Human MT6-Matrix Metalloproteinase: Identification, Progelatinase A Activation, and Expression in Brain Tumors

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Abstract

The localization of proteolytic enzymes at the cell surface is a widely used strategy for facilitating tumor invasion. In this study, we have cloned a new member of the membrane-type subfamily of matrix metalloproteinases (MT-MMPs), a group of enzymes associated with tumor progression. The cloned cDNA encodes a protein of 562 amino acids with a domain organization similar to that of other MT-MMPs, including a prodomain with a cysteine switch, a catalytic domain with the zinc-binding site, a hemopexin-like domain, and a COOH-terminal extension rich in hydrophobic residues. The predicted protein sequence also contains a short insertion of basic residues located between the propeptide and the catalytic domain and involved in the proteolytic activation of MT-MMPs by furin-like enzymes. Furthermore, immunofluorescence and Western blot analysis of COS-7 cells transfected with the isolated cDNA revealed that the encoded protein is localized at the cell surface. Based on these properties, this novel human matrix metalloproteinase has been called MT6-MMP because it is the sixth identified member of this subfamily of matrix metalloproteinase. Cotransfection of expression plasmids encoding MT6-MMP and progelatinase A resulted in activation of COS-7-secreted progelatinase A, as demonstrated by gelatin zymography. In contrast, transfection of progelatinase A cDNA alone did not lead to the activation of the proenzyme. Northern blot analysis of polyadenylated RNAs isolated from human tissues demonstrated that MT6-MMP is predominantly expressed in leukocytes, lung, and spleen. MT6-MMP was also detected at high levels in SW480 colon carcinoma cells as well as in some anaplastic astrocytomas and glioblastomas, but not in normal colon or brain or in meningiomas. On the basis of these results, we propose that MT6-MMP may facilitate tumor progression through its ability to activate progelatinase A at the membrane of cells from colon carcinomas or brain tumors.

Introduction

The MMPs are a family of structurally related endopeptidases that mediate the degradation of the different protein components of the extracellular matrix and basement membranes. These proteolytic enzymes have been involved in the remodeling of connective tissue taking place in many physiological processes such as embryonic growth and development, uterine involution, ovulation, and wound healing (1). Moreover, abnormal production of these proteinases may take place in many physiological processes such as embryonic development, wound healing, and to regulate neovascularization processes by acting as pericellular fibrinolysins (13). Finally, it has been described that MT1-MMP enables invasive migration of glioma cells due to its ability to digest central nervous system myelin-inhibitory proteins (14).

Because of the importance of MT-MMPs in both normal and tumor processes, we have undertaken studies to try to identify novel members of this family produced by human tissues. In this study, we describe the isolation of a cDNA coding for a novel human MT-MMP that has been called MT6-MMP. We also perform an analysis of its potential role as a progelatinase A activator at the cell surface. Finally, we report an expression analysis of MT6-MMP in normal and tumor tissues, including a comparative study of the expression of this novel enzyme and other MT-MMPs in brain tumors.

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3 The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; HA, hemagglutinin; pol(Ala)7, polyadenylated; FMF, familial Mediterranean fever.

Materials and Methods

Materials. Restriction endonucleases and other reagents used for molecular cloning were obtained from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were synthesized in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with [32P]dCTP (3000 Ci/mmol) purchased from Amersham International (Buckinghamshire, United Kingdom) using a commercial random priming kit from the same company. A human fetal liver cDNA library constructed at the amino acid sequence level (1, 3). According to structural and functional characteristics, human MMPs can be classified into five different subfamilies: (a) collagenases; (b) gelatinases; (c) stromelysins; (d) MT-MMPs; and (e) other MMPs.

