We demonstrate that in humans, two metalloproteases, ADAMTS-9 (1935 amino acids) and ADAMTS-20 (1911 amino acids) are orthologs of GON-1, an ADAMTS protease required for gonadal morphogenesis in Caenorhabditis elegans. ADAMTS-9 and ADAMTS-20 have an identical modular structure, are distinct in possessing 15 TSRs and a unique C-terminal domain, and have a similar gene structure, suggesting that they comprise a new subfamily of human ADAMTS proteases. ADAMTS20 is very sparingly expressed, although it is detectable in epithelial cells of the breast and lung. However, ADAMTS9 is expressed in embryonic and adult tissues, and therefore we characterized the ADAMTS-9 protein further. Although the ADAMTS-9 zymogen has many proprotein convertase processing sites, pulse-chase analysis, site-directed mutagenesis, and amino acid sequencing demonstrated that maturation to the active form occurs by selective proprotein convertase (e.g. furin) cleavage of the Arg1557-Phe1568 bond. Although lacking a transmembrane sequence, ADAMTS-9 is retained near the cell surface as well as in the ECM of transiently transfected COS-1 and 293 cells. COS-1 cells transfected with ADAMTS9 (but not vector-transfected cells) proteolytically cleaved bovine versican and aggrecan core proteins at the Glu141-Ala146 bond of versican V1 and the Glu177-Ala172 bond of aggrecan, respectively. In contrast, the ADAMTS-9 catalytic domain alone was neither localized to the cell surface nor able to confer these proteolytic activities on cells, demonstrating that the ancillary domains of ADAMTS-9, including the TSRs, are required both for specific extracellular localization and for its versicanase and aggrecanase activities.

The ADAMTS (A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type I motif) family consists of secreted zinc metalloproteases with a precisely ordered modular organization that includes at least one thrombospondin type I repeat (TSR)1, 2. Important functions have been established for several members of the family. ADAMTS-4, ADAMTS-5, and (less efficiently) ADAMTS-1 degrade the cartilage proteoglycan aggrecan and are referred to as aggrecanases (3–5). They play a major role in aggrecan loss in arthritis (6, 7). ADAMTS-1 and ADAMTS-4 participate in the turnover of the aggrecan-related proteoglycans versican and brevican in blood vessels (8) and the nervous system, respectively (9). ADAMTS2 mutations cause dermatosparaxis, a recessively inherited disorder characterized by severe skin fragility that results from incomplete proteolytic removal of the procollagen I amino propeptide (N-propeptide) (10). ADAMTS-3 and ADAMTS-14 are procollagen N-propeptidases with probable roles in procollagen II processing in cartilage or procollagen I processing in tissues other than skin, respectively (11, 12). ADAMTS13 mutations lead to inherited thrombocytopenic purpura, a coagulation disorder caused by deficient proteolytic processing of von Willebrand factor (13). ADAMTS1-null mice have abnormal adipogenesis, defective angiogenesis in the adrenal gland, and a defect of ureteric ECM turnover, leading to hydronephrosis (14). ADAMTS2-null mice have fragile skin, and males are infertile (15). Many other ADAMTS enzymes have been discovered through molecular cloning, and their functions are presently unknown. Altogether, 19 human ADAMTS symbols identifying 18 distinct genes and their products have been assigned (note that ADAMTS5 (1) and ADAMTS11 (4) designate the same gene). ADAMTS are also present in invertebrates, which contain fewer ADAMTS genes than mammalian genomes. Caenorhabditis elegans ADAMTS gene, gon-1, has an essential role in reproduction (16). The protease (GON-1) encoded by gon-1 is required for migration of distal tip cells during gonadal morphogenesis. It may have a role in degradation of basement membrane or for processing of extracellular cues required for cell migration (16). GON-1 is the largest of all ADAMTS en-

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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) AF488803 (ADAMTS9) and AF488804 (ADAMTS20).

The abbreviations used are: TSR, thrombospondin type I repeat; DMEM, Dulbecco’s modified Eagle’s medium; GAG, glycosaminoglycan; ORF, open reading frame; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Gene nomenclature (ADAMTS9 and ADAMTS20) was assigned after consultation with the Human Gene Nomenclature Committee. Adamts9 and Adamts20 are the respective mouse orthologs. The protein products of these genes are designated as ADAMTS-9 and ADAMTS-20. Similar nomenclature is used for other ADAMTS genes and their products. GON-1 refers to the product of the C. elegans gon-1 gene.
zymes described to date and contains 15 TSSRs (16). In addition, it has a presumed globular domain at the C terminus without similarity to known proteins.

