We describe the discovery and characterization of ADAMTS10, a novel metalloprotease encoded by a locus on human chromosome 19 and mouse chromosome 17. ADAMTS10 has the typical modular organization of the ADAMTS family, with five thrombospondin type 1 repeats and a cysteine-rich PLAC (protease and lacunin) domain at the carboxyl terminus. Its domain organization and primary structure is similar to a novel long form of ADAMTS8. In contrast to other ADAMTS proteases, ADAMTS10 is widely expressed in adult tissues and throughout mouse embryo development. In situ hybridization analysis showed widespread expression of Adamts10 in the mouse embryo until 12.5 days of gestation, after which it is then expressed in a more restricted fashion, with especially strong expression in developing lung, bone, and craniofacial region. Mesenchymal, not epithelial, expression in the developing lung, kidney, gonad, salivary gland, and gastrointestinal tract is a consistent feature of Adamts10 regulation. N-terminal sequencing and treatment with decanoyl-Arg-Val-Lys-Arg-chloromethylketone indicate that the ADAMTS10 zymogen is processed by a subtilisin-like proprotein convertase at two sites (Arg<sup>64</sup> | Gly and Arg<sup>233</sup> | Ser). The widespread expression of ADAMTS10 suggests that furin, a ubiquitously expressed proprotein convertase, is the likely processing enzyme. ADAMTS10 expressed in HEK293F and COS-1 cells is N-glycosylated and is secreted into the medium, as well as sequestered at the cell surface and extracellular matrix, as demonstrated by cell surface biotinylation and immunolocalization in nonpermeabilized cells. ADAMTS10 is a functional metalloprotease as demonstrated by cleavage of α<sub>2</sub>-macroglobulin, although physiological substrates are presently unknown.

Proteolytic processing of structural components of the extracellular matrix (ECM)<sup>1</sup> and cell signaling-related molecules such as cytokines, growth factors, their binding proteins, receptors, and adhesion molecules has important biological consequences (1, 2). Proteases that cleave such molecules thus play important roles in tissue remodeling, morphogenesis, inflammatory and degenerative diseases, and cancer. Zinc-metalloendopeptidases (metalloproteases) comprise an important superfamily of such enzymes. Specific roles for distinct metalloprotease families and their individual members have emerged over the past decade through genetic studies in humans and mice. Matrix metalloproteases are the major ECM-degrading enzymes, but they also have a role in proteolysis of other secreted molecules and cell surface proteins (1, 2). ADAMs are primarily “shedases,” proteases that process cell surface molecules and are thought to have little, if any, direct role in ECM catabolism (3, 4). The ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type 1 motifs) family was unknown until 1997 (5), but functions for some of these enzymes are beginning to emerge (5–11). Known ADAMTS substrates include the proteoglycans aggrecan, versican, and brevican; the fibrilляр procollagens I, II, and III; and von Willebrand factor (6, 9, 10, 12–14). The processing of von Willebrand factor and the fibrilляр procollagens by ADAMTS13 and by the procollagen amino-propeptidases (ADAMTS2, -3, and -14), respectively, is essential for their maturation to fully functional molecules (6, 7, 9, 15, 16). These two processing activities appear to be highly specialized, and the enzymes responsible for them have distinct sequence and structural features not shared by the other ADAMTS proteases (17, 18). On the other hand, a number of ADAMTS with disparate domain and sequence features (such as ADAMTS1, ADAMTS4, ADAMTS5, and ADAMTS9) are known to process large aggregating proteoglycans such as aggrecan and versican (10, 12, 20, 21). Nevertheless, this is not a general property of all ADAMTS proteases, since we have shown recently that ADAMTS7 cannot process versican or aggrecan at sites cleaved by the other proteoglycan-degrading ADAMTS (22).

ADAMTS proteases are modular, consisting of a protease domain and an ancillary domain (23). The protease domain of these enzymes, like that of ADAMs, but not MMPs, is of the reprolysin (snake venom) type. The hallmark of the ADAMTS proteases is the presence of at least one thrombospondin type 1 repeat (TSR). Other highly conserved modules are arranged around this central TSR in a specific organization, and there are additional TSRs near the carboxyl terminus in all members of the ADAMTS family with the exception of ADAMTS4 (23). ADAMTS proteases are synthesized as zymogens that are targeted to the secretory pathway and activated by proprotein convertases. Zymogen processing leads to removal of a 200–220-amino acid-long podomain in the secretory pathway or at the cell surface.

