

# Identification and Characterization of ADAMTS-20 Defines a Novel Subfamily of Metalloproteinases-Disintegrins with Multiple Thrombospondin-1 Repeats and a Unique GON Domain\*

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We have cloned a mouse brain cDNA encoding a new protein of the ADAMTS family (a disintegrin and metalloproteinase domain, with thrombospondin type-1 repeats), which has been called ADAMTS-20. This protein shows a domain organization similar to that described for other ADAMTSs including signal sequence, propeptide, metalloproteinase domain, disintegrin domain, central TS-1 motif, cysteine-rich region, and C-terminal TS module. However, this last module is more complex than that of other ADAMTSs, being composed of a total of 14 repeats. The structural complexity of ADAMTS-20 is further increased by the presence of an additional domain 200 residues long and located immediately adjacent to the TS module. This domain has been tentatively called GON domain and can also be recognized in some ADAMTSs such as gon-1 from *Caenorhabditis elegans* and human and mouse ADAMTS-9. The presence of this domain is a hallmark of a novel subfamily of structurally and evolutionarily related ADAMTSs, called GON-ADAMTSs. Expression analysis demonstrated that ADAMTS-20 transcripts can be detected at low levels in several human and mouse tissues, especially in testis. This gene is also overexpressed in some human malignant tumors, including brain, colon, and breast carcinomas. Western blot analysis using polyclonal antibodies raised against recombinant ADAMTS-20 produced in *Escherichia coli* showed the presence of a 70-kDa band in mouse brain and testis extracts. This recombinant ADAMTS-20 hydrolyzed a synthetic peptide used for assaying matrix metalloproteinases. These data suggest that this novel enzyme may play a role in the tissue remodeling process occurring in both normal and pathological conditions.

family of zinc-dependent metalloproteinases that play important roles in a variety of normal and pathological conditions (1, 2). These enzymes show a complex domain organization including signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, central TS-1 motif, cysteine-rich region, and a variable number of TS-like repeats at the C-terminal region. To date, 18 genetically different ADAMTSs have been identified in human tissues (3). Structural characterization of these enzymes has demonstrated that ADAMTSs are distinct from ADAMs, a related family of metalloproteinases that exhibit a similar domain organization. However, both proteinase families differ in some important aspects. Thus, ADAMs lack TS-1 repeats but contain a transmembrane domain that mediates their anchorage to the plasma membrane and a cytoplasmic tail that can participate in signal transduction events (4, 5). By contrast, ADAMTSs display an organization of TS repeats of variable complexity and are secreted proteins devoid of transmembrane and cytoplasmic domains in their C-terminal region. The complexity of studies on ADAMTSs has further increased after the finding of a family of proteins that resemble ADAMTSs and that have been called ADAMTSLs (ADAMTS-like) or punctins (6). These proteins lack the metalloproteinase and disintegrin-like domains of ADAMTSs but contain all the remaining ADAMTS domains, including several TS-1 repeats. ADAMTSLs have been proposed to participate in the endogenous regulation of ADAMTS activity (6).

Functional analysis of ADAMTSs has demonstrated their participation in a wide diversity of processes. Thus, ADAMTS-1 (or METH-1) and ADAMTS-8 (or METH-2) have angioinhibitory properties (7). Disruption of the mouse *adamts-1* gene results in decreased growth, renal abnormalities, partial obstruction in the ureteropelvic junction, and alterations in adipose tissue and adrenal medullary architecture (8). Fertilization is also impaired in female mice deficient in ADAMTS-1, indicating that this protease is necessary for proper function of the female genital organs (8). ADAMTS-2, ADAMTS-3, and ADAMTS-14 are procollagen N-proteinases (9–11), and deficiency in ADAMTS-2 causes Ehlers-Danlos syndrome VIIC in humans (12). Mutant mice lacking ADAMTS-2 develop fragile skin as well as male sterility due to impaired spermatogenesis (13). ADAMTS-4 and ADAMTS-5/11 are aggrecanases, and their implication in aggrecan degradation in arthritic diseases has been reported (14–16). ADAMTS-1 has also been found to cleave aggrecan at multiple sites and displays all features to be classified as an aggrecanase (17, 18). ADAMTS-1 and ADAMTS-4 also have the ability to degrade versican in human aorta (19), whereas ADAMTS-4 is responsible for brevican degradation in glioma cells (20, 21), a critical aspect in the invasive capacity of these tumors. ADAMTS-13 is a von Willebrand factor-cleaving protease, and mutations in the gene encoding

The ADAMTSs<sup>1</sup> (a disintegrin and metalloproteinase domain, with thrombospondin type-1 modules) are a growing

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ512753, AJ515153, and AJ515154

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<sup>1</sup> The abbreviations used are: ADAMTS, a disintegrin and metalloproteinase domain, with thrombospondin type-1 modules; ADAMTSL, ADAMTS-like; GST, glutathione S-transferase; MMP, matrix metalloproteinase; RT, reverse transcription; Mca, 7-methoxycoumarin-4-acetyl; Dpa, L-dinitrophenyl-diamino propionic acid; Cha, cyclohexylalanine; Nva, norvaline; RACE, rapid amplification of cDNA ends.

this enzyme cause thrombotic thrombocytopenic purpura, a life-threatening disease mainly characterized by hemolytic anemia, microvascular thrombosis, low platelet count, renal failure, and neurological dysfunctions (22–25). Other ADAMTSs, such as ADAMTS-6, -7, -9, -10, -12, -15, -16, -17, -18, and -19, have only been structurally characterized, but their functional roles remain unknown (3, 26–28).

As part of our studies on the human and mouse degradomes (29), and considering the growing relevance of ADAMTSs in normal and pathological processes, we have examined the possibility that additional yet uncharacterized family members could be present in the genome of these organisms. In this work, we report the identification of a novel ADAMTS that has been called ADAMTS-20. We also report the structural characterization of both human and mouse enzymes with the finding of a novel domain present in ADAMTS-20, as well as in a long isoform of ADAMTS-9, and in ADAMTSs from other organisms including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Anopheles gambiae*, and *Fugu rubripes*. Finally, we report the tissue distribution of ADAMTS-20 and perform a preliminary analysis of its enzymatic activity.

#### EXPERIMENTAL PROCEDURES

**Materials**—Human and mouse cDNA libraries and Northern blots containing polyadenylated RNAs from different adult and fetal human and mouse tissues were from Clontech. RNAs from human tumors were obtained from patients who had undergone surgery for diverse malignancies at the Hospital Central de Asturias (Banco de Tumores, Instituto Universitario de Oncología del Principado de Asturias), Oviedo, Spain. Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. Synthetic peptides used for enzymatic analysis were from Novabiochem.

