Cloning and Characterization of ADAMTS-14, a Novel ADAMTS Displaying High Homology with ADAMTS-2 and ADAMTS-3*

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The processing of amino- and carboxyl-propeptides of fibrillar collagens is required to generate collagen monomers that correctly assemble into fibrils. Mutations in the ADAMTS2 gene, the aminopropeptidase of procollagen I and II, result in the accumulation of non-fully processed type I procollagen, causing human Ehlers-Danlos syndrome type VIIC and animal dermatosparaxis. In this study, we show that the aminopropeptidase of type I procollagen can be cleaved in vivo in absence of ADAMTS-2 activity and that this processing is performed at the cleavage site for ADAMTS-2. In an attempt to identify the enzyme responsible for this alternative aminoprocollagen peptidase activity, we have cloned the cDNA and determined the primary structure of human and mouse ADAMTS-14, a novel ADAMTS displaying striking homologies with ADAMTS-2 and -3. The structure of the human gene, which maps to 10q21.3, and the mechanisms of generation of the various transcripts are described. The existence of two sites of initiation of transcription, in two different promoter contexts, suggests that transcripts resulting from these two sites can be differently regulated. The tissue distribution of ADAMTS-14, the regulation of the gene expression by various cytokines and the activity of the recombinant enzyme are evaluated. The potential function of ADAMTS-14 as a physiological aminoprocollagen peptidase in vivo is discussed.

ADAMTS1 (A Disintegrin and metalloprotease with thrombospondin type I repeats) is a novel family of metalloproteases found in vertebrates and invertebrates. These enzymes are related to ADAMs as judged from sequence homology and conserved domains such as a characteristic metalloprotease domain and a disintegrin-like module. However, they differ from ADAMs by their domain organization and the presence of distinct features. The most specific hallmark is the presence of a central thrombospondin type I repeat (TSPI) between the disintegrin-like module and the Cys-rich domain. All ADAMTS, except ADAMTS-4, contain also TSPI-like domains in varying numbers at the COOH terminus (1, 2). Currently, 12 ADAMTS from vertebrates and a few from invertebrates (Drosophila and Caenorhabditis elegans) have been described (1, 2). ADAMTS-1, -4, and -5 are able to cleave proteoglycans and are probably involved in cartilage degradation during arthritis (3–5). ADAMTS-1 and -8 are potent anti-angiogenic molecules (6). Adamts1+/− mice display abnormal growth, defective fertility, and altered organ morphology and function (7). A. C. elegans Adams, gon-1, was found essential for gonadal morphogenesis (8). Both the metalloprotease domain and some TSPI-like repeats are required for the control of this process.

The primarily described activity of ADAMTS-2 is to excise the aminopropeptide of type I and type II procollagens, explaining its former trivial name aminoprocollagen I/II peptidase (9, 10). Removal of the N- and C-propeptide of type I procollagen is required to generate collagen monomers able to assemble into elongated and cylindrical collagen fibers. Human Ehlers-Danlos type VIIC (dermatosparactic-type, OMIM 225410) and animal dermatosparaxis are recessively inherited disorders that are caused by mutations preventing the synthesis of active ADAMTS-2 (11). As a consequence, pN-I collagen (type I collagen that still contains the N- but not the C-propeptide) accumulates (12), resulting in the polymerization of abnormal collagen fibers that appear irregular, thin, branched and “hieroglyphic” in cross-section (13). The main clinical feature of human patients and affected animals is a severe cutaneous fragility. A similar phenotype has been recently reported in transgenic mice with inactive alleles for Adamts2 (14). Other type I collagen-rich tissues, such as bone and tendon, do not seem to be functionally affected. Furthermore, a significant proportion of type I collagen extracted from skin biopsies of Ehlers-Danlos type VIIC (EDSVIIIC) patients or from dermatosparactic calves is NH2-terminal processed, at a site that remained to be determined, although no active ADAMTS-2 is synthesized (11). These observations and the fact that processing of aminopropeptide is a complex event requiring a specific three-dimensional native conformation (15) suggested that an enzyme closely related to ADAMTS-2 would be responsible for this alternative aminoprocollagen peptidase activity.

