

Given the close proximity of MTCBP-1 and MT1-MMP, we sought to functionally characterize the effects of their interaction by assessing the newly identified autophagy-mediated events triggered by MT1-MMP [13]. To this end, we overexpressed the full length Wt-MT1-MMP or the Δcyto-MT1-MMP forms alone or in combination with GFP or GFP-MTCBP-1 (Figure 3A, top three upper panels). Proper membrane location and functional validation of both MT1-MMP recombinant constructs was confirmed by gelatin zymography, where latent proMMP-2 activation into

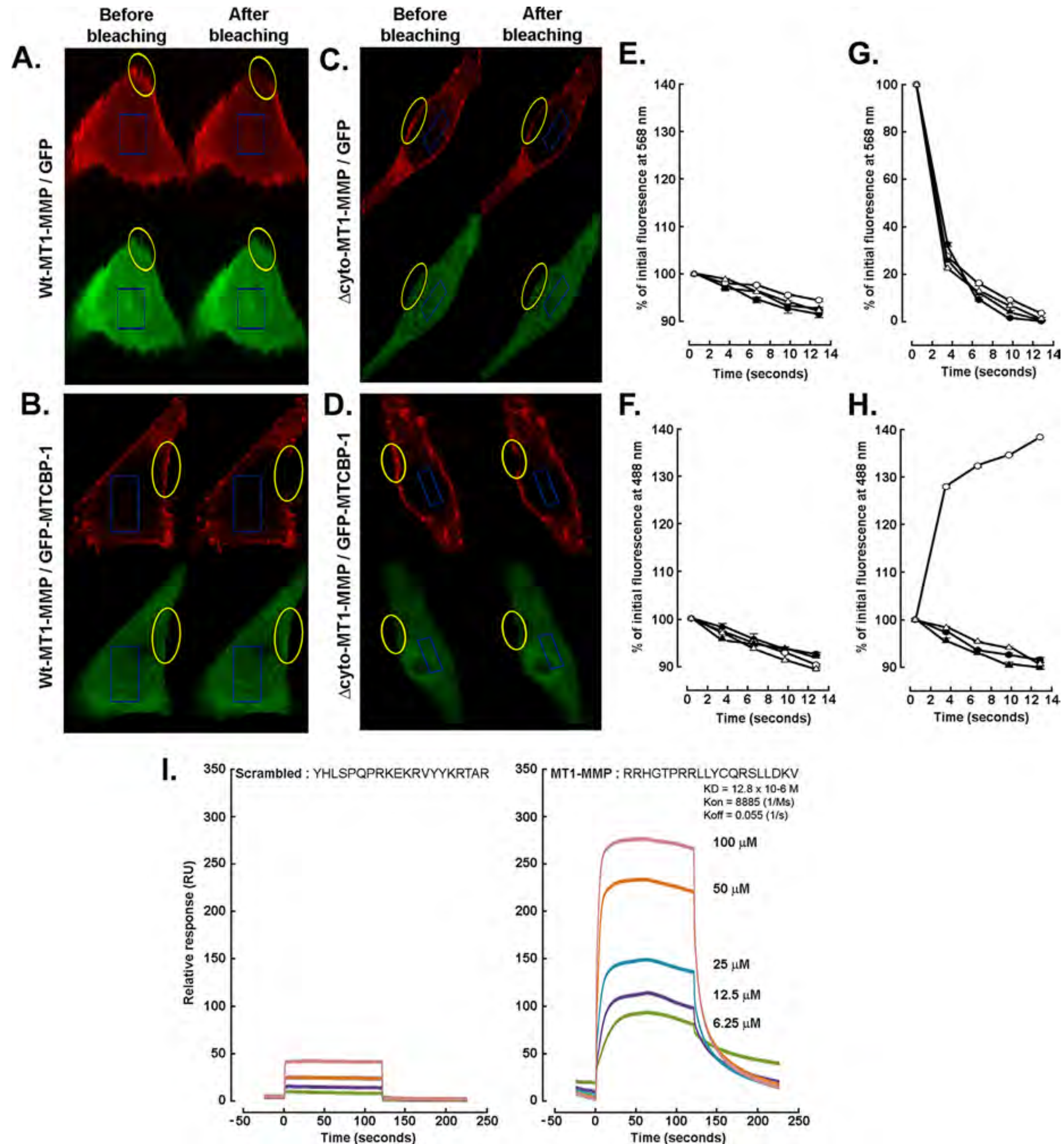

Figure 2. FRET-and SPR-based evidence of MTCBP-1 recruitment and interaction with MT1-MMP's cytoplasmic domain in U87 glioblastoma cells. U87 glioblastoma cells were transiently transfected with combinations of plasmid cDNAs encoding either a full length MT1-MMP recombinant protein (Wt-MT1-MMP), a cytoplasmic domain-deleted MT1-MMP recombinant protein (Δcyto-MT1-MMP), a GFP recombinant protein and a GFP-MTCBP-1 recombinant protein. Cells were prepared for FRET acceptor bleaching procedure as described in the Methods section. A–D) Photobleaching was performed at 561 nm and images were taken before and after acceptor bleaching in 488 nm green fluorescence and in 568 nm red fluorescence. Blue rectangles and yellow ellipses represent respectively the control region and the region of interest (ROI). E–H) Measurements of the FRET signals were taken for five cycles (see the Methods section) for the 488 nm green fluorescence and the 568 nm red fluorescence for the control region (E–F), and the ROI (G–H). The different co-transfection conditions are as follows: Δ (Wt-MT1-MMP/GFP), ▲ (Δcyto-MT1-MMP/GFP), ○ (Wt-MT1-MMP/GFP-MTCBP-1), and ● (Δcyto-MT1-MMP/GFP-MTCBP-1). Interactions of a peptide encompassing the entireMT1-MMP's intracellular cytoplasmic domain (I, right panel) and of a relative 20 amino acid scrambled sequence peptide (I, left panel) to MTCBP-1 were detected by surface plasmon resonance (SPR) analysis. MTCBP-1 was immobilized on a CM5 sensor chip and peptides were injected with concentrations ranging from 6.25 to 100 μM. The reference-subtracted response curves were analyzed using the BIAevaluation software package and fit to a steady-state affinity model.