**Isolation of cDNA Clones**—A search using the BLAST (Basic local alignment search tool) program in the public (www.ncbi.nlm.nih.gov) and private (www.celera.com) mouse genome databases allowed the identification of a mouse clone RP23–24F24 (GenBank™ accession number AC084384) containing an uncharacterized ADAMTS proteinase-like domain and regions encoding thrombospondin-like repeats. We designed specific oligonucleotides to PCR-amplify the region contained between these two putative ADAMTS domains, using several different commercially available cDNA libraries (Clontech) and the Expand High Fidelity PCR system (Roche Molecular Biochemicals). The following oligonucleotides derived from the RP23–24F24 DNA clone were synthesized: TS20F, 5'-CCAAGATACGTGGAAGTTATGGT-3'; TS20R, 5'-CTGTGCTCTTGATTCCACCTC-3', whereas the following oligonucleotides were used for the nested PCR: TS20Fnd, 5'-GTTATGGTTACAGCCGATGCT-3', and TS20Rnd, 5'-TGCTCTTGATTCCACCTCCG-3'. The PCR reaction was performed in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences for 40 cycles of denaturation (94 °C, 20 s), annealing (64 °C, 15 s), and extension (68 °C, 60 s). The amplified PCR product, of about 0.9 kb, was treated with T4 polynucleotide kinase and T4 DNA polymerase and ligated in the *Sma*I site of pUC18. The identity of the product was confirmed by sequencing using the kit DRho terminator *Taq* FS (Applied Biosystems) and the automatic DNA sequencer ABI-PRISM 310 (PerkinElmer Life Sciences). To obtain human probes for human ADAMTS-20, the following specific oligonucleotides were used in a nested PCR amplification: hTS20F, 5'-GTGAAATGCTGCC-TCAAAGG-3'; hTS20F-nd, 5'-GCTGCCTCAAAGGACCATCA-3'; hTS20R, 5'-GACATAGTAAGCAGAAAGTGG-3'; and hTS20R-nd, 5'-G-GATTTCCAATCTAAGATAGC-3'; nd indicates the primers used for the nested PCR amplification.

**5'- and 3'-Extension of Isolated cDNAs**—The 5'- and 3'-ends of cloned cDNAs were extended by successive rounds of RACE (rapid amplification of c-DNAs ends) using RNA from different mouse tissues and the Marathon™ cDNA amplification kit (Clontech), essentially as described by the manufacturer. Each cycle of RACE allowed the extension of about 200 bp toward each end. Following gel purification, the PCR products were cloned and sequenced as described above.

**Northern Blot Analysis**—Nylon filters containing 2 µg of poly(A)<sup>+</sup> RNA of a wide variety of both human and mouse tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.4), 10 × Denhardt's solution, 2% SDS, and 100 µg/ml denatured herring sperm DNA. Hybridization was performed with a radiolabeled ADAMTS-20-specific

probe (0.7 kb long) corresponding to the PCR product amplified by using the oligonucleotides TS20F and TS20R3 (5'-GTAGGGACACACTTGTGATCCA-3') as forward and reverse primers, respectively. After hybridization for 20 h under the same conditions used for prehybridization, filters were washed with 0.1 × SSC, 0.1% SDS for 2 h at 50 °C, and exposed to autoradiography. RNA integrity and equal loading were assessed by hybridization with an actin probe.

**Reverse Transcription and PCR Amplification**—To analyze the expression of ADAMTS-20 in human tumor specimens, total RNA was isolated from diverse malignant tumors by guanidium thiocyanate-phenol-chloroform extraction and used for cDNA synthesis with the RNA PCR kit from PerkinElmer Life Sciences. After reverse transcription (RT) using 1 µg of total RNA and random hexamers as primer according to the manufacturer's instructions, the whole mixture was used for PCR with the following ADAMTS-20-specific oligonucleotides (hTS20F2, 5'-CTTTACTATAGCCCATGAGC-3'; hTS20F2-nd, TTACTATAGCCCATGAGCTTGG-3'; hTS20R2, 5'-CAGGAAGTTCTGAAGCAGAT-3'; and hTS20R2-nd, 5'-TTGTCAAGAAGACATTCGGG-3'), as described above. The PCR products were analyzed in 1.5% agarose gels. cDNA quality was verified by performing control reactions with primers derived from the sequence of actin. Negative controls were also performed in all cases by omitting the template or reverse transcriptase.

**Production and Purification of Recombinant Proteins**—A cDNA construct containing the metalloproteinase domain of ADAMTS-20 was made by PCR amplification using the following two oligonucleotides containing *Bam*HI and *Eco*RI sites, respectively: TS20expF, 5'-ATCGGATCCGTTTTTATCATAC-3', and TS20expR, 5'-GAAGAATTCGAGACAGGTCATATGTTCTCC-3' (where the *Bam*HI and *Eco*RI sites are underlined). The PCR amplification was performed for 25 cycles of denaturation (95 °C, 15 s), annealing (58 °C, 10 s), and extension (68 °C, 50 s) using the Expand™ High Fidelity PCR system. The PCR product was digested with *Bam*HI and *Eco*RI and cloned between these two sites of the pGEX-3X expression vector (Amersham Biosciences). The resulting vector, pGEX-3XTS20(M), was transformed into BL21(DE3)-pLysE-competent *Escherichia coli* cells, and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration 0.5 mM). The cells were collected by centrifugation, washed, and resuspended in 0.05 volumes of phosphate-buffered saline. Then, the cells were lysed by sonication and centrifuged at 20,000 × g for 20 min at 4 °C. The soluble extract was purified using a glutathione-Sepharose 4B column (Amersham Biosciences), and the glutathione S-transferase GST-TS20(M) fusion protein was eluted with 20 mM reduced glutathione, following manufacturer's instructions. The GST-TS20(M) purified protein was used for enzymatic assays.