Human ADAMTS-9, as previously described (17) contains four TSSRs. Despite being a much smaller enzyme than GON-1, it had greater sequence similarity to it than to any other human ADAMTS (17). Here, we characterize a considerably longer ADAMTS-9 (designated ADAMTS-9B), transferred to subsequently in this paper as ADAMTS-9 that propose the authentic full-length product of ADAMTS9. In addition, we have discovered a novel enzyme, ADAMTS-20, and determined its complete primary sequence. ADAMTS-9 and ADAMTS-20 have an identical domain organization and exon structure and a very similar primary sequence, showing that they comprise a distinct subfamily of GON-1-related ADAMTS proteases in the mammalian genome. We have characterized the zymogen maturation and cellular localization of the more highly expressed of these two proteins, ADAMTS-9, and have investigated its role in proteolysis of the large aggregating proteoglycans versican and aggrecan. Our data demonstrate the critical requirement of the ancillary domains for the pro-peptidase cleavage site. These proteins are therefore of ADAMTS-9, (the signal peptide, prodomain, and catalytic domain), PCR amplification was done using the same forward primer as for the full-length ADAMTS9 cDNA, the reverse primer 5'-AACCTGAGTTTAG-GCAAAGGTTAGGTCG-3' (Xhol site underlined), and fetal heart cDNA (Clontech) as template. The resulting amplicon was cloned in pPFLAG-CMV-5a (Sigma) to generate proteins with in-frame C-terminal FLAG or myc-His tags, respectively, ADAMTS9-GFPFLAG and ADAMTS9-508MYC/HIS. Site-directed mutagenesis of the convertase (e.g. furin) sites (Arg270→Ala, Arg270→Ala, Arg270→Ala) in ADAMTS9-508MYC/HIS was done using the QuikChange site-directed mutagenesis kit (Stratagene).

The insert of the KIAA0688 gene (20) encoding ADAMTS-4 (3) in pBluescript SK (Stratagene) was excised with EcoRI and Xhol and inserted into the corresponding sites of pcDNA3.1MYC/HIS B (Invitrogen) to generate a mammalian expression vector producing untagged ADAMTS-4. The ADAMTS4 and ADAMTS5 ORFs from the convertase-processing site to the stop codon were PCR-amplified and cloned into pPFLAG-CMV-9 (Sigma) for expression in frame with a prepropeptidase leader sequence and three tandem FLAG tags present just downstream of the signal peptide cleavage site. These proteins are therefore secreted with N-terminal FLAG tag 15-FLAG, ADAMTS4-3-FLAG and ADAMTS5-3-FLAG. Site-directed expression plasmids and site-directed mutations were verified by DNA sequencing.

Northern Blot and Quantitative RT-PCR of ADAMTS9 and ADAMTS20 RNA in Situ Hybridization Analysis—Northern blot analysis of mouse and human tissues (Clontech, Palo Alto, CA) was used as the template for rapid amplification of cDNA ends as previously described (1). To confirm that the overlapping cDNA clones obtained represented a contiguous mRNA, the complete ORF was amplified by PCR. The oligonucleotide primers 5'-AACCGCGCCACCACTGATTGTTGATCC-3' (Not I site underlined and start codon italicized) and 5'-CTTCGGAATTAACACTGGCACTTCGACCC-3' (Xhol site underlined and modified stop codon italicized) were used for PCR with human fetal skeletal muscle cDNA as template and Advantage 2 polymerase (Clontech, Palo Alto, CA). The 5.8-kb PCR product was cloned into pGen-T Easy (Promega, Madison, WI) and sequenced completely. cDNA cloning of ADAMTS9 will be reported elsewhere.3 To ask whether there existed additional ADAMTS proteases with a domain organization similar to GON-1 and ADAMTS-9, the human genome sequence (Celera, Rockville, MD) was searched using the amino acid sequence of the unique C-terminal domain of ADAMTS-9, GENSCAN (available on the World Wide Web at genescan.html) to the Genes Database—Multiple tissue northern blots containing 1 μg/lane poly(A+) RNA from mouse embryos and individual adult mouse and human tissues (Clontech, Palo Alto, CA) were hybridized to [α-32P]dCTP-labeled ADAMTS9, ADAMTS20, or ADAMTS9 probes, followed by autoradiographic exposure for 3–7 days. CDNA panels derived from human adult and fetal organs normalized with respect to GAPDH mRNA levels were purchased from Clontech. Real time PCR of these cDNA templates was performed in an ABI Prism 7700 sequence detector using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA), as previously described (12). PCR amplifications were performed in triplicate for all templates, along with parallel measurements of GAPDH cDNA for normalization.

The GAPDH-normalized quantitative data for ADAMTS9 and ADAMTS20 were used to determine the ADAMTS9/ADAMTS20 transcript ratio in all templates examined. The following primers were used for amplification at a concentration of 300 nM each: ADAMTS9 forward, 5'-GGCAAGGGAGGAGCACCTC-3'; ADAMTS9 reverse, 5'-ATCCATC-GAATTCCCTC-3'; ADAMTS20 forward, 5'-GGTGGCAGTTATTGGCAGAAA-3'; ADAMTS20 reverse, 5'-CACGATCCATGCGAACATG-3'. GPHO primers were described previously (12). RT-PCR performed in the absence of template was negative with all primer pairs.

RNA in situ hybridization was performed essentially as previously described (19), using [35S]-labeled antisense and sense RNA probes transcribed from a 600-nt cDNA template encoding the unique domain of ADAMTS-20. Normal human breast and lung tissues, as well as samples of squamous cell carcinoma of breast and adenocarcinoma of lung were obtained under a Cleveland Clinic Foundation Institutional Review Board-approved protocol and fixed in formalin (tissue samples were provided by the Cooperative Human Tissue Network). 5-μm-thick paraffin sections were hybridized to the probes prior to dipping in photographic emulsion (Eastman Kodak Co.) and followed by autoradiographic exposure for 7 days. Nuclei were stained with 4',6-diamidino-2-phenylindole.