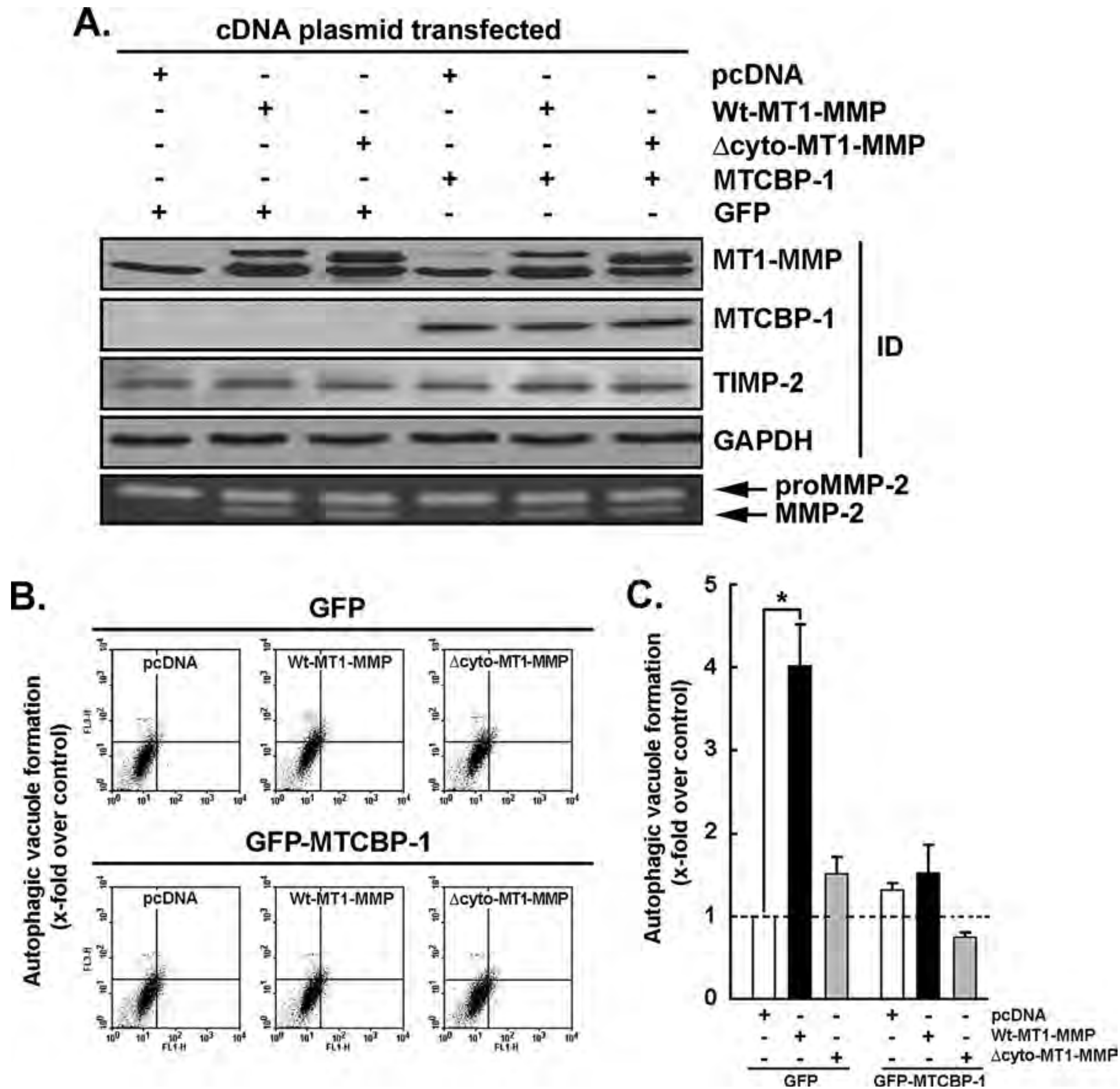

Figure 3. MT1-MMP overexpression triggers autophagy events and is abrogated by MTCBP-1. A) U87 glioblastoma cells were transiently transfected with combinations of plasmid cDNAs encoding either a full length MT1-MMP recombinant protein (Wt-MT1-MMP), a cytoplasmic domain-deleted MT1-MMP recombinant protein (Δcyto-MT1-MMP), a GFP recombinant protein, a GFP-MTCBP-1 recombinant protein, or with an empty pcDNA vector (Mock). Cell lysates as well as conditioned media were isolated as described in the Methods section in order to perform MT1-MMP, MTCBP-1, and GAPDH immunodetections or gelatin zymography to assess proMMP-2 activation status (cropped blots/gels are shown for the 4 panels and full-length blots/gels can be seen at supplemental Figure S2). B) Cells were stained with Acridine Orange, while acidic vacuole formation (FL3-H) and GFP-associated fluorescence (FL1-H) were examined by flow cytometry as described in the Methods section. C) A representative histogram of the extent of autophagic vacuole formation is shown.

MMP-2 was visualized (Figure 3A, lowest panel). Recombinant MTCBP-1 protein expression was also only observed in GFP-MTCBP-1-transfected cells but not in GFP-transfected cells (Figure 3A) and did not alter MT1-MMP's capacity to activate proMMP-2, in accordance with previous reports [29]. Given its role in