**Enzymatic Assays**—Enzymatic activity of the purified recombinant GST-TS20(M) protein was assayed using the synthetic fluorescent substrates QF-24 (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub>), QF-35 (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH<sub>2</sub>), and QF-41 (Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH<sub>2</sub>). Routine assays were carried out at 37 °C at substrate concentrations of 1 µM in a buffer containing 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.05% Brij-35, pH 7.5, with a final concentration of Me<sub>2</sub>SO of 1%. The fluorimetric measurements were made in an LS 50-B PerkinElmer Life Sciences spectrofluorimeter (λ<sub>ex</sub> = 328 nm, and λ<sub>em</sub> = 390 nm). The pH optimum analysis was carried out in assay buffer as above but using 50 mM Bis-Tris and Tris (pH 6–9), or 50 mM glycine (pH 10) as buffer for the indicated pH range and containing 2 µM fluorogenic QF-35 substrate and 29 nM enzyme. The resulting data were fit to Equation 1, describing two pK<sub>a</sub> values (pK<sub>a1</sub> and pK<sub>a2</sub>) and one limiting k<sub>cat</sub>/K<sub>m</sub> value.

$$\left(\frac{k_{\text{cat}}}{K_M}\right)_{\text{obs}} = \frac{\left(\frac{k_{\text{cat}}}{K_M}\right)_{\text{lim}}}{10^{\text{pK}_{a1} - \text{pH}} + 10^{\text{pH} - \text{pK}_{a2}} + 1} \quad (\text{Eq. 1})$$

Kinetic studies were performed using different concentrations of the fluorogenic peptide (0.5–4 µM) in 2 ml of assay buffer containing 48 nM recombinant ADAMTS-20, and peptide hydrolysis was measured as the increase in fluorescence at 37 °C for 5 min. Initial velocities were calculated using the analysis package FL WinLab 2.01 (PerkinElmer Life Sciences), and the k<sub>cat</sub>/K<sub>m</sub> ratio was calculated as described previously (30). For inhibition experiments, the reaction mixture was preincubated for 30 min at 20 °C with EDTA, BB-94, E-64, or 4-(2-aminoethyl)-benzenesulfonyl fluoride, and the hydrolyzing activity of ADAMTS-20 against QF-35 was determined by fluorimetric measurements as above. Purified recombinant GST was used in all the experiments as a negative control.

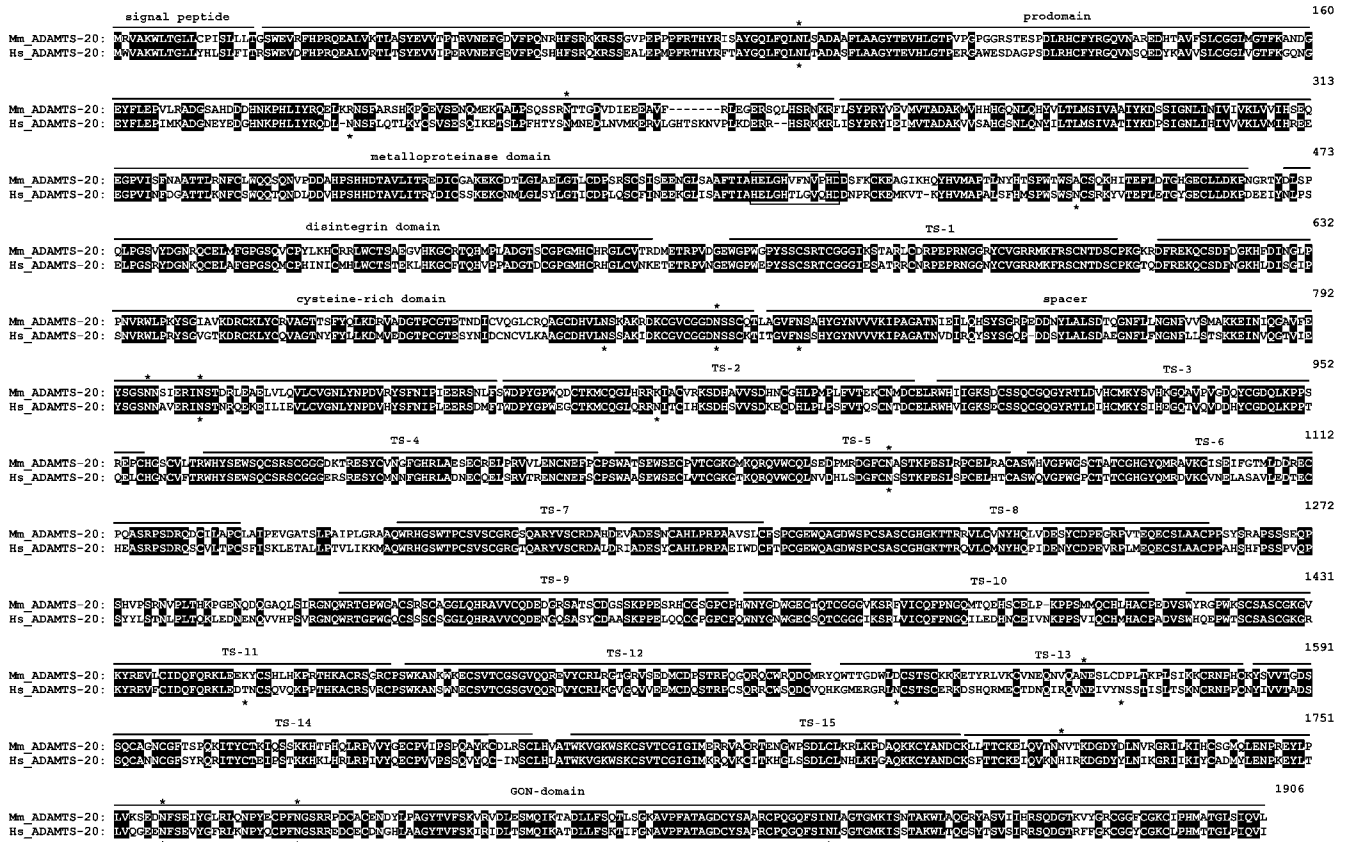


FIG. 1. Amino acid sequences of mouse and human ADAMTS-20. The different domains are *overlined*, and the potential sites of *N*-linked glycosylation indicated by *asterisks*. The zinc-binding site characteristic of metalloproteinases is *boxed*. (*H\_ADAMTS-20* indicates human ADAMTS-20; *M\_ADAMTS-20* indicates mouse ADAMTS-20; *TS* indicates thrombospondin domains).