ADAMTS9, ADAMTS4, and ADAMTS8 Expression Plasmids—The ADAMTS9 cDNA was excised as a NotI-Xhol fragment and cloned into the NotI and SalI sites of pFLAG-CMV-5a (Sigma) to introduce an in-frame C-terminal FLAG tag (ADAMTS9-FLAG). For expression of ADAMTS9-FLAG, the complete primary sequence was PCR-amplified and cloned into pPFLAG-CMV-9 (Sigma) for expression in frame with a prepropeptidase leader sequence and three tandem FLAG tags present just downstream of the signal peptide cleavage site. These proteins are therefore secreted with N-terminal FLAG tag 15-FLAG, ADAMTS4-3-FLAG and ADAMTS5-3-FLAG. Site-directed expression plasmids and site-directed mutations were verified by DNA sequencing.

ADAMTS-9 Localization in Transfected Cells—COS-1 and 293-HEK cells were maintained and transfected with ADAMTS9-FLAG, ADAMTS9-508MYC/HIS, ADAMTS4-3-FLAG and ADAMTS5-3-FLAG, as described previously (21). Transfected cell lysates and culture medium were harvested separately after 48 h and were separated by reducing SDS-PAGE followed by Western blot analysis using the FLAG M2 monoclonal antibody (Sigma). For immunolocalization of extracellular ADAMTS9-FLAG, ADAMTS9-508MYC/HIS, ADAMTS4-3-FLAG and ADAMTS5-3-FLAG, cells were transfected with anti-FLAG M2 monoclonal antibody 48 h post-transfection without permeabilization as previously described (21). Alternatively, transfected cells were stained following fixation in 4% paraformaldehyde (permeabilization). Nuclei were stained with 4',6-diamidino-2-phenylindole. As controls, COS-1 and 293 cells were transfected with the empty FLAG vector alone, followed by the immunostaining procedure, or the primary antibody was omitted for FLAG staining.

Release of ADAMTS-9 from the cell surface, transfected 293 cells and ECM were harvested by scraping and resuspended in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl). Cells and ECM were gently agitated by end-over-end rotation in PBS alone or in PBS plus 100 mM or 200 mM NaCl at 4 °C for 30 min. ADAMTS-9-508MYC/HIS Purification and Analysis—To obtain stably transfected 293 cells expressing ADAMTS9-508MYC/HIS, selection with G418 (750 μg/ml) was applied after transfection. These cells were maintained in culture medium containing 5% serum and 250 μg/ml G418. Conditioned medium was dialyzed into binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8, containing 0.03% Brij-35 (Sigma)) prior to binding on a 5-nl Ni2+–Sepharose column (ProBond®).

3 K. A. Jungers and S. S. Apte, unpublished data.
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In vitro, the column was washed with 3 column volumes of binding buffer. A gradient of 0–42.5 mM imidazole in binding buffer was used to remove nonspecifically bound molecules from the column. Stepwise elution was done using one-column volume batches of 0–250 mM imidazole in binding buffer. Elution was monitored by Western blotting using antibody 9E10. The majority of protein was determined to elute at 50 mM imidazole. ADAMTS-9-508FLAG-Transfected cells were used to determine the GAG-bearing region, GAG-α, as a result of alternative splicing (8).

Aggrecan monomer was isolated from bovine articular cartilage as previously described (23). Aggrecan (20 μg) was incubated with transfected cells as described above. Neoeptipe Western blot analysis was performed for versican (above), except that the proteolytic cleavage at the Glu1771-Ala1772 bond of aggrecan was detected using anti-Ala1772-Glu-Gly-Gly (AGEG) antisera (24) (provided by Micky Tortorella).

RESULTS

Cloning of ADAMTS9 and ADAMTS20 cDNAs—Our search for novel ADAMTS proteases identified a human expressed sequence tag (GenBank™ accession number AA205581) encoded by IMAGE clone 646675 from neuroepitheliomorph-derivated NT2 cells treated with retinoic acid. The ORF of this expressed sequence tag was homologous to ADAMTS proteases and encoded four TSRs followed by a C-terminal domain containing 10 cysteines that was similar to the C terminus of a polypeptide predicted by the C. elegans F25H18.3 cosmide (C. elegans protein data base Wormpep, www.sanger.ac.uk/Projects/C_elegans/wormpep) and subsequently identified as GON-1. The novel human ORF was designated ADAMTS-9. Completion of the full-length protein coding sequence to the putative start codon required several rounds of rapid amplification of cDNA ends. Together, the cloned cDNA sequences represent an mRNA of 8 kb (Fig. 1a). The 3’ untranslated region in IMAGE clone 646675 contained a consensus polyadenylation signal (AATAAA) 15 nucleotides upstream of the poly(A) tail. The most 5’ clone obtained (TS9-B10) contained 32 bp of the 5’ untranslated region. The putative signal peptide coding sequence was preceded by a methionine codon within a satisfactory Kozak consensus sequence (A at –3 relative to ATG), but there was no upstream, in-frame stop codon.