19 mammalian ADAMTS proteases are known, and all except ADAMTS10, the subject of this article, have previously...
been described in the literature. Within the ADAMTS family, subsets of proteases have highly conserved domain organization, primary sequence and gene structure, suggestive of a close evolutionary and perhaps functional relationship (7, 20, 22, 23). In this context, determination of the primary structure of ADAMTS10 led to realization of a putative long form of ADAMTS6, whose domain organization and primary structure support the contention that it forms a phylogenetic subset with ADAMTS10. Unlike most other ADAMTS proteases, including ADAMTS6 (24), ADAMTS10 is widely expressed. We investigated the developmental regulation of the ADAMTS10 gene in mice and the activation mechanism and localization of the enzyme in cultured cells. ADAMTS10 is shown to be a functional metalloprotease, although its physiological substrates are presently unknown.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning of Human and Mouse ADAMTS10**—Using the tBLASTn (Basic Local Alignment Search Tool) program at the National Center for Biotechnology Information, we searched the data base of expressed sequence tags (dBEST), using the protein sequences of a number of ADAMTS proteases, and identified similarities in a human EST (GenBank accession number A588434) derived from the human prostate-derived IMAGE clone 1101403. The IMAGE clone was purchased (Research Genetics, Huntsville, AL), and the insert was sequenced in its entirety. Oligonucleotide primers based on the sequences at the ends of this clone were used with human fetal brain cDNA (Marathon cDNA, Clontech, Palo Alto, CA) as a template to perform iterative rapid amplification of cDNA ends by PCR at 52°C, 60°C, and 68°C for 30 s, and 6 min by PCR at 95°C for 30 s, and 68°C for 5 min. The resulting 3.2-kbp amplicon was gel-purified and ligated into the pGEM-T easy vector (Promega Corp., Madison, WI). The insert of a sequence-verified clone was then ligated into the EcoRI and XhoI site of pcDNAmyc His A+ (Invitrogen) for expression of ADAMTS10-(1–463) with a C-terminal tandem myc and His tag. The full-length ADAMTS10 cDNA described above was cloned in frame with a C-terminal tandem myc and His tag.

**Transfection and Selection of Stable Cell Lines**—HEK293F cells (Invitrogen) at 80% confluence were transfected in 6-well plates with 100 ng of full-length ADAMTS10 or ADAMTS10-(1–463) expression plasmid DNA using Fugene 6 (Roche Applied Science) as per manufacturer's instructions. At the first medium change, it was supplemented with 2 μg/ml puromycin (Mediatech, Herndon, VA). Disks were isolated using cloning discs (PGC Scientific, Frederick, MD) and expanded. Western blotting with anti-myc monoclonal antibody 9E10 (Invitrogen) was used to determine the level of protein expression in the media of these clones. Western blotting with anti-myc monoclonal antibody 9E10 (Invitrogen) was used to determine the level of protein expression in the media of these clones.

**Expression and Characterization of ADAMTS10 and ADAMTS10-(1–463)**—Stably transfected cells expressing full-length ADAMTS10 and ADAMTS10-(1–463) were cultured in three-tier flasks (Nunc, Rochester, NY) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. When cultures were 80% confluent, the serum-containing medium was replaced with serum-free 293CD medium (Invitrogen) followed by further culture at 37°C in the presence of 8% CO2 for 5 days. Conditioned medium was collected, centrifuged briefly to remove cellular debris, and supplemented with NaCl to a final concentration of 0.5 M. ProBond resin (Invitrogen) was prepared by washing with 1 bed volume of binding buffer (0.5 M NaCl, 20 mM sodium phosphate, pH 7.8). The media and resin were mixed overnight at 4 °C in a 1:1 (v/v) ratio in binding buffer. After this binding step, the resin was pelleted by centrifugation at 1000 × g and then washed five times with 10 bed volumes of binding buffer. Bound proteins were eluted by sequential washes with high pH acetate buffer containing 50, 100, and 250 mM imidazole. The washes and eluted protein fractions were assayed for the presence and purity of desired proteins by Western blotting (using anti-myc monoclonal antibody) and by reducing SDS-PAGE with Coomassie Blue staining, respectively. Maximal yield was obtained on elution in 100–250 mM imidazole.

Following purification of ADAMTS10-(1–463), major bands of ~52, ~50, and ~29 kDa were excised after electrophoretic analysis of a polyvinylidene difluoride membrane. N-terminal sequence was determined by Edman degradation at the National Institutes of Health-supported Biotechnology Core of the Lerner Research Institute.

For identification of the zymogen-processing enzyme, ADAMTS10-(1–463) expressing cells were treated with increasing concentrations (1–100 μM) of the lipid-permeable furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (deca-BrK-ckn) (Calbiochem) for 24 h, and secreted protein was detected by Western blot analysis of conditioned medium as previously described (22). Purified ADAMTS10-(1–463) was deglycosylated with PNGase F (New England Biolabs, Beverly, MA) and detected by Western blotting with anti-myc antibody 9E10 as previously described (20, 22). Characterization of full-length ADAMTS10 was done using stably transfected HEK293F cells or transiently transfected COS cells or substantially purified protein. Western blotting was done with anti-myc monoclonal antibody 9E10. Protein deglycosylation was done as previously described using purified protein (20, 22). Processing of α2-macroglobulin (α2-M) was tested by incubation with purified protein as described previously (22). Proteolysis of the aggrecan core protein using ADAMTS10- and ADAMTS4-transfected cells was evaluated as described.