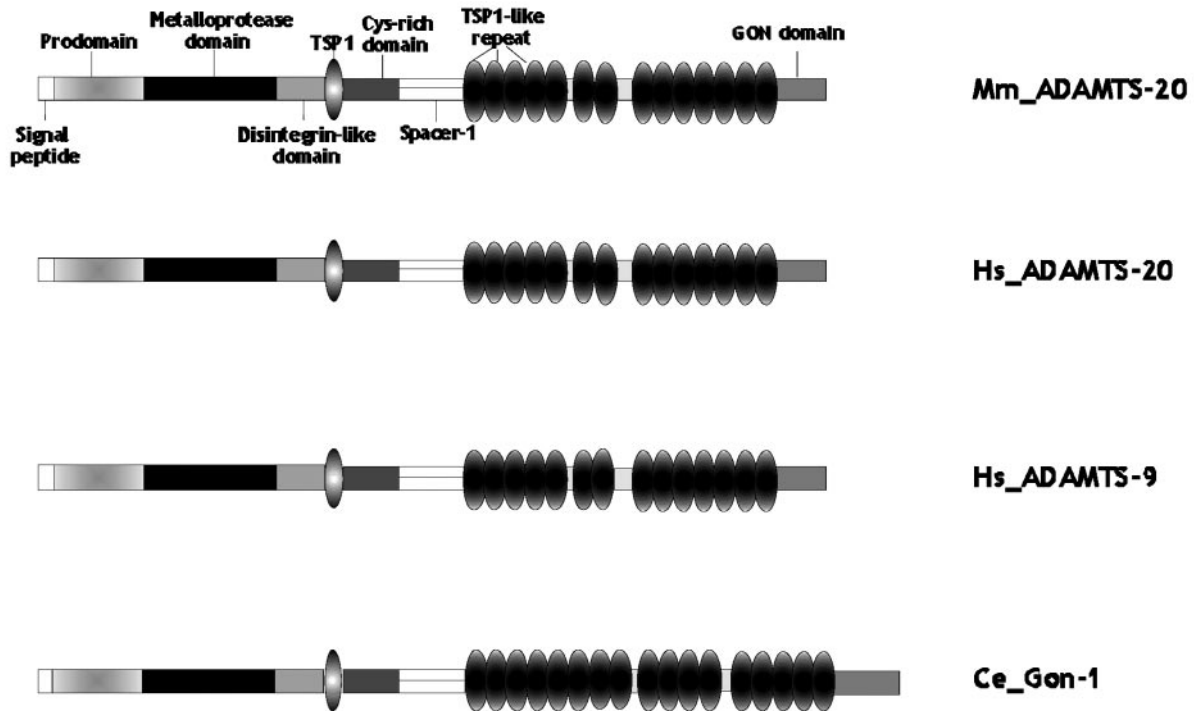
**Antibody Production and Western blot Analysis**—Purified ADAMTS-20 was injected into rabbits, and the animals were bled 6 weeks after the injection. The serum was then dialyzed for 24 h at 4 °C against 20 mM phosphate buffer, pH 7.2. After dialysis, the material was chromatographed in a column of DEAE-cellulose equilibrated and eluted in the same 20 mM phosphate buffer. IgG-containing fractions were collected and stored at -20 °C until used. Western blots were blocked in 5% milk in PBT (phosphate-buffered saline containing 0.1% Tween 20) and then incubated for 1 h with 1 µg/ml rabbit antiserum in PBT. After three washes in PBT, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:20,000 and developed with the Renaissance chemiluminescence kit (PerkinElmer Life Sciences).

RESULTS

**Identification and Characterization of ADAMTS-20**—An extensive search for DNA genomic sequences encoding uncharacterized ADAMTS-like domains allowed us to identify a mouse clone (RP23-24F24, GenBank™ accession number AC084384) that fulfilled the premises to be considered as a region containing a new *adamts* gene. These features included the presence of putative exons encoding metalloproteinase and thrombospondin-like domains similar to those found in previously described ADAMTSs. To generate a cDNA for this region, we carried out PCR amplification experiments using mouse brain cDNA libraries and specific oligonucleotides derived from the identified metalloproteinase and thrombospondin-like domains. After cloning a PCR product of about 0.9 kb, we confirmed by conceptual translation that this sequence was distinct from the equivalent regions present in all previously identified ADAMTS family members. To obtain the full-length cDNA for this putative new metalloproteinase, we performed successive rounds of 5'- and 3'-RACE experiments using specific oligonucleotides deduced from the end of the previously

obtained sequences. This strategy allowed us to extend the original cDNA clone toward both ends, and finally, to identify the corresponding start and stop in-frame codons. Computer analysis of the obtained sequence (Fig. 1 and GenBank™ accession number AJ512753) revealed an open reading frame coding for a protein of 1906 amino acid residues with a predicted molecular mass of 212,040. The sequence also contains 10 potential sites of *N*-glycosylation (Fig. 1) that can contribute to increase the predicted molecular mass for this protein. This structural analysis also showed that the overall domain organization of this protein was very similar to that of previously characterized ADAMTSs, containing signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, central TS1 motif, cysteine-rich region, and a C-terminal module with several TS1 submotifs (Fig. 2A).

Further analysis of the identified amino acid sequence showed a significant similarity with other ADAMTSs, the maximum percentage of identities (52%) being with human ADAMTS-9. Additional hallmarks of the ADAMTS family were also apparent from this analysis. Thus, the putative prodomain contains three conserved Cys residues found in other ADAMTSs (2, 3). The first two Cys are within the conserved sequences CFYRGQV (positions 129–135, consensus sequence CXYXGXV), and CGGLMG (positions 148–153, consensus sequence CXGLXG). The third Cys residue is located close to the prodomain end (position 202) and lies within a sequence (KPCEVSE) that does not resemble the Cys switch consensus sequence (PRCGVPD) present in matrix metalloproteinases (MMPs) (31, 32) and in some ADAMTSs such as ADAMTS-12 and ADAMTS-15 (3, 28). The propeptide ends in a basic region RNKR (positions 246–249), which could correspond to a pro-

**A****B**

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Mm ADAMTS-20: KLLTTCRKLQVNTNVTKGGDIDNVRERILKIHSSGVOLENPREELPQVKSE-DNPESEHYGLRLOQNYECCFNGSRRPDCAENIDYLP-AGYIVRSKVRVDLE : 101
Hs ADAMTS-20: KSFTTCRKLQVKNHIRKGGDIDYINIKSRIIKIYCADYILENPKBELTIVQGE-ENGSEVYCFRLKKNFYCCFNGSRRREDCEEDNGHLA-AGYIVRSKIRIDLT : 101
Hs ADAMTS-9 : ELPQNCRQVVKLLKGAASEDGEYFMIRSKLLKIFCAGMHSDFKREYVTVHGDSENGSEVYGHRIHNETCFYNGSRRDDCGRRKDYTA-AGFSSFCGRIDLT : 102
Mm ADAMTS-9 : ELPQNCRQVVKLLNSASVDGEYFAVRKPLKVFCAGMNSDYKREYVTVHGDSENGSEVYGHRIHNETCFYNGSRRDDCGRRKDYTA-AGFSSFCGRIDLT : 102
Fr GON-D : PLFASCRQVQVRRGVKLDGEFYFRVNSRILQVYCADMN-TDPRFVTVHRSQMDNYSEVYGHRIHNETCFYNGSRRDDCGRRKDYTA-AGYILPFRVRLDLD : 101
Ce GON-1 : -LPSTCCQLKSNVKAQGNQNTLLDQPTIETVYGHRIHNETCFYNGSRRDDCGRRKDYTA-AGYILPFRVRLDLD : 100
Dm AY094716 : GTPRSCADLKEMHGYNKGNQIQEVRSRMVHLYCHGNSRTECFYVNV--DPQENYSIYVEYTKQTNSCGQRRRRRSGRRDY----GRHHRKRLNIT : 97
Ag_AGCP2529 : PVYRNCNDARRKH--KTDCGEMMHVNETKAKIYCHGQATDTTELELS-PAGPTENYAIYINRAADANK--ENSQDWDADSSISV----GAIHYRKRIRINVS : 95