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1 The abbreviations used are: ADAMTS, A disintegrin-like and metalloprotease with thrombospondin type I repeats; EDS, Ehlers-Danlos syndrome; RT, reverse transcription; FCSC, fetal calf serum; TSPI, thrombospondin type I repeat; pN collagen, procollagen that still contains the amino- but not the carboxyl-propeptide; DMEM, Dulbecco’s modified Eagle’s medium; IL, interleukin; RACE, rapid amplification of cDNA ends.
In this study, the specificity of the processing of the aminopropeptide of type I collagen in absence of ADAMTS2 is demonstrated for the first time. The cloning of the cDNA and characterization of human and mouse ADAMTS-14, a novel ADAMTS with striking homologies with ADAMTS-2 and -3, are also reported. Finally, the aminoprocollagen peptidase activity of the recombinant enzyme, the structure of the gene, and the regulation of its expression are described.

**EXPERIMENTAL PROCEDURES**

**Analysis of Procollagen Processing in Vivo**—For evaluating the level of aminoprocollagen I (pNPI) processing in Adams2−/− mice (14), various organs and tissues were ground at liquid nitrogen temperature and extracted with 0.1 M acetic acid for 16 h at 4°C. After centrifugation, the supernatants were neutralized and ammonium sulfate was added (40% saturation). After centrifugation (8000 × g, 30 min), the pellets were washed in an ammonium sulfate solution (20% saturation) and finally dissolved in 0.1 M acetic acid. After precipitation at 33% ethanol (final concentration) at neutral pH, the pellets containing collagen were denatured in Laemml sample buffer. Similar amounts of protein were separated by electrophoresis on a 7.5% SDS-PAGE and stained with Coomassie Blue.

The cleavage site of aminoprocollagen I in the absence of Adams-2 activity was determined on dermatosparatic calf tendon. Collagen was purified from 1 kg of cartilage extracts by sequential steps of precipitation and solubilization as described earlier (16). The collagen preparation was then treated or not with prolylglutamate aminopeptidase before electrophoresis on a pre-run 7.5% acrylamide/piperazine diacrylamide gel in SDS. For evaluating the level of mRNA from cultured fibroblasts was dephosphorylated and then amplified by 5′-H11032

**PCR Amplification and Sequencing of ADAMTS-14 cDNA**—Three large overlapping cDNA fragments covering the ADAMTS14 cDNA sequence corresponding to exons 2 to 22 of ADAMTS2 were PCR-amplified from fibroblasts cDNA (37 cycles consisting of 94°C for 30 s, 66°C for 30 s, 72°C for 90 s) using Tag DNA polymerase (Takara) and three ADAMTS14 primer pairs (5′-CTATGGTGTCAGATCCTGCTCTC-A3′ and 5′-GAGCCTGCCGACTGTCGCTC-3′). The PCR products were then gel purified and sequenced using the manufacturers recommended protocols (Thermo-sequenase automated thermal cycler (GeneAmp PCR System 2400 or 9600, PerkinElmer Life Sciences, Norwalk, CT) using the GeneAmp Thermotable rTh Reverse Transcriptase RNA PCR kit (PerkinElmer Life Sciences), 10 ng of total RNA, a known copy number of internal standard RNA, when available, and specific pairs of primers (5 pmol each).

**Effects of Cytokines and Growth Factors**—Human dermal fibroblasts, at passages 4 to 12, were plated at a density of 8 × 10^4 cells/cm² and cultured for 1 or 2 days in Dulbecco’s modified Eagle’s medium (DMEM). The medium, supplemented with 10% dialyzed and decomponent FCS, contained or not 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml), IL-1β (100 units/ml), tumor necrosis factor-α (5 ng/ml), epidermal growth factor (20 ng/ml), or transforming growth factor-β (5 ng/ml). Total RNA was purified using the High Pure RNA Isolation kit (Roche Molecular Biochemicals).

**Electron Microscopy**—Fragments of skin and tendon from wild type and Adams2−/− mice were fixed for 60 min at room temperature in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed for 30 min in 0.1% osmium tetroxide in Sörensen’s buffer, dehydrated in a series of ethanol concentrations and embedded in epoxy resin (Epox 812, Fluka). Ultrathin sections were stained with uranyl acetate and lead citrate before being examined using a Jeol electron microscope CX100 II at 60 kV.
restriction enzymes (1, NotI/EcoRI; 2, EcoRI/HindIII; 3, HindIII/Nhel) and ligation of the PCR products, a cDNA fragment covering the entire coding sequence was created in a pCDNA4 vector (Invitrogen). A sequence verified full-length insert was then cloned in the NotI/Nhel sites of a pCEP4 expression vector (Invitrogen) containing a modified multiple cloning site and an additional sequence coding for a “Flag” (DYKDDDDK) at the COOH terminus of the expressed recombinant protein. This ADAMTS-14 expression vector, a similarly constructed ADAMTS-2 vector and the modified empty pCEP4 vector were then used for transfection of 293 EBNA cells (Invitrogen) by electroporation (20 μg of plasmid; 220 V and 960 μF in 4-mm cuvettes). Stably transfected cells were then selected in DMEM culture medium supplemented with 10% FCS and 200 μg/ml hygromycin B.