In recent years, the number of known members of the MMP family has grown rapidly, mainly due to the discovery of a series of membrane-bound enzymes belonging to the MT-MMP subfamily (3–7). To date, this MMP subfamily is composed of five members that are structurally characterized by having a hydrophobic region downstream of the hemopexin-like domain present in most MMPs. MT-MMPs have raised additional interest for their role as cell surface activators of progelatinase A, an enzyme widely assumed to play an important role in the invasive phenotype of tumor cells (2). Progelatinase A activation by MT-MMPs involves a two-step mechanism, analogous to that operating in other MMPs. The initial cleavage is mediated by direct action of MT-MMPs in a region of the progelatinase A propeptide domain that is exposed to solvent, whereas the secondary cleavage is autoproteolytic. This activation process appears to involve the formation of a trinolecular complex between progelatinase A, MT1-MMP, and tissue inhibitor of metalloproteinase 2 that acts as a concentration mechanism on the cell surface that is crucial for the efficiency of activation (8). More recently, MT-MMPs have been reported to participate in the activation of other MMPs such as procollagenase 3 that are also associated with a number of human malignant tumors (9–11). Moreover, these membrane proteases have the ability to directly degrade a variety of extracellular matrix proteins such as vitronectin, fibronectin, fibrillar collagens, or aggrecan (12) and to regulate neovascularization processes by acting as pericellular fibrinolysins (13).
in Agt10 and Northern blots containing poly(A)+ RNAs from different human tissues and cell lines were obtained from Clontech (Palo Alto, CA).

Screening of a Human Fetal Liver cDNA Library. A computer search of the different human DNA sequence databases for entries with similarity to previously described MMPs led us to find a genomic sequence identified by Bernot et al. (15) in the course of their studies directed at cloning the gene responsible for the inherited disease called FPF. To isolate a full-length cDNA corresponding to the putative MMP identified by Bernot et al. (15), we first performed PCR amplification of DNAs prepared from cDNA libraries available in our laboratory, using two primers (5′-TACCGTCTGAGCGGCAAGCTGGTCG-3′ and 5′-CTCATCGTCAAAAGTGATGTTCC-3′) derived from the genomic sequence. The PCR reaction was carried out in a GeneAmp 2400 PCR system from Perkin-Elmer/Cetus (Norwalk, CT) for 35 cycles of denaturation (94°C, 15 s), annealing (62°C, 15 s), and extension (72°C, 15 s).

A DNA fragment of 315 bp was amplified from human fetal liver cDNA. This PCR-generated product was phosphorylated with T4 polynucleotide kinase and cloned into a EcoRV-cut pBluescript vector. The cloned cDNA was then excised from the vector, radiolabeled, and used to screen a human fetal liver cDNA library according to standard procedures. After plaque purification, the cloned inserts were excised by EcoRI digestion, and the resulting fragments were subcloned into pBluescript.

Nucleotide Sequence Analysis. DNA fragments of interest were cloned into pBluescript and sequenced by the dyeodeoxy chain termination method using the Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH). All nucleotides were identified in both strands. Computer analysis of DNA and protein sequences was performed with the Genetics Computer Group software package of the University of Wisconsin Genetics Computer Group.

Northern Blot Analysis. Nylon filters containing 2 μg of poly (A)+ RNA of human tissues or tumor cell lines or 10 μg of total RNA from brain tumors were prehybridized at 42°C for 3 h in 50% formamide, 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH2PO4, and 1 mM EDTA (pH 7.4)), 10× Denhardt’s solution, 2% SDS, and 100 μg/ml denatured herring sperm DNA and then hybridized for 20 h under the same conditions with a probe specific for MT6-MMP. Filters were washed with 0.1× SSC, 0.1% SDS for 2 h at 30°C and exposed to autoradiography. RNA integrity and equal loading were assessed by hybridization with actin or 18S RNA probes.

Gelatin Zymography. Samples were mixed with SDS sample buffer in the absence of a reducing agent and subjected to electrophoresis without boiling on 10% acrylamide gels containing 0.2% gelatin. Gels were run at 40 mA, washed in 2.5% Triton X-100 for 3 h, and incubated at 37°C for 20 h in reaction buffer [20 mM Tris-HCl (pH 7.4), 5 mM CaCl2]. After incubation, gels were stained with Coomassie Brilliant Blue R-250. The gelatinolytic activities were detected as clear bands in the blue background.