Mm ADAMTS-20: SMCQKATADLLESOTLSCKAVPPATAGDCYS-AARCPQGGPFSINLSTCMKISNTAKMLAQRVASVITHRSQDGI--KVYERCGGFCGKLIHMANGLSIOVL : 201
Hs ADAMTS-20: SMCQKATADLLESKTIIFGNVPPATAGDCYS-AFRCPQGGPFSINLSTCMKISNTAKMLTQGSYTSVSIIRRSQDGI--RPFKCGGFCGKLIHMTIGLPIQVI : 201
Hs ADAMTS-9 : SMCQITTLQARTSPCHVPPATAGDCYS-AAKCPQGGPFSINLSTCLSLTESARMTISQENNAVSDIKKSPDGI--RVVYKCGGFCGKLTSSCTGLEVRNL : 202
Mm ADAMTS-9 : SMCQITADLLESARTSPCHVPPATAGDCYS-AAKCPQGGPFSINLSTCLSLTESARMTISQENNAVSDIKKSPDGI--RVVYKCGGFCGKLTSSCTGLEVRNL : 202
Fr GON-D : SLRQITVTLQCSQTLGRVPPATAGDCYS-AAKCPQGGPFSINLSTCLKVAESTTHTTSQENNVSIKIVHRSQDGI--RIYERCGGFCGKLTQAHNGLLQV : 200
Ce GON-1 : NRKPHLADYTFQAKREYGVHLYGTAGDCYS-MKDCPQGGPFSINLSTKSAELKLVDDLMDQCHRTSSRIDRFYNNA--KVYERCGGFCGKLSERYKGLIFEVN : 200
Dm AY094716 : DLRQMDNPKFADSR-GLAQKLGSAAGDCYNRIGCPQGGPFSINMKDDFSIRPGTVRMHCOQSVMKRISEFTLITQMRRFQGGFCGGSYIAPDSHLYLDL : 199
Ag_AGCP2529 : TLQVHTMFPRTNNS-CKKQEPGSAAGDCYSNTGRCPQGGPFSINLSTKSAELKLVDDLMDQCHRTSSRIDRFYNNA--KVYERCGGFCGKLSERYKGLIFEVN : 199

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FIG. 2. Domain organization of ADAMTS-20 and related proteins. A, the domain structure of the indicated members of the ADAMTS family. B, sequence of the GON domains found in identified or predicted ADAMTS proteins of different organisms. *Mm*, *Mouse musculus*; *Hs*, *Homo sapiens*; *Fr*, *F. rubripes*; *Ce*, *C. elegans*; *Dm*, *D. melanogaster*; *Ag*, *A. gambiae*.

protein-convertase recognition sequence for generation of the mature enzyme (consensus sequence RX(K/R)R). The catalytic domain includes the sequence HELGHVFNPHD (positions 399–410) closely related to that involved in the coordination of the catalytic zinc atom at the active site of metalloproteinases (consensus sequence HEXGHXXGXH). This motif ends in an Asp residue, which distinguishes ADAMs and ADAMTSs from other metalloproteinases, such as MMPs that contain a conserved Ser residue at this position. A few residues C-terminal to this site, there is a Met residue (position 427) that forms the

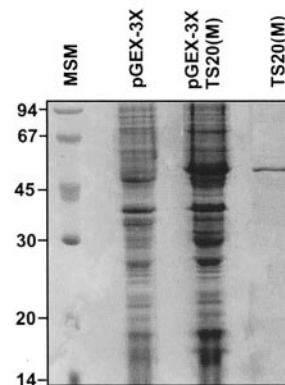
“Met turn” characteristic of ADAMs, ADAMTSs, and MMPs. The catalytic domain also contains the eight conserved Cys residues present in the corresponding region of all ADAMTSs.

The metalloproteinase domain is followed by a disintegrin domain, which is very similar in size (76 residues) to that of other ADAMTSs and includes the 8 conserved Cys residues characteristic of this region. Furthermore, a central TS-1 motif, a Cys-rich domain, and a spacer region can be clearly recognized after the disintegrin domain. These regions are also very similar in size and structural features to the equivalent ones

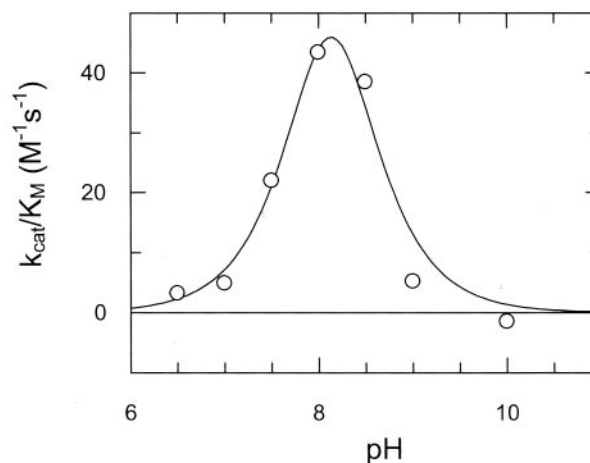
from other ADAMTSs. Following the spacer region, the characteristic C-terminal TS-1 module of these enzymes can be found. It is remarkable that the number of TS-1 repeats located at this region of the identified protein is higher than that present in all human or mouse ADAMTSs whose sequences have been reported to date. Thus, a total of 14 TS-1 repeats can be distinguished in this module, whereas most vertebrate ADAMTSs have just three or four repeats, the maximum number corresponding to ADAMTS-12, which contains seven TS-1 repeats in this C-terminal module. Interestingly, the identified sequence ends with a region of about 200 amino acids, which is not present in any other sequence described to date, with the exception of some ADAMTSs such as gon-1 (33), an ADAMTS family member from *C. elegans*. Computer analysis of the human and mouse genome sequences has also allowed us to identify enlarged isoforms of human and mouse ADAMTS-9 containing this additional domain present in ADAMTS-20 and gon-1. These enlarged ADAMTS-9 variants have not been described yet in the literature and are distinct from those reported previously for this enzyme (27). Putative proteins containing this domain can also be deduced from the genome sequence of other organisms whose sequence is available, including *D. melanogaster* (GenBank<sup>TM</sup> accession number AY094716), *A. gambiae* (GenBank<sup>TM</sup> accession number AGCP2529), and *F. rubripes* (wrongly annotated as being part of two independent proteins with GenBank<sup>TM</sup> accession numbers SINFRUP 00000055939 and 00000062760). This conserved domain present in all these proteins is rich in Cys residues (Fig. 2B) and represents a particular structural hallmark for these ADAMTSs. Because the first published sequence containing this motif is that of gon-1, we propose the name GON domain for this newly identified domain present in a subset of ADAMTSs.