the regulation of MT1-MMP auto-proteolytic activity, the tissue inhibitor of metalloproteinase-2 (TIMP-2) expression profiling was monitored and found not to be altered (Figure 3A). Moreover, the auto-proteolytic activity of MT1-MMP was also found unaltered given similar levels of the 43-kDa inactive fragment of MT1-MMP were assessed (Figure S3, in the supplemental data section). Altogether, this suggests that the MT1-MMP/TIMP-2 complex is unaltered and functional in any given conditions tested. When autophagy acidic vesicles formation was assessed (Figure 3B), we found that Acridine Orange staining significantly increased upon Wt-MT1-MMP overexpression, and this was abrogated by co-expressing MTCBP-1 (Figure 3C). Neither Δcyto-MT1-MMP nor MTCBP-1 overexpression triggered significant increases in Acridine Orange staining, confirming the absolute requirement for the intracellular domain of

MT1-MMP. Measurement of GFP-LC3 puncta formation in cells overexpressing a combination of Wt-MT1-MMP, Δcyto-MT1-MMP, GFP, or GFP-MTCBP-1 was also performed and visualized either by fluorescent microscopy (Figure 4A and B) or measured by a decrease in fluorescence by flow cytometry (Figure 4C). In support of the Acridine Orange staining, we found that puncta formation occurred only in those cells overexpressing Wt-MT1-MMP, and that overexpression of MTCBP-1 abrogated Wt-MT1-