Construction of Eukaryotic Expression Vectors, Cell Transfection, and Immunolocalization. A modified MT6-MMP cDNA encoding an open reading frame comprising amino acids Met1-Arg562 and containing an internal 24-bp sequence coding for the HA epitope of human influenza virus was generated by PCR and cloned in the EcoRV site of a pcDNA3 vector. Thus, the resulting MT6-MMP protein was HA-tagged between the hemopexin domain and the COOH-terminal extension rich in hydrophobic residues present in this protein. Expression plasmids for progelatinase A were kindly provided by Drs. G. Murthy and V. Knüper (University of East Anglia, Norwich, United Kingdom). COS-7 cells were transfected with 1 μg of plasmid DNA, using Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Forty-eight h after transfection, cells were fixed for 10 min in cold 4% paraformaldehyde in PBS, washed in PBS, and incubated for 10 min in 0.2% Triton X-100 in PBS. Fluorescence detection was performed by incubating the slides with monoclonal antibody 12CA5 (Boehringer Mannheim) against HA (diluted 1:2500), followed by another incubation with goat antimouse fluoresceinated antibody (diluted 1:50). After washing in PBS, slides were mounted with vectashield (Vector, Burlingame, CA) and observed using a BioRad confocal laser microscope. COS-7 extracts were also obtained for Western blot analysis of the MT6-MMP-HA protein.

Western Blot Analysis. COS-7 cells were transiently transfected with the pcDNA3 MT6-MMP-HA plasmid as described previously. Cells were rinsed in PBS and scraped from the plates. Membrane fractions were prepared essentially following the procedure described by Zucker et al. (16). Extracts were separated by SDS-PAGE, analyzed by Western blotting with an anti-HA monoclonal antibody, and detected with an enhanced chemiluminescence kit (Amersham).

Results

Isolation and Characterization of a Human Fetal Liver cDNA Coding for a New MT-MMP. Recently, Bernot et al. (15) have reported the discovery of a genomic sequence located in the region containing the FMP gene and potentially encoding a member of the MMP family of proteolytic enzymes. To further examine this possibility and to define the precise structure of this putative MMP, studies were undertaken to isolate a full-length cDNA encoding this proteinase. To do that, we first prepared a probe specific for this enzyme by PCR amplification of total λ-phage DNA obtained from a human fetal liver cDNA library. The PCR-amplified product was cloned, and its identity was confirmed by nucleotide sequencing. The cloned fragment was then radiolabeled and used as a probe to screen the same fetal liver cDNA library used for the previous PCR amplification experiment. Upon screening of approximately 1 × 105 plaque-forming units, two positive clones named 1.3.5 and 3.10.2 were identified and characterized. DNA was isolated from these positive clones, and their nucleotide sequence was determined by standard procedures. This sequence analysis revealed that one of these clones (3.10.2) had an insert of 1 kb, which was entirely contained in the 3-kb coding sequence determined for clone 1.3.5. A detailed analysis comparing the sequence obtained for the largest clone with those corresponding to other MMPs suggested that it contained the entire coding sequence for an archetypical MMP. Computer analysis of the obtained sequence (European Molecular Biology Laboratory accession number AJ 239053; Fig. 1) revealed an open reading frame coding for a protein of 562 amino acids with a predicted molecular mass of 63 kDa.