The search for ADAMTS-9-related proteins led to identification of a polypeptide (Celera hCP1629711) predicted by exons on human chromosome 12. The complete 5733-nl-long ADAMTS-20 ORF was assembled from overlapping cDNA clones (Fig. 1a). The ADAMTS20 mRNA was found in low quantities, routinely requiring 35 cycles of PCR or nested PCR for visualization of the PCR products on a gel. Because of the rarity of ADAMTS20 transcripts as well as the presence of numerous regions that are difficult to PCR-amplify, we have been so far unable to obtain the complete ORF in a single PCR reaction.

Identical Domain Organization and Similar Primary Structure of ADAMTS-9 and ADAMTS-20—ADAMTS-9 and ADAMTS-20 are similar in length, containing 1935 and 1911 amino acids, respectively (Figs. 1b and 2). Each contains a C-terminal array of 14 TSRs (15 TSRs/enzyme) that is interrupted by short “linker” peptides located between TSR-6 and -7 and TSR-8 and -9 that do not have similar sequences. ADAMTS-9 and ADAMTS-20 are very similar to each other, with 48% identity and 64% similarity. The cysteine signatures of individual modules in ADAMTS-9 and ADAMTS-20 are identical to those of most other ADAMTS enzymes, with the exception of the procollagen aminoproteinidases (ADAMTS-2, ADAMTS-3, ADAMTS-14) and ADAMTS-13, which have distinctive prodomains and catalytic domains (12). Each module in ADAMTS-9 and ADAMTS-20 (with one exception, described below) contains an even number of cysteines, suggesting participation in internal disulfide bonds. There are 126 cysteines in mature ADAMTS-9, predicting 63 intrachain disulfide bonds. ADAMTS-20 has a Cys to Tyr substitution in TSR-13 (Fig. 2). Since the substituted Cys is the fourth of six conserved cysteines in TSRs, TSR-13 in ADAMTS-20 may contain two intrachain disulfide bonds instead of three and have an unattached cysteine.

The predicted molecular mass of the full-length enzymes is
Both enzymes contain consensus sites for ADAMTS-9, which is predicted to have a molecular mass of 185,000. The mass will decrease by 3 kDa following signal peptide processing. In addition, both enzymes have a prodomain that is likely to be proteolytically processed prior to or during secretion. ADAMTS-9 contains five consensus furin cleavage sites in its prodomain, whereas ADAMTS-20 contains three (Figs. 1a and 2). Two sites, those corresponding to Arg74 and Arg287 in ADAMTS-9, are conserved with ADAMTS-20. Following processing at the furin recognition sequence closest to the C terminus, mature ADAMTS-9 is predicted to have a molecular mass of 184,000 and mature ADAMTS-20 a molecular mass of 185,000. Both enzymes contain consensus sites for N-linked glycosylation (Asn-X-Ser/Thr, where X is any amino acid except Pro), 9 in ADAMTS-9 and 15 in ADAMTS-20 (Fig. 1b). Five such sites, including three in the unique C-terminal domain, are conserved in ADAMTS-9 and ADAMTS-20. Because of the high likelihood of utilization of these sites, the molecular mass of ADAMTS-9 and ADAMTS-20 will probably be in excess of that predicted (i.e., >185,000). Although there are a number of Ser-Gly or Gly-Ser motifs in both ADAMTS-9 and ADAMTS-20, most are within presumed disulfide-bonded domains and lack the expected sequence context for xylosyltransferase recognition (25, 26). However, one motif in the middle of the ADAMTS-9 spacer domain with the sequence Glu-Tyr-Ser\[sup]330\]-Gly-Ser\[sup]332\]-Glu-Thr-Ala-Val-Glu lies within a sequence context that is compatible with GAG attachment to Ser\[sup]330\] or Ser\[sup]332\]. A similar sequence is present at this location in ADAMTS-20 (Fig. 2). ADAMTS-9 and ADAMTS-20, respectively, contain three and two Cys-Ser-Val-Thr-Cys-Gly (CSVTCG) motifs that are believed to mediate binding to the cell surface molecule CD36 (27, 28) (Fig. 2). In addition, each enzyme contains two BBXB motifs (where B represents basic amino acid and X represents any amino acid) that have been shown to mediate heparin and sulfatide binding (27, 29) (Fig. 2). Neither enzyme contains an Arg-Gly-Asp motif.

**ADAMTS-9 and ADAMTS-20 Do Not Have Identical Zinc-binding Catalytic Site Motifs**—The ADAMTS-9 catalytic site is identical to that of ADAMTS-1 and ADAMTS-15 and very similar to that of ADAMTS-4 (Fig. 3a). The unique feature of ADAMTS-9, ADAMTS-1, and ADAMTS-15 is the presence of a proline residue preceding the third zinc-coordinating histidine (Fig. 3a). The corresponding amino acid is leucine in ADAMTS-4, the next most closely related enzyme. The ADAMTS-20 zinc-binding site is not identical to that of any other ADAMTS but is most closely related to that of ADAMTS-7 and ADAMTS-12 with 4/12 variant amino acids (Fig. 3a). All of the substitutions in the ADAMTS-20 active site relative to ADAMTS-7 and ADAMTS-12 are conservative ones. Alignment and clustering of the published ADAMTS proteases confirm the unique place of ADAMTS-9 and ADAMTS-20 in the ADAMTS family (Fig. 3b) and indicate that they constitute a distinct subfamily of proteases.