2 Gene nomenclature has been assigned in agreement with the Human Gene Nomenclature Committee. ADAMTS10 and ADAMTS10 are human and mouse orthologs. The protein products of both genes are designated as ADAMTS10. Similar nomenclature is used for other ADAMTS genes and their products.
FIG. 1. A, domain structure of ADAMTS10 and ADAMTS6B. The key to the domains depicted in the schematic diagram is at the bottom. B, alignment of predicted amino acid sequences of mouse (Adams10) and human ADAMTS10 with ADAMTS6B. Three potential furin cleavage sites (e.g., RQRR) are indicated by brackets. The asterisk over two sites indicates that these were confirmed by N-terminal sequencing. The zinc-binding histidine triad of the active site and the "Met-turn" (LMA) are in boldface type and boxed. The disintegrin-like domain is in black type against a gray background, and the cysteine-rich domain is in white type against a gray background. The spacer domain is indicated by a dashed
were stained with 4′,6-diamidino-2-phenylindole as previously described (26).

ADAMTS10 Localization in Transfected Cells—These studies examined the distribution of full-length ADAMTS10 in vitro, in regard to the cells expressing it. COS-1 cells (ATCC, Manassas, VA) were transiently transfected with 1 μg of full-length ADAMTS10 prior to immunofluorescence localization of secreted protein in nonpermeabilized cells, essentially as previously described (27). ADAMTS10 was detected using antibody Y10 and Alexa-488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc., Eugene, OR) in an indirect immunofluorescence method that does not detect intracellular protein. Following staining for tagged ADAMTS10, cells were permeabilized and nuclei were stained with 4′,6-diamidino-2-phenylindole as previously described (26) following reducing SDS-PAGE (27). Stably transfected HK293F cells expressing ADAMTS10 in suspension were biotinylated as previously described (22). Isolation of biotinylated proteins from the cell surface and their analysis by electrophoresis was as previously described (22). As a control, cells were treated with trypsin to eliminate all cell surface proteins prior to biotinylation, essentially as previously described (22). Isolation of biotinylated proteins from the cell surface and their analysis by electrophoresis was as previously described (27), followed by fluorescent microscopy. Medium, cell lysate, and extracellular matrix from these cultures and from stably transfected HK293F cells were collected as previously described and subjected to immunoblotting with antibody Y10 following reducing SDS-PAGE (27).

RESULTS
cDNA Cloning of Human and Mouse ADAMTS10—Using the tBLASTn algorithm to scan dbEST for novel ESTs that were homologous to cognate ADAMTS proteins, we identified the EST 1101403. This EST was identified when the database was screened with the sequence of ADAMTS6 but not with other ADAMTS proteins. Following extension of the EST by rapid amplification of cDNA ends in both directions, we generated an amplicon of 3.2 kbp from human fetal lung and the bovine genome sequence in GenBankTM was used to predict the ORF encoding the cysteine-rich domain through to the 3′-untranslated region of mouse ADAMTS10. Mouse chromosome 17 genomic sequence in GenBankTM was used to predict the likely amino acid sequences upstream of this clone that were then validated by cDNA cloning (by PCR of the complete ORF using mouse embryo cDNA) and sequencing. The human and mouse nucleotide and predicted amino acid sequences have an overall identity of 86 and 91%, respectively (Fig. 1B), although mouse ADAMTS10 is one amino acid longer (1104 aa). The putative start codon of human ADAMTS10 is the N-terminalmost ATG codon in the predicted ORF and is within an appropriate Kozak consensus sequence (although there is not a purine nucleotide at position –3 relative to A (position 1) of ATG, there is a G at +4 constituting an acceptable consensus start context) (28). The mouse ADAMTS10 start codon is also within a good Kozak consensus sequence.

Features of the Primary Structure of ADAMTS10—Numerous sequence features in mouse and human ADAMTS10 are very highly conserved (Fig. 1B), and the discussion that follows pertains to human ADAMTS10, mentioning mouse ADAMTS10 only where it differs. Overall, the domains of ADAMTS10 are very similar to those of other ADAMTSs, and each of its domains is comparable in length and number of cysteine residues with those of the other ADAMTS proteases (24).

The start codon is followed by a signal peptide containing a region of 11 hydrophobic residues (Trp17–Phe26), suggesting that ADAMTS10 is a secreted protein (Fig. 1B). According to consensus observations made for a number of proteins (the so-called –1, –3 rule) (29), it can be predicted that signal peptidase cleavage probably occurs following Ala25, and the secreted form has the N terminus Phe26–Arg-Ser-Gln.