Pairwise comparisons for sequence similarities between the identified amino acid sequence (Fig. 1) and those determined for other human MMPs showed that the maximum percentage of identities (48%) was found with MT4-MMP. In addition, the deduced amino acid sequence from the human cDNA isolated in this work displays a number of structural features characteristic of MMPs. Thus, it contains a stretch of hydrophobic amino acids close to the initial methionine that likely corresponds to the signal peptide that targets these proteinases to the secretory pathway. Computer analysis using the algorithm developed by Nielsen et al. (17) revealed that the Ala21-Pro28 peptide bond in the sequence Arg-Ala-Pro-Lys presented the highest probability to be the processing site of the leader sequence found in this MMP. Assuming that the signal peptidase cleaves at this position, the resulting protein would be composed of 541 residues. The multiple alignment of this sequence with those determined for other MMPs also allows us to identify a prodomain region with the Cys residue (at position 90) essential for maintaining the latency of these enzymes, a catalytic domain of about 170 residues including the consensus sequence HEXGHXXGXXH involved in zinc binding, and a fragment of approximately 200 amino acids with sequence similarity to hemopexin. In addition, this novel sequence contains a 10-amino acid insertion located between the propeptide and catalytic domains that ends in a stretch of basic amino acid residues. This insertion is present in all MT-MMPs and has been proposed to mediate intracellular activation of these proteinases by furin-like enzymes. Finally, the identified sequence contains a COOH-terminal extension rich in hydrophobic residues that could correspond to the transmembrane domain present in the different MT-MMPs characterized to date. According to these structural features, we suggest that the isolated fetal liver cDNA codes for a novel human MT-MMP that we propose calling MT6-MMP. Nevertheless, this protein presents a structural peculiarity shared with MT4-MMP, consistent in the absence of the short cytoplasmic tail present in the remaining MT-MMPs. This fact,
together with the above-mentioned data indicating that the highest percentage of MT6-MMP amino acid sequence identities was found with MT4-MMP, suggests that both proteins may form a subgroup within the MT-MMP group of membrane metalloproteases. On the other hand, following the nomenclature system proposed for vertebrate MMPs, we would assign number 25 to this novel MT-MMP, because MMP-24 corresponds to MT5-MMP (3). It should be noted that although Bernot et al. (15) assigned number 20 to the putative MMP derived from the genomic sequence identified by them, this number had been previously used to designate enamelysin, a MMP family member produced by odontoblastic cells (18). The discovery of some differences between the cDNA sequence identified herein and data derived from genomic clones is also remarkable (15). These differences were likely due to errors in the sequence of genomic clones because cDNA sequences reported in this study were confirmed in a number of clones from different sources. We also observed that some of these differences were located at the region coding for the COOH-terminal extension characteristic of MT-MMPs and originated a series of open reading frame changes that hampered the identification of the encoded MMP as a member of the MT-MMP subfamily on the basis of data derived from the available genomic sequences.

**MT6-MMP Localization at the Cell Surface.**

To provide additional support for the subcellular distribution of MT6-MMP, COS-7 cells were transfected with pcDNA3 MT6-MMP-HA, a construct containing the HA epitope at the end of the hemopexin domain of MT6-MMP. Transfected cells were then analyzed by immunofluorescence with a mouse monoclonal antibody (12CA5) specific for this viral epitope. As shown in Fig. 2A, a fluorescence pattern surrounding the cell was visualized in a serial optical section obtained by the confocal microscope. This observation provides strong evidence that human MT6-MMP is a membrane-bound MMP, meeting the requirement for an activator of progelatinase A at the cell surface. To further verify the membrane localization of MT6-MMP, cell lysates from COS-7 cells transfected with MT6-MMP-HA were analyzed by SDS-PAGE, followed by Western blotting detection with anti-HA monoclonal antibody. A band of about 63 kDa corresponding to MT6-MMP was detected in the membrane enriched fractions, but not in the cytoplasmic fraction or in the conditioned medium, reinforcing its membrane-bound localization (Fig. 2B; data not shown).

**Characterization of the Catalytic Activity of MT6-MMP.**

Because MT6-MMP has a number of structural features characteristic of MT-MMPs, we next evaluated its potential ability to induce activation of progelatinase A, a functional characteristic of members of this MMP subfamily. To examine this question, we first cotransfected expression plasmids encoding MT6-MMP and progelatinase A into COS-7 cells transfected with MT6-MMP-HA were analyzed by SDS-PAGE, followed by Western blotting detection with anti-HA monoclonal antibody. A band of about 63 kDa corresponding to MT6-MMP was detected in the membrane enriched fractions, but not in the cytoplasmic fraction or in the conditioned medium, reinforcing its membrane-bound localization (Fig. 2B; data not shown).