**ADAMTS-9 and ADAMTS-20 Are Related to GON-1**—The domain organization and primary sequence of ADAMTS-9 and ADAMTS-20 have a greater similarity to GON-1 than any other mammalian ADAMTS enzyme (Figs. 1b and 2). ADAMTS-9 and ADAMTS-20 are equally related to GON-1 in paired BLAST comparisons. The percentage identity of ADAMTS-9 protein to GON-1 is 33% (that of ADAMTS-20 is 32%), and the percentage similarity (including conservative substitutions) is 46% for both ADAMTS-9 and ADAMTS-20 relative to GON-1. The zinc-binding active site sequence of GON-1 resembles ADAMTS-9 more closely than ADAMTS-20, with just 2 of 14 variant amino acids (Fig. 3a). The conserved C-terminalmost convertase-processing site is at an identical location in ADAMTS-9, ADAMTS-20, and GON-1. The unique C-terminal domain varies slightly in length but nevertheless is highly similar in the three enzymes, including an identical cysteine signature (Fig. 2). TSR-1 is well conserved in these ADAMTS enzymes, but there is less similarity between TSRs 2–15 of...
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ADAMTS-9 and ADAMTS-20 are linked to the carboxyl-terminal domain of GON-1 (amino acids 384–1954) and the carboxyl domain of GON-1 (amino acids 1943–2165). The entire sequence of GON-1 (GenBank™ accession number NP501792) and its corresponding mRNA are not aligned, because it has more TSRs than ADAMTS-9 and ADAMTS-20. Map of GON-1 shows the N terminus of mature ADAMTS-9. Consensus sequences for metalloprotease ADAMTS-9 were indicated by filled asterisks.

Fig. 2. Alignment of primary sequences of ADAMTS-9, ADAMTS-20, and GON-1 with the TSRs of ADAMTS-9 and ADAMTS-20 are boxed. The exposed amino acids are underlined and numbered consecutively. Two linker peptides between TSR-6 and -7 and between TSR-8 and -9 are indicated by filled circles. Cysteine in the carboxyl-terminal domain (but not elsewhere) is indicated by filled asterisks. A Cys→Tyr change in TSR-13 of ADAMTS-9 is demonstrated.

ADAMTS-20 is indicated by the filled circle. Prospective GAG attachment sites are indicated by open asterisks. Exon junctions are indicated by open circles. Sense probe showed no hybridization (Fig. 4, i).
ADAMTS-9 is located near the cell surface but not in conditioned medium—ADAMTS-9-pH101 was detected in lysates of transiently transfected COS-1 and 293 cells as two major anti-FLAG reactive bands migrating at ~180 and ~250 kDa under reducing conditions (Fig. 5a), although the 250-kDa band was inconsistently seen. In addition, a number of smaller FLAG-tagged bands, presumably derived from the full-length ADAMTS-9 were also seen (Fig. 5a, upper panel). Treatment of ADAMTS-9-expressing cells with an increasing concentration of NaCl demonstrated a concentration-dependent release of ADAMTS-9 from the cells (Fig. 5a, lower panel). Due to the unfavorable effects of supraphysiological salt concentrations on cell viability, concentrations higher than 340 mM were not tested.

To identify the cellular or extracellular location of ADAMTS-9 and contrast it with ADAMTS-4, ADAMTS-5, and the ADAMTS-like protein, punctin (21), transiently transfected COS-1 and 293 cells were immunostained with anti-FLAG M2 antibody. ADAMTS-9-transfected cells produced a stronger immunoreactive band than ADAMTS-9-transfected cells. The ADAMTS-9 catalytic domain without the ancillary domains (encoded by ADAMTS-9-pH101) did not process aggrecan or versican at these sites (data not shown).

Intracellular Maturation of ADAMTS-9 Involves N-Glycosylation of the Prodomain and Furin Processing at the Arg287-Phe288 Bond—The predicted molecular masses of signal peptide processed and ADAMTS-9-pH101 that is processed at the consensus proprotein convertase sites are shown in Fig. 6a. Transient expression of ADAMTS-9-pH101FLAG in 293 cells followed by pulse-chase analysis, immunoprecipitation using anti-FLAG M2 antibody, and fluorography identified three major immunoreactive bands in cell lysates with molecular masses of ~66, 56, and 54 kDa, respectively. The relative intensity of these bands varied with the duration of pulse and chase. After a 15-min pulse and 60-min chase, the amount of the 66-kDa protein seen was significantly greater than that seen after a 15-min chase (Fig. 6b). Conversely, the 54–56-kDa doublet was more prominent after a 15-min chase (Fig. 6b). The 66-kDa band intensified substantially after a 135-min chase with very little of the 54–56-kDa doublet being detectable. When cell lysate and culture medium were immunoprecipitated and immunoblotted with anti-FLAG M2 antibody 48 h following transfection of QBI 293A cells, the cells contained the 66-kDa band and essentially no 54–56-kDa doublet (Fig. 6c). When these cell lysates were treated with PNGase F, this 66-kDa band was reduced to a doublet of ~54–56 kDa (Fig. 6c). Collectively, these observations suggest that the 66-kDa band is derived from a 54–56-kDa precursor by N-linked glycosylation. N-Glycosylation of ADAMTS-9-pH101FLAG was confirmed by culture of stably transfected cells in the presence of the tunicamycin A homolog (data not shown). Under the pulse-chase conditions used, no labeled protein could be immunoprecipitated from the conditioned medium (Fig. 6b), and protein corresponding in size to the active form (28 kDa) was not seen in cell lysate. However, in stably transfected cells (not shown) or immunoprecipitation 48 h after transfection, the mature, tagged protein could be detected in culture medium (Fig. 6c). Deglycosylation did not alter the migration of the secreted mature enzyme (Fig. 6c). N-terminal sequencing of the secreted mature ADAMTS-9-pH101FLAG gave the sequence Phe-Ser-Leu-Tyr-Pro-Arg-Phe.