The prodomain, extending from Phe26 to Arg233, by analogy with other ADAMTS proteases is somewhat unusual in the ADAMTS family in containing only one sequence motif, in complete agreement with the proproteinase conversion recognition sequence Arg-Xaa-Arg/Lys-Arg (Fig. 1B) (35–37). Most ADAMTS proteases have multiple proprotein convertase recognition sites, with the most C-terminal of these undergoing the processing that yields the final processed form (16, 20, 22, 30). At the expected location corresponding to the final processing site (i.e. Arg233), the sequence (Gly-Leu-Lys–Arg233) does not match the optimal furin consensus (Fig. 1B). There is a dibasic motif encompassing the P1 and P2 residues, but the P4 residue is Gly, and there is not a compensating Arg residue at the P6 position (37). However, there is a Ser residue at the putative P1 position, which is found in over 50% of processed proproteases (37).

The ADAMTS10 catalytic domain contains a typical zinc-binding active site sequence (Fig. 1B) that is not different from other ADAMTSs. The TS domains are underlined by a thick line and are numbered sequentially, and the PLAC domain is enclosed in a rounded box, and its cysteines are shown as white type on a black background. Putative heparin and GAG-binding sites in TSR1 are indicated as white on black rectangles and white on black circles, respectively. A possible GAG attachment site in the ADAMTS6B spacer domain (Ser-Gly-Ser) is indicated by boldface type. Exon junctions are indicated by arrowheads and are identical in both ADAMTS10 and ADAMTS6.
identical to any other ADAMTS protease. The catalytic domain, disintegrin-like domain, and cysteine-rich domain have the typical sequence layout and number of cysteine residues (8, 8, and 10, respectively) seen in other ADAMTS and are predicted to be internally disulfide-bonded. The central TSR of ADAMTS10 contains a possible sulfatide/glycosaminoglycan (GAG) binding motif (Trp550-Thr-Pro-Trp) at its N terminus and also contains a basic region (Arg591-Arg-Arg-His-Arg) that could mediate GAG binding (Fig. 1B). The four C-terminal TSRs do not have these motifs and do not resemble each other substantially, although each has the conserved N-terminal Trp residue and the signature six-cysteine arrangement typical of TSRs. At the carboxyl terminus of ADAMTS10, a cluster of six cysteine residues has the hallmark arrangement of a PLAC (protease and lacunin) domain (Fig. 1B). This domain was first described in an ADAMTS-like ECM protein, lacunin (31), and is also found toward the carboxyl terminus of some proprotease convertases and ADAMTS proteases.

In addition to glycosylation within the prodomain, other consensus N-linked glycosylation sites are present and conserved within mouse and human ADAMTS10, predicting that activated ADAMTS10 is likely to be a glycoprotein. Two such sites are located in the spacer and one within TSR3. An N-linked glycosylation site in the catalytic domain of human ADAMTS10 is absent in the mouse. The predicted molecular mass of the human and mouse ADAMTS10 zymogen and fully processed forms are 118 and 95 kDa, respectively.

Homology to ADAMTS10 Uncovers a Longer Form of ADAMTS6 (ADAMTS6B)—The close sequence similarity of ADAMTS10 to ADAMTS6 led us to ask whether there existed a longer form of this protease with the same modular organization as ADAMTS10. Analysis of 3’ genomic sequence of ADAMTS6 revealed previously unknown exons that could splice to a putative splice donor site 150 bp upstream of the previously identified ADAMTS6 stop codon (24). The conceptual translation product that included the new exons added three additional TSRs and a C-terminal PLAC domain to the cognate ADAMTS6 protein, mirroring precisely the structure of ADAMTS10. This conceptual product of ADAMTS6 is designated ADAMTS6B (Fig. 1B), and its existence is supported by numerous ESTs in GenBank™.

ADAMTS10 and ADAMTS6B have an identical domain organization and amino acid identity and similarity (includes conserved amino acid substitutions) of 59 and 73%, respectively. The conservation extends to the positioning of two ADAMTS6B proprotein convertase processing sites (Arg-Arg-Arg and Arg-Gln-Lys-Arg), a highly similar zinc binding motif (Arg-Arg-Arg-Gln-Lys-Arg-Glu) in the ADAMTS6 spacer (Fig. 1B), highly similar zinc binding motif in the ADAMTS6 spacer (Fig. 1B) relevant in light of the recent discovery of GAG attachment in ADAMTS6B (Fig. 1B). A Gly-Ser-Glu motif in the ADAMTS6B spacer (Fig. 1B) is relevant in light of the recent discovery of GAG attachment in ADAMTS6B (22). The two adjacent acidic residues (italicized above) may favor GAG attachment to the boldface Ser residue (32). This sequence motif is missing in ADAMTS10 (Fig. 1B). ADAMTS17 and ADAMTS19 (33) also have five TSRs and a C-terminal PLAC domain, but they have less homology to ADAMTS10 (e.g. ADAMTS19 has 32% amino acid sequence identity and 44% similarity) and are thus less closely related to ADAMTS10 than is ADAMTS6.