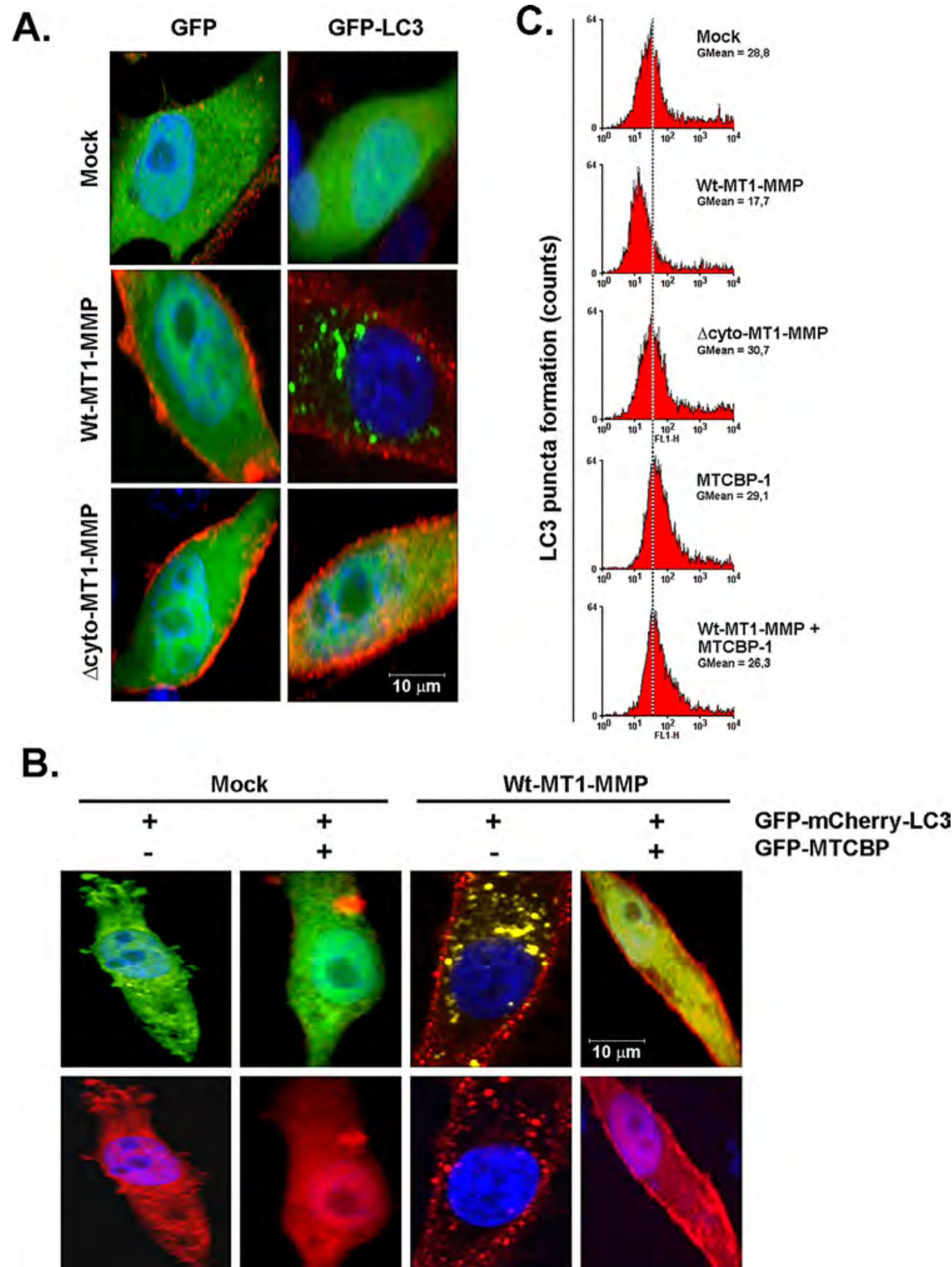

Figure 4. Overexpression of MTCBP-1 inhibits MT1-MMP-mediated induction of LC3 puncta. A) U87 glioblastoma cells were transiently co-transfected with cDNA plasmids encoding either a full length Wt-MT1-MMP, a cytoplasmic-deleted Δcyto-MT1-MMP, or empty pcDNA (Mock) in combination with either a plasmid encoding GFP or a plasmid cDNA encoding pEGFP-LC3. Samples were fixed 24 h later, immunostaining was performed for recombinant MT1-MMP expression at cell surface membranes (in red), and nuclei counterstained with Hoechst 33342 (in blue). Photomicrographs showing the intracellular distribution of exogenous LC3 (in green) were taken using a fluorescence microscope. Characteristic punctate of pEGFP-LC3 staining can be observed in Wt-MT1-MMP-transfected. B) Similar transient co-transfections were performed with combinations of plasmid cDNAs encoding Wt-MT1-MMP, GFP-MTCBP-1, or GFP-mCherry-LC3. C) Puncta formation was also assessed by flow cytometry and reflected by a decrease in the geometric mean of fluorescence as compared to the Mock condition.