Furin-deficient CHO-RPE 40 cells did not process ADAMTS-9 (Fig. 7a). Processing was rescued by transfection with furin (Fig. 7a). In QBI 293A cells, the Arg287 → Ala, Arg288 → Ala, or Arg289 → Ala mutants did not affect the appearance of...
the mature protein in the medium, but abrogation of the most C-terminal processing site (Arg$^{287}$ → Ala) resulted in failure of processing to the mature form (Fig. 7b). Expression of the Arg$^{287}$ → Ala mutant resulted in anomalous bands of 40 and 45 kDa in conditioned medium in addition to the mature protein (Fig. 7b). Instead of the mature 28-kDa form, expression of the Arg$^{287}$ → Ala mutant resulted in the appearance of 37- and 42-kDa proteins in culture medium whose identity is not known (Fig. 7b).

### DISCUSSION

**Identification of the Full-length Product Of ADAMTS9**—Although a report of the ADAMTS9 mRNA (GenBank™ accession number AF 261918) and ADAMTS9 chromosomal localization was published (17) while our work was in progress, the novel sequence data we report here extend the predicted C terminus of that protein further to include an additional 10 TSRs and the unique C-terminal domain. Our data suggest that the ADAMTS9 transcript presented here encodes the full-
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length, authentic product of this gene for several reasons. First, the previously described ADAMTS9A cDNA diverges from our ADAMTS9 sequence at an unspliced intron (deduced by comparison of the ADAMTS9A and mRNA sequence with the ADAMTS9 genomic sequence). Another ADAMTS9 product predicted by the sequence of the KIAA1233 gene (GenBank™ accession number AB037733) is incomplete at both the amino- and carboxy termini. Comparison of this sequence with the cDNA sequence reported here and the ADAMTS9 genomic sequence suggests the inclusion of an unspliced intron leading to a premature stop codon. Intron inclusion suggests cloning of partially processed pre-RNA, not authentic mRNA. Second, the ADAMTS9-9A transcript does not contain a consensus polyadenylation sequence upstream of the poly(A) tail, in contrast to the ADAMTS9 transcript reported here. Third, Northern analysis demonstrated that probes from the novel sequences we describe here, as well as a probe from the region shared by all transcripts (data not shown), hybridized to the same major 8-kb band on Northern blots, suggesting that the dominant transcript in most tissues encodes the longer form, ADAMTS-9B.

Previous studies of ADAMTS9 had shown widespread expression in fetal and adult tissues by RT-PCR (18). Our studies using Northern blots and quantitative RT-PCR are in agreement with this and provide additional information about the mRNA size. The 8-kb ADAMTS9 mRNA is compatible with the long ORF we have cloned. The identity of the smaller mRNAs found in kidney and ovary is presently unclear. These could represent alternative splice forms or unrelated transcripts that cross-hybridize with the probe. Since ADAMTS20 mRNA is undetectable on Northern blots, both its size and the existence of alternative forms is unknown. In fact, ADAMTS20 transcripts are extremely rare in all of the tissues we have examined, and there are only two human ADAMTS20 expressed sequence tags (AU132653 and BG212007) reported in GenBank™. Nevertheless, a sensitive RNA in situ hybridization approach did demonstrate low levels of expression in epithelial cells of breast and lung origin. The prevalence and biological significance of this low level expression is unknown. Therefore, at the protein level, detailed characterization of the more abundantly expressed enzyme, ADAMTS-9, was subsequently undertaken.

ADAMTS9 and ADAMTS20 Constitute a Distinct Subfamily of ADAMTS Proteases—ADAMTS proteases can be clustered into subfamilies of closely related enzymes on the basis of their domain organization and primary sequences. The procollagen aminopropeptidase subfamily (ADAMTS-2, -3, and -14) represents the most striking example, and other enzymes such as ADAMTS-7 and -12 and ADAMTS-6 and -10 occur in closely related pairs. The ADAMTS-9 and ADAMTS-20 subfamily is particularly interesting, because it is the first such ADAMTS subfamily with a closely related ortholog in invertebrates, indicating, perhaps, a highly conserved physiological role. However, unlike the other ADAMTS subfamilies, ADAMTS-9 and ADAMTS-20 do not have identical zinc-binding active site sequences. Furthermore, their expression patterns are quite different, suggesting they may have nonredundant biological roles.