For evaluating the production of recombinant ADAMTS-14, cells were cultured at confluence, scraped, and rotated for 2 h at 4 °C in an extraction buffer (50 mM Tris, pH 7.5, 200 to 500 mM NaCl, 2 mM CaCl2, 25 mM NEM, 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the cell extracts were collected and the pellets were solubilized in SDS-PAGE denaturation buffer containing 100 mM dithiobisretol. Conditioned culture medium and the two cell-associated fractions were then assayed by Western blotting by using the anti-FLAG™ M2 monoclonal antibody (Sigma) and ECL Western blotting detection reagents (Amersham Biosciences Inc.).

Aminoprocollagen Peptidase Activity of Recombinant ADAMTS-14—The stably transfected 293 cells amplified from two separate transfections were grown to confluence in DMEM supplemented with 10% FCS and 20% ascorbic acid (50 μg/ml). Aminoprocollagen peptidase activity of ADAMTS-14 are reported in % of the empty vector were considered as background and subtracted from the culture medium by differential salt precipitation with 1 M NaCl and from the culture medium by 6.25% SDS-PAGE in nonreducing conditions.

RESULTS

Procollagen Processing in Dermatosparaxis—The level of type I aminoprocollagen (pNI) processing in the skin of wild type (WT) or ADamts-2−/− mice was investigated by SDS-PAGE (Fig. 1A). Only a1 and a2 chains were observed in tissues of WT mice, illustrating the complete processing of the aminoproteptide of type I collagen. In ADamts2−/− mice, pNI11 and pNI21 chains were detected, as expected from an animal lacking Adamts-2 activity. However, mature a1 and a2 chains were present, suggesting the existence of an alternative aminoprocollagen peptidase activity. Similar observations were made in bovine dermatosparaxis and human Ehlers-Danlos syndrome type VIIC (12). The level of processing varied from tissue to tissue (Fig. 1B) and was not related to the collagen content in the various organs. Skin contains mostly unprocessed pNI-collagen (60 to 70%) while, in tendons, it represents only 20 to 25%. When most of the pN-I collagen is not processed, such as in the skin of ADamts2−/− mice, collagen fibers are deeply altered (Fig. 2, compare panels A and B). On the opposite, in tendons, where a high proportion of collagen is correctly processed, fibers look almost normal in terms of diameter, shape, and supramolecular organization (Fig. 2, panels C and D). Their mechanical resistance is also normal (not shown).

The specificity of the processing of pNI collagen in the absence of Adamts-2 activity was assessed by amino acid sequencing of the NH2 terminus of processed a1 and a2 chains isolated from dermatosparactic calf tendon. A first attempt failed to provide sequence information, suggesting that their amino terminus was blocked, possibly by modification of the glutamine residue which is the first expected amino acid of a1 and a2 when the processing occurs at the site normally cleaved by Adamts-2. After digestion with pyrogglutamate aminopeptidase, a LSYGGY sequence was obtained for the a1 chain and a FDKKGG sequence for the a2 chain. In bovine type I collagen, these two sequences immediately follow the Gln residue in position 1 of correctly processed a1 and a2. These features demonstrate that, even in absence of Adamts-2 activity in vivo, procollagen processing can occur at the cleavage site for Adamts-2.