MMP-induced LC3 puncta (Figure 4B), and asreflected by a decrease in the geometric mean (GMean) of fluorescence (Figure 4C). The quantification of total punctae/cells has also been performed for all the experimental conditions and the analysis provided in the supplemental figure S4. Altogether, these data suggest that the balance between MT1-MMP-mediated signaling and MTCBP-1 expression may contribute to the autophagic index of the cancer cell.

Concanavalin-A-induced Autophagy Requires MT1-MMP and is Abrogated By MTCBP-1 Overexpression

Concanavalin-A (ConA) is a lectin well documented to trigger MT1-MMP expression and to induce autophagy [31,32]. We, therefore, evaluated the impact of MT1-MMP gene silencing in control and ConA-treated cells. Effective silencing of MT1-MMP altered ConA's capacity to trigger proMMP-2 activation (Figure 5A) confirming its involvement in ConA functions, and was further found to abrogate ConA induction of autophagic vacuole formation (Figure 5B and C). We next assessed the impact of MTCBP-1 overexpression upon ConA's capacity to induce autophagic acidic vesicles. Cells were, therefore, transiently-transfected with cDNA plasmids expressing either GFP or GFP-MTCBP-1, then treated or not with various concentrations of ConA (Figure 5D). We found induction of autophagic acidic vesicles in all cells which had not incorporated GFP (Figure 5E, upper panel, closed circles) or which had not incorporated GFP-MTCBP-1 (Figure 5E, upper panel, and open circles). On the other hand, while fluorescent cells which expressed GFP were still found sensitive to autophagic acidic vesicle formation (Figure 5E, lower panel, closed circles), those which had overexpressed GFP-MTCBP-1 became resistant to ConA-mediated induction of autophagy (Figure 5E, lower panel and open circles). Interestingly, several main regulators of autophagy (LC3, ATG3, ATG5, ATG12, ATG16L1, BNIP3), which expression was tested by qRT-PCR, were not found induced by ConA, neither affected by MTCBP-1 in U251 cells, an alternate glioblastoma cell line which gene and protein profiles differ from those of U87 cells (data not shown).

DISCUSSION

Resistance to apoptotic cell death, a hallmark of most cancers, has driven the search for novel targets in cancer therapy. The autophagy pathway is among such targets currently being explored in multiple cancers including gliomas, and is a promising avenue for further therapeutic development [33]. A better understanding of autophagy regulation and of autophagy-inducing mechanisms is therefore an emerging area of interest in brain cancer research. Given that progression of astrocytic tumors into more aggressive and chemoresistant phenotypes is, in part, related to their decreased autophagic capacity [3], appropriate modulation of autophagy may therefore sensitize tumor cells to anticancer therapy [34]. In this study, we highlight novel MT1-MMP-to-MTCBP-1-mediated mechanisms, showing enhanced MT1-MMP expression in high-grade tumors and enhanced MTCBP-1 expression in low-grade tumors (Fig.1A). Since we have also shown that MTCBP-1 abrogates MT1-MMP-mediated signaling that triggered autophagy, our data suggest that high-grade brain tumors may possibly exhibit unregulated MT1-MMP-mediated autophagy processes that may enable these cancers to escape cell death-inducing therapeutic modalities that trigger, in part, cell autophagy. Together with the fact that cellular stresses such as hypoxia induce both autophagy [35] and MT1-MMP [36,37], these adaptive mechanisms may help established tumors to survive [38].

Our study further sheds light on the dual pro-apoptotic and autophagic capacities of biomarkers, such as MT1-MMP, that exhibit both properties. For instance, small-interfering RNA-mediated gene silencing of CHOP/GADD153 resulted in increased cell viability, decreased upregulation of LC3II and of cleaved caspase-3, and inhibition of apoptosis and autophagy [8]. In support of this, the dual role of MT1-MMP in apoptosis as well as in autophagy was shown through direct and indirect evidence [11,13,16,20]. Beyond the evidence that MT1-MMP bears combined pro-apoptotic and pro-autophagic transducing properties, our study highlights an unreported role for MTCBP-1, in addition to its ability to inhibit MT1-MMP-mediated cell invasion [29], in abrogating MT1-MMP's ability to trigger autophagy. Given that MTCBP-1 expression is low in highly invasive tumors, this observation strengthens the concept that high-grade brain tumors possess cell death-escaping properties, possibly through dysregulated autophagy. In support of this, an oligonucleotide microarray of metastasis-related genes in genistein-treated HCC1395 breast cancer cells demonstrated upregulated expression of MTCBP-1 [39]. Given that genistein can induce both apoptotic and autophagic cell death [40]; it has the potential to circumvent chemoresistance due to alterations in apoptotic signaling. Whereas a variety of other food components including vitamin D, selenium, curcumin, and resveratrol have also been shown to stimulate autophagy vacuolization [41], it remains difficult to determine if induction of autophagy is a pro-tumorigenic or antitumorigenic response.