ADAMTS6B and ADAMTS10 have identical gene structures, each having 25 exons with conserved splice boundaries (Fig. 1B). ADAMTS10 maps to human chromosome 19 and mouse chromosome 17. Gene location has been experimentally validated by interspecific backcross analysis in the mouse and radiation hybrid mapping in humans. ADAMTS6 maps to human chromosome 6 and mouse chromosome 13 (24).

ADAMTS10 mRNA Is Widely Expressed—Adamts10 was expressed at all four mouse developmental ages examined. Maximal expression was seen in 15- and 17-day-old embryos (Fig. 2A, left panel), and the lowest levels were present in 7-day-old embryos. A single mRNA of ~5 kb was detected in the mouse. A similar sized mRNA of comparable or greater intensity was seen in some adult mouse tissues such as the heart and lungs (Fig. 2A, right panel). Fainter bands were seen in kidney, liver, spleen, brain, and testis, and no Adamts10 message was detectable in adult skeletal muscle. The ADAMTS10 (human) mRNA differed in that two species of 5 and 8 kb were detected on Northern blots from human organs and cell lines (Fig. 2B). Widespread expression was also seen in human tissues, and in the mouse, skeletal muscle had the lowest levels of expression (Fig. 2B, left panel). Of the human cancer cell lines examined (Fig. 2B, right panel), the highest expression was seen in the A549 cell line, a lung-carcinoma-derived line with characteristics of a type II alveolar cell.

Adams10 Is Dynamically Expressed during Mouse Embryogenesis—In situ hybridization of mouse embryos at different stages revealed a highly dynamic pattern of expression with widespread low level expression in 9.5-, 10.5-, and 12.5-day-old embryos and increasingly tissue-specific gene expression in 14.5-, 15.5- (Fig. 3A), and 17.5-day-old embryos (Fig. 3B). Expression was found in most tissues of the embryonic day 9.5-12.5 mouse embryos except ectoderm (Fig. 3A). Since organogenesis has not yet advanced substantially at this age, the broad tissue distribution was uninformative. At embryonic days 14.5 and 15.5, the expression pattern was identical, and the mRNA distribution was relatively organ- and cell type-specific (Fig. 3A). In the craniofacial region, strong expression was noted in the craniofacial mesenchyme (Figs. 3A and 4E), submandibular gland (Figs. 3A and 4A), mesenchyme sur-

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3 M. F. Seldin, K. Peterson, G. Wistow, and S. S. Apte, unpublished data.
ranging the cochlear neuroepithelium (Fig. 4C), developing cerebral cortex (Figs. 3A and 4J), newly formed bone in the mandible (Fig. 3A), and tongue musculature (Fig. 3A). Strong expression was seen in perichondrium and periostium but not in cartilage (Fig. 3A). Expression was essentially mesenchymal and was not seen in developing mandibular, tongue, or nasal epithelium, surface ectoderm, or cochlear neuroepithelium (Figs. 3A and 4, A, C, and E). Of the thoracic organs, the lungs had prominent expression restricted to the mesenchymal cells between the developing bronchial tree (Fig. 4K), and blood vessels expressed Adams10 (Fig. 4F), but the heart was negative (Fig. 3A). In the abdomen, putative mesenchymal cells in the media of the stomach and duodenum and the pancreas were positive. The liver did not have levels of autoradiographic signal above background (signal seen in Fig. 3, A and B, is from red blood cells). In the dorsal regions, there was strong expression in the dorsal root ganglia and primary vertebral ossification centers of vertebrae (Fig. 4L). Unidentified cells in adrenal and renal cortex and the developing gonad were positive (Fig. 4, B, H, and L). High expression was noted in the connective tissue mesenchyme between the cartilaginous metacarpals of the hand and in the foot (data not shown). In addition, dense connective tissue such as joint capsule, tendons and ligaments (e.g. around the hip joint) (Fig. 4D) were strongly positive. Sense cRNA probe did not give signal above background levels, indicating that the antisense probe was specific (Fig. 4G).

In the 17.5-day-old embryo, the tissue-specific expression pattern (Fig. 3B) was essentially similar to that at embryonic days 14.5 and 15.5, with some notable differences. The expression levels in the lung mesenchyme, craniofacial mesenchyme, and the developing bone were of higher relative intensity than at preceding stages (Fig. 3B). The relative expression level was decreased in the developing brain, and for the first time, prominent expression was seen in chondrocytes in the developing cartilaginous skeleton (Fig. 4M). Rapid ossification of the skeleton at this developmental stage with strong expression in bone and cartilage and lung may explain why 17-day-old embryos had the highest expression levels on Northern blot. In addition, strong expression was seen in the walls of large arteries and around large vascular structures in the liver, which are precursors of the hepatic veins and the inferior vena cava.