Taking together these structural comparisons, we can conclude that the identified murine sequence corresponds to a *bona fide* member of the ADAMTS family of metalloproteinases whose officially approved name is ADAMTS-20. In this work, and by using the mouse ADAMTS-20 sequence as query, we have also deduced the complete sequence of its human orthologue (Fig. 1, and GenBank<sup>TM</sup> accession number AJ515153). The gene encoding human ADAMTS-20 is located in chromosome 12q12, syntenic to a region of mouse chromosome 15, where the mouse *adamts-20* gene is located. Detailed pairwise comparisons revealed that human ADAMTS-20 is 69% identical to its mouse counterpart and also contains all structural hallmarks characteristic of this family of metalloproteinases. Human ADAMTS-20 contains 15 potential sites of *N*-glycosylation, 6 of them being conserved with those predicted for mouse ADAMTS-20 (Fig. 1). Interestingly, bioinformatic analysis of the sequence of human *ADAMTS-20* gene led us to predict the presence of an alternative exon that was not found in the mouse orthologue. To provide experimental support to this prediction, we performed PCR amplifications of this region using the specific oligonucleotides hTS20F, hTS20F-nd, hTS20R, and hTS20R-nd and a human brain cDNA library. These experiments allowed us to amplify a cDNA sequence that confirmed the occurrence of an alternative splicing event in the analyzed region. This event would take place after the exon encoding the TS-1 repeat number 10 and would lead to the synthesis of a short form of human ADAMTS-20 with a total of 11 TS-1 repeats, due to the presence of an in-frame stop codon at the end of this region (GenBank<sup>TM</sup> accession number AJ515154). These splicing events are common in the 3'-end of *ADAMTS* genes and can be responsible for the previously observed variations in the C-terminal end of these metalloproteinases (3, 26–28).

A



B



**FIG. 3. Production and enzymatic characterization of the metalloprotease domain of ADAMTS-20.** As shown in A, 5  $\mu$ l of bacterial extracts transformed with pGEX-3X (lane 2) or pGEX-3XTS20(M) (lane 3) and purified catalytic domain of ADAMTS-20 (TS20(M)) (lane 4) were analyzed by SDS-PAGE. The sizes of the molecular size markers (MWM, lane 1) are indicated on the left. As shown in B, the fluorogenic peptide QF-35 (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH<sub>2</sub>) was incubated with 10 nM purified catalytic domain of mouse ADAMTS-20 using 50 mM Bis-Tris, Tris, or glycine as buffer (see "Experimental Procedures"). The fluorimetric measurements were made at  $\lambda_{ex}$  = 328 nm, and  $\lambda_{em}$  = 393 nm.

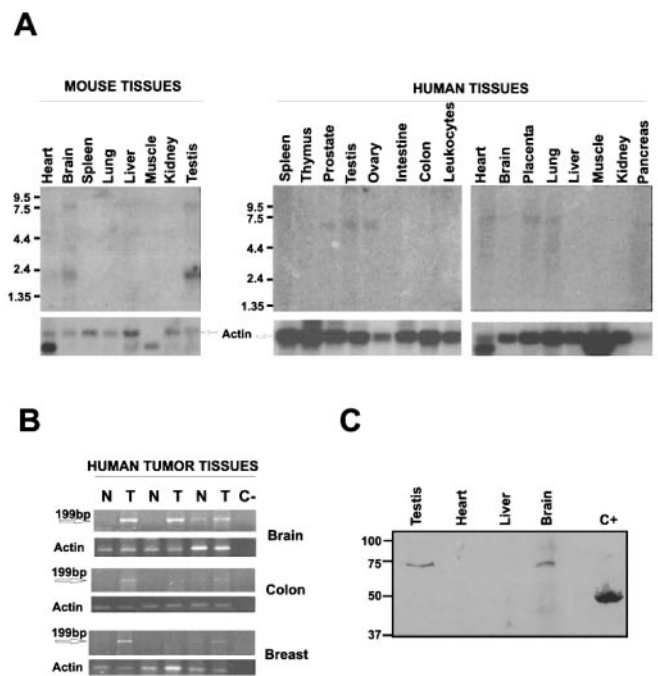
**Production of Recombinant ADAMTS-20 in *E. coli* and Analysis of Its Enzymatic Properties**—We have expressed the metalloprotease domain of mouse ADAMTS-20 in bacterial cells to analyze its activity. To do this, a cDNA coding for this catalytic domain was subcloned into the expression vector pGEX-3X, and the resulting plasmid, called pGEX-3XTS20(M), was transformed into *E. coli*. After induction with isopropyl-1-thio- $\beta$ -D-galactopyranoside, a protein band of the expected size (51 kDa) was detected by SDS-PAGE analysis of the protein extract (Fig. 3A). The recombinant fusion protein was purified using glutathione-Sepharose chromatography as described previously (34) (Fig. 3A). The soluble GST-TS20(M) fusion protein eluted from the column was directly used for enzymatic analysis, employing as putative substrates a series of synthetic quenched fluorescent peptides commonly used for assaying other metalloproteinases, including MMPs. The recombinant catalytic domain of ADAMTS-20 exhibited significant proteolytic activity against the fluorogenic peptide QF-35 (38  $\mu$ M product/min).