While the Kd constants, as determined by SPR, may appear insufficient to infer efficient interaction between MTCBP1 and MT1-MMP in vivo, we still provide evidence for MTCBP-1 and MT1-MMP interaction using immunoprecipitation approaches. The SPR-derived constants most likely relate to the fact that the peptide derived from MT1-MMP does not necessarily adopt a precise and optimal secondary structure in the absence of the remaining protein. Moreover, the interaction observed in vivo between

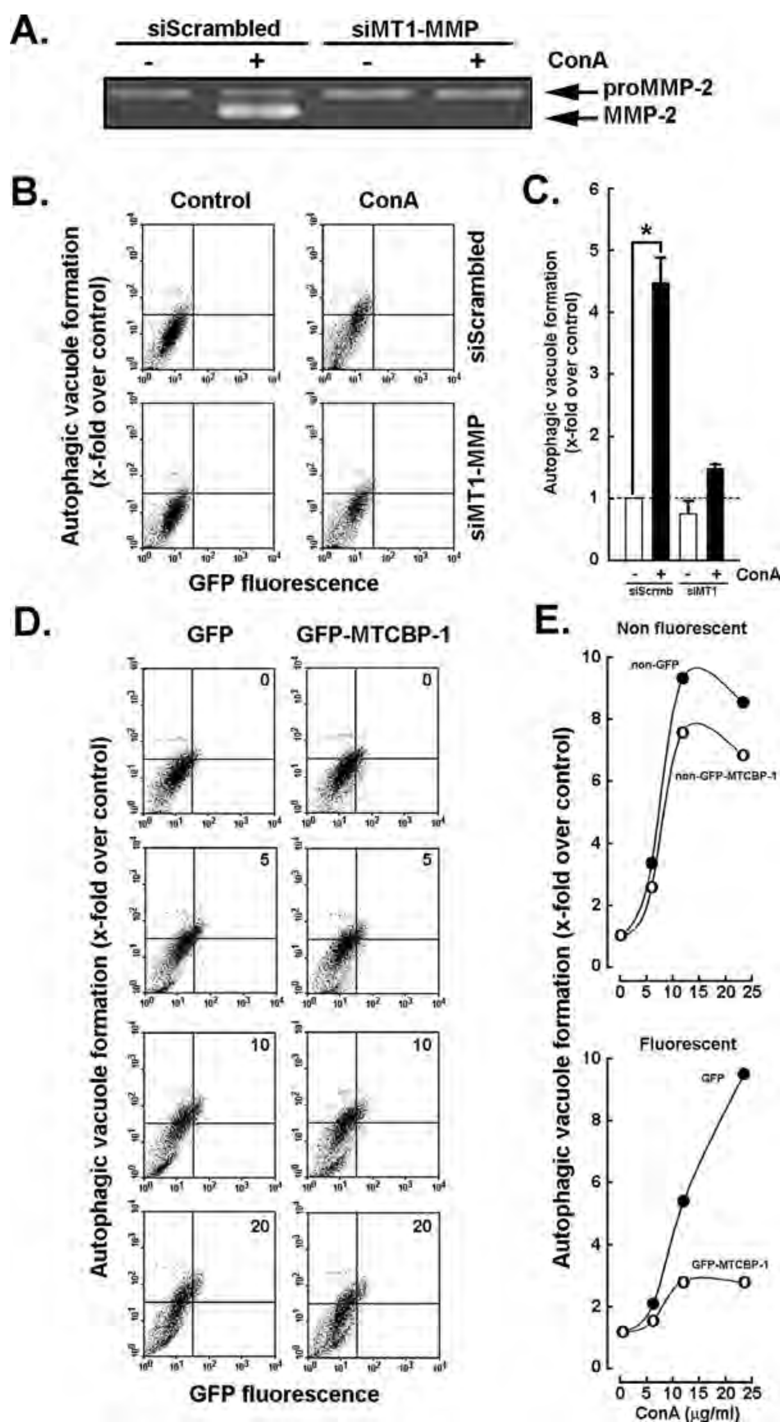

Figure 5. Concanavalin-A-induced autophagy requires MT1-MMP and is abrogated by MTCBP-1 overexpression. (A) Conditioned media was isolated from serum-starved U87 cells transiently transfected with either siScrambled or siMT1-MMP siRNAs as described in the Methods section in order to assess ConA-mediated proMMP-2 activation. (B) Gene silencing was performed with either siScrambled or siMT1-MMP in U87 glioblastoma cells. Cells were then serum-starved in the presence or absence of 30 μg/ml ConA for 24 h, stained with Acridine Orange and acidic vacuole formation (FL3-H) examined by flow cytometry as described in the Methods section. (C) A representative histogram of the extent of autophagic vacuole formation is shown. (D) U87 glioblastoma cells were transiently transfected with cDNA plasmids encoding either recombinant GFP or recombinant GFP-MTCBP-1, then treated with various concentrations of Concanavalin-A (ConA concentrations in μg/ml indicated in the upper right quadrants). Cells were then stained with Acridine Orange and acidic vacuole formation (FL3-H) examined by flow cytometry as described in the Methods section. GFP-positive cells were assessed in FL1-H upper right and lower right quadrants, while acidic vacuole formation was assessed in upper left FL3-H quadrants. (E) Representative plots of acidic vacuole formation in GFP-negative (non-fluorescent) cells that remained untransfected are shown in the upper panel from GFP-transfected (closed circles) and from GFP-MTCBP-1-transfected (open circles) cells. Acidic vacuole formation was also monitored in GFP-positive cells shown in the lower panel from GFP-transfected (closed circles) and from GFP-MTCBP-1-transfected (open circles) cells.