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expression plasmid for progelatinase A alone did not result in the activation of the proenzyme (Fig. 3, Lane 4). It is noteworthy that when an expression plasmid for MT1-MMP was cotransfected with progelatinase A cDNA, the activation rate of progelatinase A secreted by COS-7 cells was significantly higher than that observed after cotransfection of MT6-MMP cDNA (Fig. 3, Lanes 5 and 7). Taken together, these functional analyses suggest that the cloned human fetal liver cDNA for MT6-MMP encodes an archetypical MT-MMP with the ability to mediate the activation of progelatinase A. Nevertheless, the relatively low efficiency of MT6-MMP in this activation process when compared to MT1-MMP suggests that this novel membrane protease may also participate in the cell surface activation of other substrates. Preliminary studies have shown that MT6-MMP has no significant effect on the shedding of HER2, pro-transforming growth factor-α, or heparin-binding EGF-like growth factor (data not shown). Additional studies will be required to identify the nature of putative substrates other than pro-gelatinase A that could be target of the proteolytic activity of this novel membrane-bound enzyme.

Analysis of Expression of MT6-MMP in Human Tissues, Cancer Cell Lines, and Brain Tumors. Analysis of expression of MT6-MMP in human tissues was performed by hybridization of Northern blots containing a variety of human poly(A)+ RNAs, with a specific probe for this protease derived from the 5′-end region of the isolated cDNA (from Met1 to Glu212). As shown in Fig. 4, a transcript of about 4 kb was predominantly detected in leukocytes, lung, and spleen. A second transcript of about 6 kb was also clearly detected in leukocytes and spleen. Interestingly, when a full-length probe was used, a transcript of about 1.5 kb was detected in the heart as well as in other human tissues including the colon, intestine, ovary, and prostate (data not shown). This mRNA transcript likely corresponds to a second transcriptional unit identified by Bernot et al. (15) in this region of the human genome that overlaps with that of MT6-MMP. This additional transcriptional unit derives from the strand opposite to that encoding MT6-MMP and contains stop codons in all three frames (15). We also examined the possibility that MT6-MMP could be expressed by human cancer cells from different sources, as already shown for other MMPs. To this purpose, we hybridized a Northern blot containing poly(A)+ RNAs extracted from different cancer cell lines with the same MT6-MMP probe as described above. As shown in Fig. 4, the 4-kb MT6-MMP transcript was clearly identified in colorectal adenocarcinoma SW480 cells. Reverse transcription-PCR analysis also revealed the expression of MT6-MMP transcripts in some primary colon carcinomas, but not in the adjacent normal mucosa (data not shown). Finally, we examined the possibility that MT6-MMP could be overexpressed in brain tumors as described for other members of the family (3, 19). As can be seen in Fig. 5, MT6-MMP transcripts were detected in some anaplastic astrocytomas and glioblastomas, but not in normal brain or in meningiomas. Interestingly, when the same filter was hybridized with probes specific for other MT-MMPs, including MT1-MMP, MT4-MMP, and MT5-MMP, different patterns of expression were observed, with MT1-MMP being more restricted in its tumor expression than other MT-MMPs (Fig. 5). These results suggest that overexpression of some of the diverse members of the MT-MMP family by brain tumors may be a general strategy for the cell surface focusing of degradative processes presumably linked to the progression of these tumors.

Discussion

The identification of expanding roles for MMPs in a wide variety of normal and tumor processes has stimulated the search for new family members by using a variety of cloning strategies. Recently, different groups have used PCR-based methods with degenerate oligonucleotides or expressed sequence tag-based approaches for cloning several human MMPs from both normal and tumor tissues (3–7, 9). In this study, we have used information derived from large-scale genome sequencing projects to characterize a new human MMP, MT6-MMP,
belonging to the membrane-type subfamily. Pairwise comparisons for structural similarities between the identified amino acid sequence for this protein and those for the remaining MMPs confirmed that it contains all protein domains characteristic of MMPs, including a signal sequence, a propeptide, a catalytic domain with the Zn-binding site, a hinge region, and a COOH-terminal hemopexin-like domain organized in four recognizable repeats. In addition, this sequence contains all features characteristic of members belonging to the MT-MMP subfamily, including the furin-like cleavage site at the end of the propeptide domain and the COOH-terminal extension rich in hydrophobic residues and involved in the membrane anchoring of these proteases (3–7). According to all of these structural data, we conclude that the identified protein is a member of the MT-MMP subfamily. Consistent with this conclusion, immunofluorescence analysis of COS-7 cells transfected with a MT6-MMP expression vector and Western blot analysis of cell membrane fractions provided additional evidence on the plasma membrane localization of this enzyme.