Other peptides, including QF-24 and QF-41, were not significantly hydrolyzed by this recombinant protein. The proteolytic activity of ADAMTS-20 against QF-35 was substantially abolished by EDTA and BB-94 but not by inhibitors of other classes of proteolytic enzymes distinct of metalloproteinases (data not shown). To further examine the catalytic activity of ADAMTS-20, we performed an analysis of the pH profile of activity of this enzyme. As shown in Fig. 3B, recombinant ADAMTS-20 exhibited a pH optimum of 8.1. Finally, we also carried out a kinetic study using QF-35 as substrate. To this purpose, the recombinant protease was incubated with different concentrations of fluorogenic substrate, and the  $k_{cat}/K_m$  was deduced as described previously (30). The observed  $k_{cat}/K_m$  of ADAMTS-20 for substrate QF-35 at pH 8.1 was  $46 \text{ M}^{-1} \text{ s}^{-1}$ , similar to that calculated for some MMPs such as Mcol-A with the ability to hydrolyze the same substrate ( $k_{cat}/K_m = 58 \text{ M}^{-1} \text{ s}^{-1}$ ) but substantially lower than values determined for most members of this family of metalloproteinases (35). Finally, it is remarkable that preliminary experiments aimed at evaluating the ability of the recombinant catalytic domain of ADAMTS-20 to hydrolyze diverse endogenous substrates, including several proteoglycans targeted by other ADAMTSs, have not revealed any significant degrading activity against them. These results suggest that the presence of ADAMTS-20 ancillary domains may be necessary for the *in vivo* function of this enzyme, although the possibility that it could target a novel substrate cannot be ruled out.

**Expression Analysis of Mouse and Human ADAMTS-20**—To study the expression pattern of mouse and human ADAMTS-20, we have hybridized Northern blots containing poly(A)<sup>+</sup> prepared from a variety of human and mouse tissues. As shown in Fig. 4A, two transcripts of about 7.5 and 2.5 kb were detected in mouse brain and testis. In the case of human ADAMTS-20, a single transcript of about 7 kb was observed in testis, prostate, ovary, heart, placenta, lung, and pancreas (Fig. 4A). RT-PCR amplification and nucleotide sequencing of the amplified products confirmed the expression of ADAMTS-20 in all these human tissues in which transcripts were detected at low levels by Northern blot (data not shown). To examine the possibility that ADAMTS-20 was produced by human tumors, we performed RT-PCR amplification with RNAs obtained from a panel of paired primary tumors and adjacent normal tissue. As illustrated in Fig. 4B, which shows some representative cases, ADAMTS-20 was overexpressed in several brain, colon, and breast carcinomas when compared with the low or undetectable levels observed in the paired adjacent normal tissues. Finally, we performed Western blot analysis of protein extracts from different mouse tissues with polyclonal antibodies against the purified recombinant ADAMTS-20. As can be seen in Fig. 4C, a major band of about 70 kDa was observed in testis and brain but not in other tissues in which the expression of this gene had not been detected. These findings demonstrate the presence of the ADAMTS-20 protein in these tissues. Furthermore, the absence of significant amounts of a putative immunoreactive band of about 212 kDa, which could correspond to the intact ADAMTS-20, strongly suggests that as demonstrated previously for other ADAMTSs (28), this novel enzyme is subjected to several proteolytic processing-mediated events to generate the final active molecule.

#### DISCUSSION

In this work, we describe the identification and characterization of a novel member of the ADAMTS family of secreted metalloproteinases with disintegrin and thrombospondin domains. The approach to identify ADAMTS-20 involved a search of human and mouse genome databases followed by a combination of RT-PCR amplifications of cDNA libraries and succes-



**FIG. 4. Analysis of the expression of ADAMTS-20 in normal and tumor tissues.** A, Northern blot analysis of ADAMTS-20 expression in mouse and human tissues. About 2  $\mu\text{g}$  of polyadenylated RNA of the indicated mouse and human tissues were hybridized with specific probes isolated from the mouse and human ADAMTS-20 cDNAs. The position of the size markers is shown. The filters were subsequently hybridized with a mouse and a human actin probe, respectively, to ascertain the differences in RNA loading among the different samples. B, RT-PCR analysis of ADAMTS-20 expression in paired normal and tumor tissues. A 199-bp fragment corresponding to a segment of human ADAMTS-20 was amplified with primers indicated under "Experimental Procedures" in a volume of 50  $\mu\text{l}$ , and 10  $\mu\text{l}$  of the reaction were separated on a 1.5% agarose gel run in Tris borate-EDTA buffer. Amplification of  $\beta$ -actin was used to ascertain RNA integrity and equal loading. C- indicates negative control. C, Western blot analysis of protein extracts from the indicated mouse tissues with 1  $\mu\text{g}/\text{ml}$  polyclonal antibody against ADAMTS-20 in PBT. As a positive control (C+) we used purified recombinant ADAMTS-20. The sizes of the molecular size markers are shown to the left.

sive 5'- and 3'-RACE experiments to extend the originally amplified cDNA fragments. This strategy allowed us to isolate a full-length cDNA for mouse ADAMTS-20 and to deduce the complete sequence of its human orthologue. Both proteins exhibit an identical domain organization that is similar to that of previously described ADAMTSs. Thus, they harbor signal sequence, propeptide, metalloproteinase-, disintegrin-, central TS-, and cysteine rich-domains and a complex C-terminal TS-like module. Likewise, mouse and human ADAMTS-20 show several conserved residues and motifs characteristic of each of these domains, including a propeptide convertase activation sequence at the end of the propeptide, a zinc-binding site with the reprolysin signature in the catalytic domain, and conserved patterns of cysteine arrangements in the disintegrin and cysteine-rich regions. However, ADAMTS-20 also exhibits some characteristic features that allow us to distinguish this enzyme from other family members as well as to define a novel subfamily of ADAMTSs. Thus, it contains an unusually complex organization of TS repeats at the C-terminal module, being composed of a total of 14 repeats, the highest number among all equivalent modules present in vertebrate ADAMTSs identified to date. Interestingly, we have previously reported that although ADAMTS-9 has been described to possess three TS repeats at the C-terminal module (27), information retrieved from databases reveals the occurrence of an alternative tran-

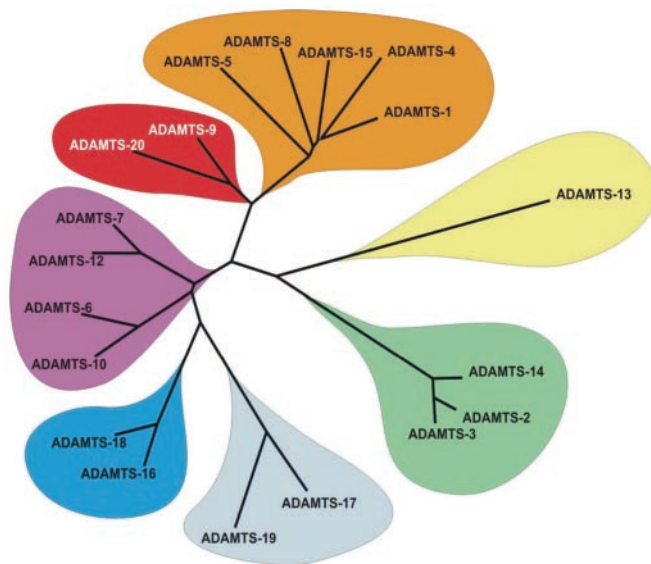


FIG. 5. **The human ADAMTS subfamilies.** A phylogenetic tree of the human ADAMTS family was generated using the amino acid sequences of their metalloprotease domains and the program supplied by the Human Genome Mapping Project ([www.hgmp.mrc.ac.uk](http://www.hgmp.mrc.ac.uk)).