Characterization of ADAMTS10 in Transfected HEK293F Cells—Western blotting of serum-containing conditioned medium from HEK293F cells stably transfected with myc-tagged human ADAMTS10 revealed a single band sized approximately at 130 kDa (Fig. 5A) or two closely approximated bands of nearly the same size (Fig. 5B). The ~130-kDa immunoreactive bands significantly exceeded the predicted size of the ADAMTS10 zymogen (118 kDa) or mature enzyme (95 kDa). When stably transfected cells were cultured in serum-free medium, a number of immunoreactive bands were detectable by Western blotting using the anti c-myc antibody (Fig. 5A); this indicated that ADAMTS10 undergoes proteolysis in the absence of serum.

To determine whether the unexpected mass increase of ADAMTS10 was a result of N-linked glycosylation, we deglycosylated it using PNGase F. This resulted in faster migration on SDS-PAGE, and the deglycosylated ADAMTS10 now had an apparent molecular mass of 120 kDa (Fig. 5B, left panel), which is close to the predicted size of the zymogen. A fainter band of ~100 kDa also emerged after deglycosylation, which may represent the mature ADAMTS10 enzyme (Fig. 5B). Other bands were smaller than expected and may be derived by proteolytic degradation of ADAMTS10. The secreted product of ADAMTS10-(1–463) transfected cells was also deglycosylated using PNGase F, but as predicted by the primary sequence, glycosylation was restricted to the prodomain, since migration of the 29-kDa fully processed catalytic domain was unaffected by PNGase F treatment (Fig. 5B, right panel). Four major myc-reactive bands were present in the medium of ADAMTS10-(1–463)-transfected cells, namely, a pair of bands (52 and 54 kDa), possibly representing the zymogen and Arg54-deleted processed zymogen, an uncharacterized intermediate of 37 kDa, and the 29-kDa mature derivative of ADAMTS10-(1–463). The failure of PNGase F to alter the relative size difference between the paired ~130-kDa (full-length ADAMTS10) and 52–54-kDa (ADAMTS10-(1–463)) bands suggested that they have different N termini. ADAMTS10 was able to cleave the broad spectrum protease substrate/inhibitor α2-M (Fig. 5C). Cleavage of α2-M within its bait region results in the entrapment of the cleaving protease in an irreversible complex with this protease inhibitor (34). Thus, an apparent size shift of myc-tagged ADAMTS10 is observed under nonreducing conditions, indicating cleavage of the inhibitor and entrapment of the enzyme (Fig. 5C). Pretreatment of the enzyme with 10 mM EDTA resulted in the abolition of the size shift, confirming the proteolytic activity of ADAMTS10 as a metalloprotease (Fig. 5C).

To assess whether ADAMTS10 was able to cut the large aggregating proteoglycan aggrecan, we used an antibody that detects a neoepitope generated by ADAMTS4/5/9 cleavage of the Glu1771-Ala1772 peptide bond of the aggrecan core protein...
ADAMTS10 digestion produced no detectable immunoreactivity of aggrecan to the neoepitope antibody on Western blots (Fig. 5D). In contrast, ADAMTS4 digestion did generate the neoepitope, as has been previously described (Fig. 5D) (20, 26).

Characterization of ADAMTS10 Zymogen Processing—Because of its smaller size, which allows for easier discrimination of processed species from the zymogen, ADAMTS10-(1–463) was used for studies of zymogen processing. ADAMTS10-(1–463) was substantially purified from stably transfected HEK293F cells by chromatography on nickel-Sepharose (Fig. 6A, left panel). Bands visible on Coomassie Blue-stained gels (Fig. 6A, left panel) corresponded to bands prominent on Western blotting of the purified protein using anti-myc (Fig. 6A, right panel), although the smaller amount of protein in Fig. 5B better permits distinction of the 52- and 54-kDa bands. N-terminal sequencing indicated the origin of the bands as follows: secreted zymogen (54 kDa; sequence NH₂-Phe²⁶-Arg-Ser-Gln-Asp), thus confirming the predicted signal peptidase cleavage site; partially processed zymogen (52 kDa; NH₂-Gly⁶⁷-Thr-Gly-Ala-Thr); and fully processed catalytic domain (29 kDa; NH₂-Ser²₃⁴-Val-Ser-Arg-Glu), respectively (Fig. 6A). Incubation of cells with the lipid-permeable furin inhibitor dec-RVKR-cmk showed that formation of the 29-kDa form was suppressed when cells were incubated in the presence of 10–100 μM inhibitor, but the levels of the larger forms or of the 37-kDa intermediate were affected only at correspondingly higher concentrations.

Some Secreted ADAMTS10 Localizes to Cell Surface and ECM—ADAMTS10 could be extracted from COS-1 or HEK...