Identification of ADAMTS-14 cDNA and Gene—Based upon the hypothesis that the specific aminoprocollagen peptidase activity observed in animals lacking Adamts-2 results from the expression and the activity of a closely related enzyme, we scanned nucleic acid data bases from GenBank™ using the human ADAMTS2 cDNA sequence (accession number AJ003125). Besides homologies with ADAMTS2 and ADAMTS3 cDNA and genes (on chromosome 5 and 4, respectively), homology was also found between portions of exons 6, 7, 8, 10 of ADAMTS2 and sequences from chromosome 10 (accession numbers: AC069538, AC016043, AC007484, AC018979, and AL358817). A second homology search using less stringent parameters and the sequence of individual ADAMTS2 exons revealed that, with the exception of exons 1, 5, and 21, each ADAMTS2 exon had partial sequence homology with sequences in chromosome 10. This suggested the existence of a gene coding for a new ADAMTS closely related to ADAMTS2.

By RT-PCR, we found that this new ADAMTS gene was actually expressed in human skin fibroblasts in culture and in placenta, although at a lower level. Region of the mRNA corresponding to exons 2 to 22 of ADAMTS2 was then RT-PCR amplified and sequenced. This allowed to determine that sequences corresponding to exons 5 and 21 of ADAMTS2 were present in the new ADAMTS but displayed very low homology, explaining why they were not detected by scanning of the data bases. We also found an alternative splicing mechanism leading to the removal of the last 9 bases of exon 6 (Table 1). The ADAMTS14 name was assigned to this new cDNA, in agreement with the Human Gene Nomenclature Committee (GenBank™ accession number AF366351). Comparison of the ADAMTS14 cDNA sequence with the draft sequence of the human genome revealed that the gene is located on chromosome 10 (q21.3). The exon/intron structure has been determined (Table 1). A 5' RACE method was used for the determination of sequences located upstream exon 2. Two different sequences were identified. They represent 2 alternative exons 1 that were named exon 1A and 1B (Fig. 3, Table 1). Comparison with genomic sequences showed that the beginning of exon 1B (5'-TATTG) starts at a tgtgTATTTT potential Cap signal located 17 base pairs (bp) downstream a potential TATA-box (tggtat) (19). This suggests that the TATTT sequence represents the actual start of transcription of this transcript. For exon 1A, the 5'-end sequence that was determined (TTGC) does not start after a typical Cap site, perhaps suggesting that the actual 5’
end of the transcript had not been cloned. A tracg Cap signal 27 bp upstream and adequately preceded (82 bp) by a typical GC-box could be the actual start of transcription. As a confirmation of the presence, at the 5’ end of the mRNA, of the alternative exons 1A and 1B, we performed a RT-PCR assay using a common antisense primer specific of exon 2 and one

FIG. 1. Accumulation of pNα1 and pNα2 type I collagen in mouse tissues. A, the pattern of type I collagen polypeptide purified from skin and tendon of “wild type” (WT) and Adamts2−/− (TS2−/−) mice was determined after SDS-PAGE and Coomassie Blue staining. In WT, only α1 and α2 mature chains are visible. In TS2−/−, absence of Adamts-2 activity results in the accumulation of pNα1 and pNα2 chains. Higher proportions of pNα chains were observed in skin. B, type I collagen was extracted from various tissues of TS2−/− mice. After electrophoresis on SDS-PAGE and staining, pNα1, pNα2, α1, and α2 type I collagen bands were quantified. For each tissue, the proportion of pNα chains is expressed as a percentage of total type I collagen. Mu, muscle; K, kidney; He, heart; Lu, lung; St, stomach; Ey, eye; Bo, bone; Te, tendon; Sk, skin.

FIG. 2. Structure of collagen fibrils. Collagen fibrils from skin and tendons of wild type (WT) and Adamts2−/− (TS2−/−) mice were observed by electron microscopy. In TS2−/−, skin collagen fibrils are strongly disorganized while they display an almost normal shape in tendon. Bars represent 0.3 μm. Top panels, collagen fibrils (longitudinal and cross-section) in skin from a WT mouse (A) or a TS2−/− mouse (B). Bottom panels, collagen fibrils, in longitudinal (C) or cross (D) section, in tendon from a TS2−/− mouse.
sense primer specific of exon 1A or exon 1B. Products of the expected size (204 and 241 bp, respectively) and sequence were obtained. However, another product (∼700 bp) was also RT-PCR amplified using the exon 1B-specific primer. In absence of the RT step, this product was not detected, demonstrating that it did not result from the amplification of genomic DNA. Sequencing revealed that this product was generated from a large exon, named 1C, consisting of exon 1B, intron 1B, and exon 2 fused together (Fig. 3, Table I). In the following, the mRNA beginning with exon 1A, 1B, or 1C will be named transcript A, B, or C, respectively. RT-PCR evaluation of the relative level of the three transcripts in cultured fibroblasts revealed that transcript A is more abundant than transcript C that is much more abundant than B. Moreover, while transcript A is expressed in placenta, skin, and fibroblasts, transcripts B and C are found at a significant level only in fibroblasts (not shown).