script of ADAMTS-9, which encodes a protein isoform also containing 14 TS-1 repeats (Fig. 1 and data not shown) (3). The second distinctive feature of ADAMTS-20 is the presence of an additional domain in its C-terminal region, immediately adjacent to the TS-terminal module. This structural motif, which we have designated as GON domain, is characterized by the presence of several conserved cysteine residues and can be recognized in gon-1 from *C. elegans*, in the large isoforms of human and mouse ADAMTS-9, and in proteins predicted from sequence analysis of the genomes of *D. melanogaster*, *A. gambiae*, and *F. rubripes*. On the basis of these structural features, we propose that all these proteins define a novel subset of ADAMTSs that could be known as GON-ADAMTSs.

The dendrogram shown in Fig. 5 confirms the structural relationships among these ADAMTS family members and allows the classification of the family of human ADAMTSs into seven subfamilies of closely related members. The first of these subfamilies should be that of hyaluronanases (36), comprising ADAMTS-1, -4, -5/11, -8, and -15 and characterized by structural and enzymatic similarities including proteoglycanase activities, and in some cases, angioinhibitory properties (7, 14–21). The second subfamily should be that of procollagen N-propeptidases and includes ADAMTS-2, -3, and -14 (9–12). ADAMTS-9 and ADAMTS-20 should conform to the subfamily of GON-ADAMTSs. ADAMTS-13, with unique properties among all described ADAMTSs, should be the only representative of von Willebrand factor-cleaving proteases (22–25). Finally, ADAMTS-6, -7, -10, and -12; ADAMTS-16 and -18; and ADAMTS-17 and -19 form groups of structurally related family members that might also be indicative of putative common enzymatic and functional properties. Further studies aimed at identifying the substrates targeted by the proteases belonging to these three last ADAMTS subfamilies will be necessary to confirm that the structural similarities here defined are also supported by functional relationships between them. In this regard, the structural similarities between vertebrate and invertebrate members of the GON-ADAMTS subfamily of ADAMTSs may also allow us to speculate about putative functional roles for ADAMTS-20. To date, no physiological role has been ascribed to ADAMTS-9 nor to those related proteins identified in *Drosophila*, *Anopheles*, or *Fugu*; however, gon-1 is an

active metalloproteinase essential for controlling morphogenesis in *C. elegans* (33). Thus, mutagenesis studies have suggested that this ADAMTS permits and directs expansion of the gonad by remodeling the extracellular matrix and basement membrane. Interestingly, the region containing TS-1 repeats is critical for gon-1 activity because some mutations inactivating this gene are located in regions encoding these repeats (33). Since it has been suggested that similar activities may control organ morphogenesis throughout the animal kingdom, it is tempting to speculate that ADAMTS-20 may play similar roles in vertebrates to those played by gon-1 in nematodes.

As a previous step to evaluate this hypothetical function of ADAMTS-20 as an extracellular matrix remodeling enzyme, we have performed a preliminary analysis of the catalytic properties of a recombinant form of this protease produced in bacterial cells. Interestingly, ADAMTS-20 is able to hydrolyze a synthetic peptide used for analysis of vertebrate MMPs, and this hydrolyzing activity is abolished by inhibitors of metalloproteinases, demonstrating that the identified protein is an active member of this class of proteolytic enzymes. To our knowledge, this is the first report showing that a member of the ADAMTS family is able to degrade peptides such as QF-35 commonly used to assay the activity of MMPs, thus confirming the connections between both proteolytic systems (reviewed in Ref. 32). Nevertheless, it is remarkable that kinetic analysis has revealed that the catalytic efficiency of ADAMTS-20 against QF-35 is much lower than that of most MMPs with the ability to hydrolyze this peptide, suggesting the occurrence of important differences in the active site of both types of metalloproteinases. Consequently, studies of substrate specificity and resolution of the three-dimensional structure of the ADAMTS-20 catalytic domain will be required to clarify the similarities and differences of this novel enzyme with members of the MMP family.

Previous studies have shown that ADAMTSs may be of relevance in tumor processes (20, 28). Therefore, in this work, we have also explored the potential significance of ADAMTS-20 in human cancer through analysis of its expression pattern in a panel of malignant tumors. These studies have shown that ADAMTS-20 is overexpressed in several brain, colon, and breast carcinomas when compared with the paired adjacent normal tissues, suggesting that this protease could play some role in the progression of these tumors. Also, in this regard, it is interesting that the region containing the human ADAMTS-20 gene (12q12) has been found to be a recurrent site of translocations and other alterations in human malignancies (37–40). Genetic lesions in this region have also been linked to several diseases, including a new locus for Parkinson's disease (41), whose responsible gene remains to be characterized. It will be interesting to examine the possibility that ADAMTS-20 could be a target of some of these genetic abnormalities, as already demonstrated for other ADAMTS family members linked to relevant genetic diseases (12, 22). To this purpose, as well as to clarify the role of ADAMTS-20 in physiological processes, it will be very helpful to create a mouse deficient in this protease. This work is currently in progress in our laboratory and has been facilitated by the availability of cDNA and genomic clones for mouse ADAMTS-20 generated in the present study.

In conclusion, we have cloned and characterized ADAMTS-20, a protease that, according to our exhaustive analysis of both mouse and human genomes, represents the only member of the ADAMTS family that remained to be identified in these organisms. ADAMTS-20 is an active protease with a profile of activity and sensitivity to inhibitors characteristic of metalloproteinases. However, it also exhibits a series of structural

peculiarities including the presence of the newly identified GON domain, which has allowed us to define the occurrence of a novel subfamily of ADAMTSs: the GON-ADAMTSs. This structural analysis, together with that performed with other family members, has also prompted us to propose that ADAMTSs can be organized into seven different subfamilies. Hopefully, this classification may facilitate future studies aimed at exploring the multiple roles that this large and complex family of proteases may play in processes involving cell migration, tissue remodeling, and changes in cell adhesion.

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