The genomic organization of ADAMTS9 and ADAMTS20 bears little resemblance to other genes in the family. ADAMTS-1 is encoded by nine exons, and the prodomain, disintegrin-like domain, and central TSR are each encoded by single exons, whose boundaries coincide with the domain boundaries (30). In ADAMTS-1, a single terminal exon encodes the spacer and two C-terminal TSRs (30). This is clearly not the case with ADAMTS9 and ADAMTS20, where few domains other than the TSRs are encoded by single exons. ADAMTS13 (13) has 29 protein-coding exons whose boundaries are different from ADAMTS-1, -9, and -20. The procollagen aminopropeptidases share a different genomic organization (12). Therefore, gene structure may be conserved in ADAMTS subfamilies, but there is not a characteristic gene structure that is shared by the entire family.

The Cys to Tyr substitution in TSR-13 is not an artifact of cloning, because we found it both in the genomic DNA (in Celera and GenBank™ databases), in the cloned cDNA, and in a small number of normal human alleles in which the corresponding exon was subjected to PCR-direct sequencing (data not shown). It may represent a non-synonymous single nucleotide (4715A→G) polymorphism, since TSR-13 in mouse ADAMTS-20 has the typical six-cysteine signature. The preva-
ence and significance of this amino acid change in humans is not presently known and will be investigated further.

Intracellular Maturation Of ADAMTS-9 Involves Glycosylation of the Prodomain and Processing at a Single Proprotein Convertase-processing Site—Following removal of the signal peptide and entrance into the secretory pathway, ADAMTS proteases, like ADAMs and some MMPs, are processed further by one or more proprotein convertases to remove the prodomain and undergo additional post-translation modification such as glycosylation. Proprotein convertases (e.g. furin) are serine proteases present in the Golgi apparatus or at the cell surface that typically cleave immediately following a consensus recognition sequence rich in basic residues (31). Our studies showed that processing did not occur in the absence of furin but could be rescued by transfection of furin, demonstrating that proprotein convertases were essential for pro-ADAMTS-9 maturation.

Our studies suggest that there is rapid glycosylation of the ADAMTS-9 prodomain following synthesis that is essentially complete in about 2 h. There is no N-glycosylation of the catalytic domain, consistent with the observation that the prodomain contains three consensus N-glycosylation sites, whereas the catalytic domain has none. Our data indicated processing of the Arg287–Phe288 peptide bond, whereas none of the other furin sites appear to be used for enzyme maturation. We should emphasize that the Arg280 mutation would abrogate two furin sites, since this residue serves as the P1 Arg for the Arg-Glu-Lys-Arg287 site as well as the P4 residue for the Arg-Thr-His-Arg283 site. We could detect the 28-kDa mature form intracellularly in the wild-type and Arg33Ala mutant. This then accumulates in the medium following secretion through the constitutive secretory pathway. On the other hand, in the Arg287Ala mutant, the precursor is not processed intracellularly and accumulates in the medium along with other unidentified bands. The N terminus of mature ADAMTS-9 determined by amino acid sequencing was in agreement with the location of the N terminus of mature ADAMTS-1, ADAMTS-4, and ADAMTS-13, suggesting that although more than one processing site may be present, the C-terminal furin-processing site is generally used for production of the mature ADAMTS enzymes.

Western blotting of full-length ADAMTS-9 suggested that it undergoes substantial post-translational modification. In keeping with the number of consensus sites for N-linked glycosylation and the large number of serine and threonine residues, glycosylation of full-length ADAMTS-9 has also been noted (data not shown), as is shown in the prodomain. Expression of full-length ADAMTS-9 demonstrated the existence of a number of smaller FLAG-tagged fragments that were presumably derived from it by proteolysis. Regulated processing has been noted in ADAMTS-1 (32), ADAMTS-4 (33), and ADAMTS-12.

**FIG. 6.** a, scheme of the protein encoded by ADAMTS91–508FLAG. The domains included in the expressed proteins and the locations of N-linked sugar attachment (lollipops) and FLAG tag are shown. Below this are the protein species predicted following signal peptidase cleavage or cleavage at each of five consensus furin cleavage sites. The expected molecular mass of each unmodified protein species is shown at the right. b, pulse-chase analysis of ADAMTS91–508FLAG–transfected QBI 293A cells. Cells were pulsed with radiolabeled amino acids and chased for varying times as indicated. Control cells were transfected with empty expression vector. Cell extracts and media were immunoprecipitated with anti-FLAG M2 monoclonal antibody and detected by fluorography. The arrowhead indicates a doublet at 54–56 kDa, and the arrow indicates a major N-glycosylated band at 66 kDa. C, cell lysates; M, medium. Molecular mass markers are shown at left. c, deglycosylation of ADAMTS91–508FLAG by PNGase F. Transiently transfected QBI 293A cell lysates and culture medium were immunoprecipitated with anti-FLAG M2 48 h after transfection. Western blot analysis was done using anti-FLAG-M2. Ig, the immunoglobulin heavy chain. The arrow indicates the mature form in culture medium, and the arrowheads indicate the intracellular zymogen form.
(34) and is a potentially intriguing phenomenon because the released ancillary domains could have interesting biological functions or modify the function of ADAMTS-9 (33). Proteolytic fragments of the native enzyme will be sought in tissues and cells once specific high affinity antibodies are available.