MTCBP1 and MT1-MMP may probably also mostly occur in the vicinity of the plasma membrane, a somewhat hydrophobic environment. In sharp contrast, the SPR assays are required to be performed in aqueous solution. These conditions may, therefore, prevent the MT1-MMP-derived peptide to adopt a precise 3D conformation allowing for perfect positioning of the residues side chains involved in the interaction. Nevertheless, our current SPR data show that a short peptide derived from MT1-MMP can interact specifically with a relatively good affinity with MTCBP1, in contrast to a scrambled sequence peptide with similar net charge and molecular weight.

Our study also supports the possible development of therapeutic strategies targeting the transducing events mediated by MT1-MMP's intracellular domain. Structure-function studies have already confirmed that MT1-MMP induces the expression of biomarkers such as cyclooxygenase-2 in inflammation [16,42], BNIP-3 in autophagy [13], endothelial tubulogenesis [43], and apoptosis [20]. ConA was also confirmed to trigger autophagy [32] and to, in part, require MT1-MMP-mediated signaling (this study). Interestingly, inhibition of the intracellular domain Tyr573 phosphorylation of MT1-MMP by either genistein [44,45] or through site-directed mutagenesis approaches [21], inhibited ConA-mediated or recombinant MT1-MMP's transducing functions. Furthermore, ConA-induced signaling cascades, in which MT1-MMP served as an intermediate, were also recently found to be triggered and highlighted a role for transcription factors including NF-κB [23], STAT3 [46], and HIF-1α [36]. Pharmacological strategies targeting MT1-MMP functions recently included the use of tetra- and hexavalent mannosides which inhibited the pro-apoptotic effects of ConA [47], epigallocatechin-3-gallate which inhibited colony-stimulating factor-2, and -3 expression [21]. Whether these agents also modulate MT1-MMP-mediated autophagy remains to be explored. Finally, gene silencing of p130cas, another MT1-MMP interacting partner in endothelial cells [27] whose expression is associated with poor clinical outcome in human ovarian carcinoma [48], decreased tumor growth through stimulation of combined apoptotic and autophagic cell death [48].