Primary Structure of ADAMTS-14—For transcript A, the most 5′-in-frame ATG found in exon 1A (Fig. 3) is located in a conserved Kozak consensus sequence (20). Its use as a Start codon would lead to the presence of a moderately hydrophobic NH₂-terminal domain (amino acids 52 to 76) that could serve as a signal peptide (Fig. 4A). Low homology was found between part of this sequence and the NH₂ terminus of ADAMTS-2 (amino acids 42 to 59). For transcripts B and C, the first ATG triplets that are found are not in a perfectly conserved Kozak consensus sequence and are followed by Stop codons. The first ATG in a suitable Kozak motif for translation (A at position −3 and G at position +4, with respect to the A (+1) of the ATG Start codon) is found in exon 2 (Fig. 3). The predicted protein from these transcripts starts at Met⁴⁰ of the sequence reported for transcript A (Fig. 4A) and does not contain an obvious signal peptide. Besides this difference, the predicted proteins trans-

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### Table I

ADAMTS14 Gene Structure

| Exons | Introns | Splice sites |
|-------|---------|-------------|
| No. | Size | Position* | No. | Size | 5′ Donor | 3′ Acceptor |
| 1A* | 135 | −53 to 82 | 1A | 1668 | CCCAG/ctgct | tacctecaag/AGCTG |
| 1B* | 156 | − − − − | 1B | 468 | TCTAG/ctgct | tacctecaag/AGCTG |
| 1C* | 1064 | − − − − | 2 | >27000 | GATTC/ctaggg | tectgcttagg/GCGGG |
| 2 | 440 | 83 to 522 | 2 | >27000 | GATTC/ctaggg | tectgcttagg/GCGGG |
| 3 | 157 | 523 to 679 | 3 | 6119 | TGAAG/ctaggg | tcttgcttagg/UCTTT |
| 4 | 191 | 880 to 870 | 4 | 20549 | ATACT/ctaggg | tcttgcttagg/GTACG |
| 5 | 84 | 871 to 954 | 5 | 724 | GACAG/ctaggg | gcggctgctagg/TCCCT |
| 6d | 157 | 955 to 1111 | 6d | 1995 | GCCAG/ctaggg | tcttgcttagg/GTATTA |
| 7 | 106 | 1112 to 1217 | 7 | 1525 | CACCT/ctaggg | tccctegaag/GCTGC |
| 8 | 144 | 1218 to 1361 | 8 | 1141 | CTCCC/ctaggg | tccctegaag/CTCCT |
| 9 | 133 | 1362 to 1494 | 9 | 1378 | TGGCA/ctaggg | tcttgcttagg/TTTAG |
| 10 | 114 | 1495 to 1608 | 10 | 2046 | GCCAG/ctagct | gcrgctgccg/aGGGT |
| 11 | 149 | 1609 to 1757 | 11 | 2146 | CCCC/ctgctgag | gctgtgcggc/GCCAG |
| 12 | 176 | 1758 to 1933 | 12 | 2385 | CGATG/ctagctgag | cctcctgaag/GCCGC |
| 13 | 134 | 1934 to 2067 | 13 | 384 | GTGAG/ctagctgag | tctgcttagg/CTGAG |
| 14 | 124 | 2068 to 2191 | 14 | 1036 | GGGCC/ctagctgag | tcttgcttagg/GAGCT |
| 15 | 81 | 2192 to 2272 | 15 | 4515 | CATTG/ctagctgag | gctgcttagg/GTGGG |
| 16 | 164 | 2273 to 2436 | 16 | 1492 | TCTGG/ctagctgag | atctcctgaag/GCTCT |
| 17 | 169 | 2437 to 2605 | 17 | 448 | TGGAG/ctagctgag | cccaccccgag/GGAATC |
| 18 | 133 | 2606 to 2738 | 18 | 1572 | CCTG/ctagctgag | cctgctttagg/GTGAG |
| 19 | 208 | 2739 to 3046 | 19 | 3955 | CCAAG/ctagctgag | cccaccccgag/GGCTC |
| 20 | 130 | 2947 to 3076 | 20 | 83 | TGGG/ctagctgag | tctgcttagg/GAATAT |
| 21 | 111 | 3077 to 3187 | 21 | 2075 | ATCAA/ctagctgag | tcttgcttagg/GCGAG |

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*a* A of the ATG start codon in exon 1A is considered as +1.