In this study, we have also shown that MT6-MMP is a functionally active member of this group of cell surface MMPs, as assessed by evaluating its ability to act as a progelatinase A activator, which is a
characteristic feature of MT-MMPs. Thus, cotransfection of expression plasmids encoding MT6-MMP and progelatinase A resulted in activation of COS-7-secreted progelatinase A, as demonstrated by gelatin zymography. In contrast, we did not detect any significant effect of MT6-MMP on the ectodomain shedding of a variety of transmembrane proteins including pro-transforming growth factor α, HER2, and pro-heparin-binding EGF-like growth factor. Furthermore, and in marked contrast with all of these biochemical properties of MT6-MMP that unequivocally include this protease within the MT-MMP subfamily, chromosomal mapping and expression analysis of the MT6-MMP gene (MMP-25) have revealed a series of features that are unique to this novel family member. Thus, the localization of genomic sequences for MT6-MMP in the region immediately close to the FMF gene (15) demonstrates that MMP26 is located at chromosome 16p13.3, a unique position among all known MT-MMP genes, which have been localized to chromosomes 14q11 (MT1-MMP), 16q13 (MT2-MMP), 8q21 (MT3-MMP), 12q4 (MT4-MMP), and 20q11.2 (MT5-MMP, Refs. 3, 20, and 21). This situation contrasts with the case of the MMPs located at the 11q22 cluster, which contains at least eight different family members tightly linked in a small region of the human genome (18, 22). Therefore, transposition events within subfamilies have contributed to a higher diversification of this gene family, likely reflecting a strong selective pressure on the requirements to degrade distinct protein components of connective tissues or as an adaptation to cleave similar substrates in different tissues. Consistent with this proposal, the pattern of MT6-MMP expression in human tissues is completely different from those reported for the remaining MT-MMPs. Thus, in this study, we have shown that this gene is predominantly expressed in leukocytes, lung, and spleen. None of the remaining MT-MMPs exhibits a similar pattern of expression (3–7). Thus, MT1-MMP and MT2-MMP are widely expressed in a variety of adult human tissues, whereas MT3-MMP, MT4-MMP, and MT5-MMP, which display a more restricted expression pattern, are abundantly expressed in brain, a tissue lacking any significant levels of MT6-MMP transcripts. On this basis, it is tempting to speculate that this novel membrane protease could play some specific role in membrane activation of specific substrates or in any of the connective tissue remodeling processes occurring in those tissues in which its levels are higher than those of the remaining MT-MMPs. A similar situation may occur in the case of MT-MMP expression in malignant tumors. In fact, in this study, we have provided evidence that MT6-MMP is expressed at high levels in SW480 colon carcinoma cells, whereas no expression is detected in normal colon. Furthermore, MT6-MMP is also expressed in several brain tumors, including anaplastic astrocytomas and glioblastomas. In marked contrast, samples from normal brain or meningiomas did not show any significant levels of MT6-MMP RNA transcripts. These findings suggest that MT6-MMP may be somewhat linked to the malignant transformation of some cell types and provides additional interest in the further functional characterization of this protease. In this regard, it is remarkable that a comparative analysis of the expression of several MT-MMPs in the same panel of brain tumors did reveal distinctive patterns for all of them. These data suggest that different tumors may use different MT-MMPs to activate progelatinase A or other alternative substrates at the plasma membrane as part of a general mechanism to facilitate tumor progression.

In conclusion, we have identified and characterized a new MT-MMP that shows similarities and differences with the remaining members of this subfamily of MMPs. MT6-MMP exhibits all structural features characteristic of these membrane-bound proteases, as well as a profile of activity against progelatinase A, which is typical of these enzymes. However, its chromosomal location and expression pattern distinguish this enzyme from other family members. Furthermore, the pattern of expression in cancer cell lines and brain tumors is also distinct from other MT-MMPs. Additional studies will be required to elucidate the biological significance of this protein in normal processes as well as its putative implication in the cell surface focusing of proteolytic activities during invasive growth of tumor cells.

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References


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