Fig. 4. Organ-specific expression of Admats10 in mouse development. In these paired panels, coupled dark field microscopy-fluorescent microscopy overlays are shown on the left, and the corresponding fluorescent image is shown on the right (except F and G) to provide morphological correlation. In the left panels, autoradiographic signal from the hybridized probe is red (pseudocolor imparted to signal from dark field microscopy), and the cell nuclei are blue (Hoechst 33258 visualized by UV light). Lack of hybridization to the sense probe is illustrated in G. A–L are from 15.5-day-old embryos, and M is from a 17.5-day-old embryo. A, submandibular gland. The asterisk indicates an epithelial lobe. B, developing kidney (kd) and adrenal (ad) show cortical signal. C, developing cochlea. A semicircular canal is shown in cross-section to illustrate lack of signal in lining neuroepithelium (ne) and surrounding cartilage (c). D, hip joint, showing the absence of signal in the developing femoral head cartilage (fe) and strong signal in overlying ligaments and tendons (l) and joint capsule. E, snout region, showing expression of Adamts10 in mesenchyme but not in hair follicle epithelium (hf). F, blood vessel (bv) showing expression in the wall. G, lack of sense probe hybridization to lung (compare with Figs. 3, A and B, and 4K). H, expression in gonad, most likely a testis (t), from the striped appearance, and in the wall of an adjacent artery (a). I, expression in developing gonad, likely to be a prospective ovary, showing that expression is absent in tubular epithelium (asterisk). J, expression in cerebral cortex is highest in the ventricular zone (vz), v, ventricle; cp, choroid plexus of lateral ventricle. K, in the lung, expression is excluded from bronchial epithelium (bronchial tubes are marked by the asterisk). L, section through the vertebrae, showing strong expression in dorsal root ganglion (drg) and bone (b). M, embryonic day 17.5 vertebral column shows strong expression in cartilage (c) and dorsal ligament (d), but not in intervertebral disc (ivd). Compare this with the lack of cartilage expression in 14.5-day embryos (C and D).
**DISCUSSION**

ADAMTS10 is probably the last mammalian ADAMTS that will be identified, taking the total number of mammalian ADAMTS proteases to 19. Since over 99% of the human and mouse genomes have been annotated, it is very unlikely that additional ADAMTS proteases will be discovered in mammals. Although ADAMTS10 has a different domain organization from the cognate ADAMTS6, it preferentially clustered with ADAMTS6 in sequence alignment and phylogenetic analysis (23). Since all ADAMTS enzymes within a clade have identical domain structures (23), we considered the possibility that there might exist an alternative form of ADAMTS6 having the domain structure of ADAMTS10. This appears to be the case. Although ADAMTS6B has not yet been cloned as a single contiguous mRNA, the similarity of the conceptual product to ADAMTS10 was felt to be of sufficient relevance to describe it here. The genesis of the alternative transcript and its tissue-specific expression are beyond the scope of the present work and will be described elsewhere. Despite also having a domain

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4 S. A. Oblander and S. S. Apte, unpublished data.
structure similar to ADAMTS10, ADAMTS19 and ADAMTS17 are highly homologous to each other but not to ADAMTS6 or ADAMTS10 and constitute a separate subclass (23).

The primary structure of ADAMTS10 predicts many of the typical features of ADAMTS proteases. Like the majority of them, it is a secreted glycoprotein. The experimental data indicate that it is synthesized as a zymogen that is processed by proprotein convertases in the secretory pathway. However, the maturation process appears to be inefficient, since unprocessed zymogen is also secreted. In this respect, ADAMTS10 differs from other ADAMTS proteases. In HEK293F cells, both the ADAMTS10 zymogen and fully processed form are secreted from the cell, whereas ADAMTS39-transfected cells produce only the fully processed form (20). In ADAMTS7, where we previously described stepwise zymogen processing, some of the final processing to mature enzyme occurred at the cell surface and was substantially inhibited by 10 \( \mu \)M dec-RVKR-cmk with complete inhibition achieved by 25 \( \mu \)M (22). On the other hand, the final processing step in ADAMTS10 requires 100 \( \mu \)M dec-RVKR-cmk for nearly complete inhibition. This suggests that this lipid-permeable inhibitor needs to penetrate into intracellular compartments to affect the final ADAMTS10 processing step. Curiously, the processing site for production of the mature ADAMTS10 enzyme lacks the typical proprotein convertase consensus sequence Arg-Xaa-Arg/Lys-Arg ↓ (35, 36). Sequence comparison of a large number of furin substrates suggests an absolute requirement for the P1 Arg and requires that at least two of the three residues at P2, P4, and P6 must be basic for efficient cleavage (35, 36). The cleaved site in ADAMTS10 does not fulfill the second requirement, although there is a Ser residue at the P1’ position in ADAMTS10 that was noted in about 50% of furin substrates (37). Despite these discrepancies, however, both N-terminal sequencing and dec-RVKR-cmk inhibition suggest that the completely processed form is generated by a proprotein convertase, albeit inefficiently. Since ADAMTS10 is widely expressed and furin is the only ubiquitous proprotein convertase (35, 37), it is likely to be the physiological processing enzyme of ADAMTS10. The origin of the 37-kDa intermediate found in purified ADAMTS10-(1–245) preparations has not been established; however, its formation appears to be inhibited by dec-RVKR-cmk, suggesting that it may result from processing at another atypical furin cleavage site Val-Tyr-Lys-Arg^182 ▼ Ser that is conserved in mouse and human ADAMTS10. This putative site lacks both the P4 and P6 Arg residues required for optimal processing, but it has a P1’ Ser. The production of unprocessed and partly processed zymogen might imply that the furin cleavage is inefficient because of the suboptimal recognition sequences at two of three processing sites. Partial activation could be an important physiological mechanism by which this highly expressed protease is regulated post-translationally.