*b* Alternative exons 1A and 1B are separated from exon 2 by introns 1A and 1B, respectively (see Fig. 3). Alternative exon 1C, made of exon 1B, intron 1B, and exon 2, is separated from exon 3 by intron 2.

*c* This alternative exon does not contain the ATG start codon used for the numbering (see Footnote a).

*d* An alternative splicing mechanism using a CTCAG/gta/gctgag donor site causes a 9-bp deletion (see Fig. 4A).

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**Fig. 3.** Mechanisms of generation of alternative exon 1A (A), 1B (B), and 1C (C). Open rectangles representing exons and lines representing promoter or intronic sequences are not at scale. Dotted lines illustrate splicing mechanisms. For transcript A, exon 1A is joined to exon 2 by splicing of intron 1A. A potential regulatory GC-box is located upstream of the 5′-end of the transcript, as determined by 5′-RACE. The ATG triplet located in exon 1A in a suitable Kozak consensus sequence is indicated. Transcripts B and C start in exon 2 downstream of the potential TATA-box. Introns 1B and 2 are spliced during the maturation of transcript B while it is conserved in transcript C. The first ATG start codon for these two transcripts is located in the sequence corresponding to exon 2 of transcript B. The size of E1A is a minimal size evaluated from the presence in genomic DNA of a suitable CAP signal situated 27 bp upstream of the cDNA sequence determined by 5′-RACE products. The use of a more upstream CAP signal would lead to a larger E1A.
FIG. 4. Human and mouse ADAMTS-14 amino acid sequence and structure. A, the human sequence (hTS14) is reported on the middle lines. The beginning and end (|) of the partial mouse sequence (mTS14) and human ADAMTS-2 (hTS2) are reported on the upper or lower lines, respectively, but only at positions where their sequence differs from hTS14. The peptide sequence translated from transcript A only is in italics (amino acids 1 to 67). The MQG-(369–371) sequence is alternatively present due to alternative splicing mechanism at the end of exon 6. In the mouse these three amino acids are always absent. The furin cleavage site (RKRR), the catalytic site and Met-turn, the PLAC domain and the 4 TSPI are underlined. The start of the disintegrin-like, the Cys-rich, and Spacer domains are indicated (>).

B, the catalytic domain and the first TSPI module of the ADAMTS family members were compared. Length of the different members and their degree of similarity with ADAMTS-14 are reported between parentheses. Amino acid residues identical to those in ADAMTS-14 are shaded. Residues that are hallmarks of ADAMTS-2, -3, and -14 are indicated (*).
lated from the three transcripts are identical. They have a higher homology with ADAMTS-2 and -3 than with the other ADAMTS (Fig. 4B) and they display a similar domains organization consisting of a pro-domain separated from the metalloprotease domain by a furin cleavage site, a disintegrin-like domain, a central TSPI, a cystein-rich domain, a spacer domain, three additional TSPI, and a COOH-terminal tail without significant homology for ADAMTS-2 or -3, except for a highly conserved PLAC domain (Fig. 4A). Analysis of the sequence of the catalytic site and the first TSPI, two of the most conserved domains in the ADAMTS family, confirmed that ADAMTS-14 is closely related to ADAMTS-2 and -3. Specific hallmarks of these three enzymes are a Thr and a Glu at position 3 and 10 of the catalytic site and Phe (+9), Lys/Arg (+21) and Arg (+23) of the first TSPI (Fig. 4B).

A partial mouse sequence was also determined. It showed a high similarity with human ADAMTS-14, mainly in regions supposed to be critical for enzyme function such as the furin cleavage site, the metalloprotease domain and the beginning of TSPI (Fig. 4A).

Northern Analysis—As seen for other ADAMTS genes, ADAMTS14 mRNA was expressed at low level. Therefore, Northern analysis was performed on mRNA from fibroblasts in culture, the richest source of ADAMTS14 mRNA, using antisense riboprobe to increase the sensitivity of the assay. Two different probes were used, specific of either the 5′-end or the 3′-end of the mRNA. The 5′ end probe revealed two transcripts of about 4.5 and 5.7 kb (Fig. 5), which is similar to the size of other ADAMTS transcripts. The 3′ specific probe recognized these two products too but also smaller transcripts (about 2.8, 2.0, and 1.1 kb).