In conclusion, our data suggest that the signaling balance that is involved in the modulation of the autophagy index is crucial in dictating the survival or death decision that cells take during metabolic adaptation and tumor progression [49]. The MT1-

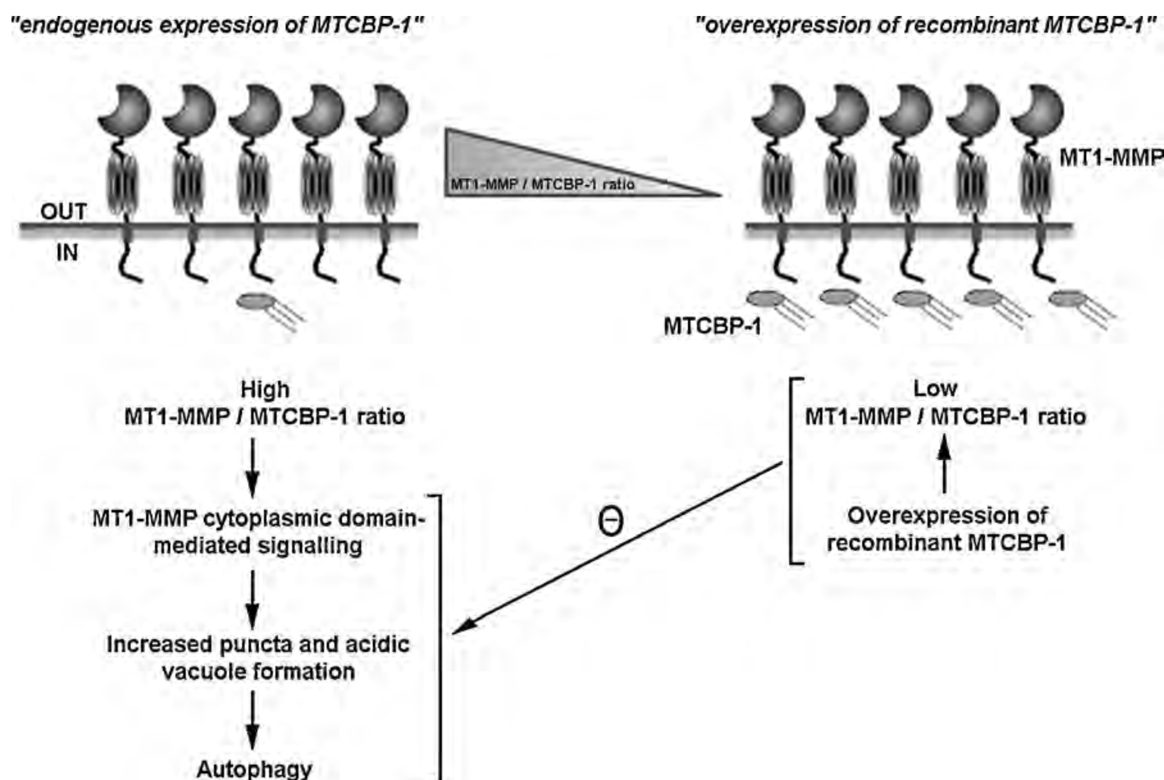

Figure 6. Overexpression of recombinant MTCBP-1 reduces the MT1-MMP/MTCBP-1 ratio and regulates MT1-MMP-mediated autophagy. Summarized scheme of the possible mechanism by which MTCBP-1 may regulate MT1-MMP-mediated autophagy. Endogenous expression in high grade brain tumours of MT1-MMP is elevated, while that of MTCBP-1 is low (Figure 1A). The consequent high MT1-MMP/MTCBP-1 ratio condition, therefore, favors disregulated MT1-MMP-mediated autophagy and potential resistance to cell death. On the other hand, overexpression of recombinant MTCBP-1 lowers the MT1-MMP/MTCBP-1 ratio which more closely approximates that condition observed in low-grade brain tumors (Figure 1A). Our data show that MTCBP-1 functionally controls (−) MT1-MMP's intracellular-mediated induction of processes that characterize autophagy, and may control brain cancer cells invasive phenotype.

MMP-to-MTCBP-1 expression ratio may be one of these "go-no-go" decision making processes (schematized in Figure 6). Although our study does not exclude the contribution of a potential yet unidentified third partner, our FRET analysis data allows us to confidently confirm the molecular proximity of MTCBP-1 and MT1-MMP intracellular domain at angstrom distances ranging from 10 to 100 Å [50], while our SPR analysis data evidences specific interaction between MTCBP-1 and the intracellular MT1-MMP domain. We believe that identification and functional characterization of intracellular MT1-MMP binding partners, such as MTCBP-1, may enable the development of future therapeutic strategies aimed at exploiting intracellular MT1-MMP transducing functions which contribute to the invasive and chemoresistant phenotype of glioblastoma.

ACKNOWLEDGEMENTS

J.P. is a Natural Sciences and Engineering Research Council of Canada (NSERC) awardee. This study was funded by a grant from the NSERC [#288249] to B.A.

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