Previous studies have demonstrated localization of ADAMTS1, ADAMTS4, ADAMTS7, and ADAMTS9 to the cell surface (20, 22, 38–40). The putative GAG-binding sequences in ADAMTS10 might mediate such localization, and as previously shown for ADAMTS1, ADAMTS4, and ADAMTS7, the cell surface may be a staging area for further proteolytic activation steps (22, 38, 39). ADAMTS10 in serum-free medium undergoes substantial proteolysis. Western blotting using monoclonal antibody 9E10 to the C-terminal myc epitope identified several myc-reactive fragments representing C-terminal processing events analogous to those reported for ADAMTS1.
and ADAMTS4. The removal of C-terminal modules in these proteases has been shown to have a profound effect on enzyme activity and specificity (19, 38, 40). Once specific ADAMTS substrates are identified, the regulatory role of C-terminal proteolysis can be studied in greater detail. The inhibition of proteolysis in the presence of serum, perhaps by broad spectrum protease inhibitors such as α-M suggests that the responsible enzymes originate in the 293 cells and that this processing of ADAMTS10 occurs extracellularly.

Few ADAMTS proteases other than ADAMTS9 have been shown to have such a broad expression profile, since constitutively active proteases such as the ADAMTS are likely to be highly regulated at the transcriptional and post-transcriptional level. An emerging theme in the ADAMTS family is that enzymes of a given subfamily appear to have very different expression patterns and levels and that biological roles for each enzyme may be determined by its nonredundant sites of expression. As a case in point, ADAMTS9 is widely expressed, but its homolog, ADAMTS20, is only sparingly expressed. Similarly, ADAMTS6 appears to be expressed primarily in the placenta (24), but ADAMTS10 is very widely expressed. Interestingly, the highest embryonic expression of ADAMTS10 is in the developing lung and among human tumor cell lines examined, in a lung carcinoma cell line. Since antibodies to ADAMTS10 are not presently available, it is not known whether all of the expressed ADAMTS10 RNA is translated into protein. Although ADAMTS10 may have a broad participation in mesenchymal and basement membrane remodeling in a variety of morphogenic processes, this does not imply that it has an essential role at all expression sites. Indeed, some widely expressed proteases have seemingly few nonredundant roles during development.

The determination of specific developmental roles in genetic models as well as screens for substrates will provide more insight to follow on this initial characterization of ADAMTS10.

Acknowledgments—Micky Tortorella kindly provided the anti-AGEG antibody. We thank Dr. Graeme Wistow and Dr. Katherine Peterson (NEI, National Institutes of Health) for sharing ADAMTS10 sequence information.

Note Added in Proof—A recent article described ADAMTS10 mutations in Well-Marchesani syndrome (Dagoneau et al. (2004) Am. J. Hum. Genet. 75, 801–806).

REFERENCES

1. Somerville, R. P., O lblander, S. A., and Apte, S. S. (2003) Genome Biol. 4, 216
2. Sternlicht, M. D., and Werb, Z. (2001) Annu. Rev. Cell Dev. Biol. 17, 463–516
3. Kheradmand, F., and Werb, Z. (2002) Biosci. Rev. 24, 8–12
4. Bialdos, C. P. (1997) Cell 80, 589–592
5. Kuno, K., Kanada, N., Nakashima, E., Fujiki, F., Ichimura, F., and Matsushima, K. (1997) J. Biol. Chem. 272, 556–562
6. Colige, A. Li, S. W., Sieron, A. L., Nusgens, B. V., Prokop, D. J., and Lapiere, C. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2374–2379
7. Fernandes, R. J., Hirohata, S., Engle, J. M., Colige, A., Cohn, D. H., Eyre, D. R., and Apte, S. S. (2001) J. Biol. Chem. 276, 31592–31599
8. Iruela-Arispe, M. L., Carvajal, D., and Lapiere, C. M. (2002) J. Cell Biol. 159, 229–231
9. von Heijne, G. (1990) Insect. Biochem. Mol. Biol. 20, 351–355
10. Wu, S., Toh, K., and Arner, E. C. (2000) J. Biol. Chem. 275, 22244–22250