ADAMTS-9 Is Located near the Cell Surface and Is Involved in Versican and Aggrecan Degradation—Neither ADAMTS-9 nor ADAMTS-20 nor any of the other known ADAMTS proteases has a potential transmembrane sequence or a glycosylphosphatidylinositol signal anchor sequence. Therefore, these are not predicted to be membrane-anchored enzymes. Accordingly, studies with various ADAMTS proteases have shown that they are soluble or associated with the ECM (3, 4, 35). ADAMTS-9 and ADAMTS-4 are therefore the first ADAMTS proteases shown to localize near the cell surface, as demonstrated by immunofluorescence microscopy, although their precise location relative to the cell membrane or the binding mechanism is presently unknown. In contrast, both the localization and appearance of ADAMTS-5 distribution are different. Furthermore, although restricted to the ECM, ADAMTS-5 presents a different distribution than punctin, an ADAMTS-like protein comprising only ancillary domains (21). Punctin localization to the cell substratum (21) and the failure of ADAMTS-91–508 or C-terminally truncated ADAMTS-1 (35) to be located in either the ECM or cell surface strongly validates the role of the ancillary domains in anchoring these enzymes near the cell. ADAMTS-9 has consensus sites for binding to heparin (and therefore to heparan sulfate proteoglycans) and CD36, and these may be candidate cell surface and pericellular ECM ligands. In support of this possibility, ADAMTS-9 was released from cells and ECM by gentle washes with low concentrations of salt.

To identify potential substrates for ADAMTS-9, we relied upon comparison of the ADAMTS active site sequences, the phylogenetic profile of the ADAMTS family, and the previous descriptions of their enzymatic activities. The ADAMTS enzymes (ADAMTS-1, ADAMTS-4, and ADAMTS-5) that process the large aggregating proteoglycans versican, aggrecan, and brevican have very similar (although not identical) active site sequences, but they have different domain structures. Because ADAMTS-9 has an active site sequence identical to that of ADAMTS-1 and similar to that of ADAMTS-4, we considered that it might be a proteoglycan core protein-degrading enzyme. Since ADAMTS-9 was not secreted into the culture medium of cells, we used a cell-based ADAMTS assay. Serum-free culture medium has the appropriate pH and salt concentration for ADAMTS activity and, when supplemented with calcium, provided the reaction conditions necessary for the versicanase and aggrecanase assays.

By analogy with aggrecanase-susceptible sites in aggrecan, Sandy et al. (8) had previously predicted two putative ADAMTS cleavage sites in human versican and had prepared polyclonal antisera recognizing one such predicted neoepitope generated by proteolysis of the V1 Glu441–Ala442 bond (8). Versican V0 and V1 forms differ in the inclusion of the GAG-α region that is present in the V0 form but missing in the V1 form. Accordingly, the peptide bond cleaved has a different location in the two forms (8). Consistent with the mixed population of versican made by smooth muscle cell cultures, two bands (70 and 180 kDa corresponding to G1 versican fragments DPEAAE (V0 form) and DPEAAE (V1 form)) were seen in previous studies of ADAMTS-4 processing of versican (8). Of these, the 70-kDa band was considerably stronger, consistent with there being more of the V1 form in the versican preparation (8). In contrast, neither ADAMTS-4 nor ADAMTS-9 proteolysis gave an anti-DPEAAE reactive band at 180 kDa in our experiments.

A refined comparison of ADAMTS-4 and ADAMTS-9 cannot be done in the cell-based assay, since transfection efficiency, expression levels, secretion, and zymogen processing may be different. Purified ADAMTS-9 is not yet available, and given its complex domain structure and cell surface localization, it may be difficult to obtain. We have purified ADAMTS-91–508, but it does not process versican or aggrecan, demonstrating the essential role of the ancillary domains in substrate recognition and/or binding. Therefore, this form cannot be used in kinetic studies to compare with ADAMTS-4. With these limitations,
The procollagen N-propeptidases have identical domain organizations and identical active site sequences (in fact, 70 amino acids around the zinc-binding site are identical in these enzymes) (12). This identity suggests that the structural requirements for procollagen processing are very stringent, and indeed, the catalytic sites of the procollagen N-propeptidases have a distinctive cysteine signature not found in other ADAMTSs. Similarly, ADAMTS-13, the von Willebrand factor protease, is unlike any other ADAMTS in its domain organization and is clearly the major, if not only, von Willebrand factor-processing enzyme (13), suggesting that the structural requirements for this function are stringent as well. In contrast, the four ADAMTS enzymes that degrade proteoglycan core proteins have neither an identical domain organization nor identical active site sequences. This dissimilarity suggests a relatively relaxed structural requirement for proteoglycan processing and supports the likelihood that ADAMTS enzymes may have activity against proteoglycans. In the future, it will be important to define the relative prevalence of each of the proteoglycan-degrading ADAMTS enzymes in different tissues as well as in diseases such as arthritis and to determine their tissue-specific role by targeted inactivation of the corresponding mouse genes. In future studies, it will also be important to ask whether ADAMTS-20 can process versican and aggrecan and to ask whether ADAMTS-9 and ADAMTS-20 have biological roles similar to GON-1.

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Characterization of ADAMTS-9 and ADAMTS-20 as a Distinct ADAMTS Subfamily Related to Caenorhabditis elegans GON-1

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