Tissue Distribution and Regulation of Expression—Tissue-specific expression was evaluated by RT-PCR. ADAMTS14 is expressed in all examined tissues, with highest levels in type I collagen-rich tissues and in fibroblasts in culture (Fig. 6). Lower levels were observed in liver, stomach, brain, and eye. This tissue distribution and the relative amount of ADAMTS14 mRNA are quite similar to those determined for ADAMTS2 (21), the only exception being the eye which has a low ADAMTS14 expression while it contains a high level of ADAMTS2. Scanning EST data bases using human and mouse cDNA sequences revealed that ADAMTS14 is also expressed in ovary (accession numbers: BF906533, BF906528, and BF906355), kidney tumor (accession number BF823025), and mammary tumor (accession number BF123774) and that it is up-regulated in larynx carcinoma (accession number AJ403134).

Regulation of expression of ADAMTS14 mRNA was investigated in culture treated with factors known to be regulators of the expression of various genes in fibroblast (Fig. 7). As a control for the efficiency of cell treatment, MMP1 expression was also measured in the various conditions. Results are expressed as a ratio of the values determined for treated on untreated cells. Modulation of MMP1 expression measured in the different conditions was in good agreement with previous reports (22, 23) confirming the efficiency of cell treatment. At the opposite, none of the five treatments was able to significantly modify the ADAMTS14 overall expression (Fig. 7), neither that of individual transcripts A, B, and C (not shown). Similar results were obtained for ADAMTS2 and -3 (not shown).

Recombinant ADAMTS-14 Analysis—Recombinant ADAMTS-14 with a COOH-terminal FLAG epitope was produced in 293-EBNA cells stably transfected with an eukaryotic expression vector containing the full-length ADAMTS14 cDNA. Cell populations were also created by transfection of, respectively, the empty vector and an ADAMTS-2 expression vector. As ADAMTS-2, ADAMTS-14 was not detected in the conditioned culture medium (not shown) but could be efficiently recovered from the cell layer, in the absence of detergent, using mild conditions of extraction such as Tris buffer containing 0.2 M NaCl. This suggests that the recombinant ADAMTS-14 is secreted but can be immobilized at the cell surface or in the extracellular matrix. By Western blotting, the recombinant ADAMTS-14 appears as a single band displaying a molecular size slightly higher than expected from the deduced amino acid sequence, suggesting that it is glycosylated (Fig. 8). The aminoprocollagen peptidase activity of recombinant ADAMTS-14 recovered from cell extracts was assayed and compared with the activity of cells containing the empty vector (background values) or recombinant ADAMTS-2, as a positive control. In the absence of any treatment, the activity of ADAMTS-14 was barely detectable suggesting either that this enzyme is not an aminoprocollagen peptidase or that it is secreted in an inactive pro-form. Various treatments known to promote the activation of pro-metalloproteases were performed. Limited trypsin digestion of recombinant ADAMTS-14 increased its aminoprocollagen peptidase activity with values ranging from 3 to 13% of the activity determined in similar conditions for ADAMTS-2 (Fig. 9A). At the opposite, p-aminophenylmercuric acetate had no significant effect. When cell cultures were treated for 2 days in the presence of dextran sulfate or concanavalin A, the aminoprocollagen peptidase activity recovered from the extracts was also significantly increased (Fig. 9B). The activity of ADAMTS-14 was also investigated, in the absence of exogenous activation, in a co-culture model. For these experiments, control 293 cells or 293 cells expressing ADAMTS-2 or -14 were plated with fibroblasts isolated from dermatosparactic calf skin. In culture, 80% of the type I collagen polypeptides secreted by these fibroblasts still contain the amino-terminal propeptide (pNoa chains) due to the absence of ADAMTS-2 activity (Fig. 10, lane 1). Co-culture of these deficient fibroblasts with 293 cells transfected with the empty vector did not modify the level of processing. By contrast, the presence of 293 cells expressing ADAMTS-2 resulted in a complete cleavage of the pNoa chains similar to that observed with normal calf skin fibroblasts (Fig. 10, lanes 3 and 6), validating this co-culture model for the evaluation of aminoprocollagen peptidase activity. When 293 cells expressing ADAMTS-14 were used, a significant conversion of both pNoa chains into α chains was observed (Fig. 10, lanes 4 and 5). A quantification of the polypeptides pattern indicated that the relative level of α chains rose from 20 to 52% in the presence of ADAMTS-14, evidencing its aminoprocollagen peptidase activity.
DISCUSSION

An intriguing observation in Ehlers-Danlos type VIIC (EDSVIIC) patients and animal dermatosparaxis is the presence of processed type I collagen in the absence of a functional ADAMTS-2 (12). This observation was confirmed in this study by using Adamts2/−/− mice. The level of processed collagen varied from tissue to tissue and could not be correlated to type I collagen content. For example, 80% of type I collagen is processed in tendon while only 30 to 40% is processed in the skin. This difference is biologically significant since, in Adamts2/−/− mice, tendon has a normal mechanical resistance and contains almost normal collagen fibers while the skin is highly fragile and contains the abnormal hyperglycopic collagen polymers seen in dermatosparactic calf and EDSVIIC patients (12, 13). Studies investigating the processing of pN-I collagen in ADAMTS-2 deficient human and animal had been performed so far by analysis of a SDS-PAGE pattern. Hence, it remained to be determined that the observed processing occurred at the cleavage site specific for ADAMTS-2 or at a close site. For example, MMP13 can cleave the aminotelopeptide of type I collagen (24), generating collagen fragments of about the same size as the products released from ADAMTS-2 digestion. Sequencing of processed pN-I collagen chains extracted from dermatosparactic calf tendon demonstrated that pN-I cleavage occurred at the bonds that are cleaved by ADAMTS-2 (Pro142-Glu143 for pN-I1 and Ala79-Glu80 for pN-I2). Processing of pN-I collagen requires a complex three-dimensional structure of the substrate in which the 3 propeptides (two pN-I1 and one pN-I2) are folded back across the major triple helix of the molecule (15). This specific requirement and the fact that the processing occurs at the ADAMTS-2 cleavage site for both pN-I1 and pN-I2 chains in dermatosparactic animal strongly suggested that a closely related enzyme could display a true aminoprocollagen peptidase activity. Because of its high homology with ADAMTS-2, ADAMTS-3 was first considered as the enzyme that could partially compensate for the ADAMTS-2 deficiency. Preliminary investigations on tissue distribution and relative level of ADAMTS3 mRNA are reported as arbitrary units of absorbance per ng of total RNA in the samples. Br, brain; He, heart; St, stomach; Li, liver; In, intestine; Mu, muscle; Ey, eye; Bo, bone; Sh, skin; Lu, lung; Sp, spleen; Ki, kidney; Te, tendon; Fb, fibroblasts in culture.

Fig. 6. Distribution pattern of Adamts14 in mouse tissues. Duplicate samples containing 10, 2, or 0.4 ng of total RNA purified from various tissues were RT-PCR amplified using specific primers for Adamts14 and 28 S RNA. After electrophoresis on 10% polyacrylamide gel and staining, the amplified cDNA products were quantified. Values obtained with 28 S primers were equivalent in all tissues (not shown). Values measured for products amplified from Adamts14 mRNA are reported as arbitrary units of absorbance per ng of total RNA in the samples. Br, brain; He, heart; St, stomach; Li, liver; In, intestine; Mu, muscle; Ey, eye; Bo, bone; Sh, skin; Lu, lung; Sp, spleen; Ki, kidney; Te, tendon; Fb, fibroblasts in culture.

Fig. 7. Regulation of ADAMTS14 expression. Fibroblasts in culture were left untreated or were treated for 1 day (black bar) or 2 days (gray bar) with 12-O-tetradecanoylphorbol-13-acetate (5 ng/ml), IL-1β (IL-1, 100 units/ml), tumor necrosis factor-α (TNF, 10 ng/ml), epidermal growth factor (20 ng/ml), or transforming growth factor β (TGF, 5 ng/ml). MMP1 (A) and ADAMTS14 (B) mRNA levels were assayed by RT-PCR. Results are expressed as the ratio of mRNA levels in treated/untreated control cultures.

Fig. 8. Western blotting analysis of the recombinant ADAMTS-14. 293 cells stably transfected with the empty vector (Cont.) or an ADAMTS-14 expression vector (TS14) were scraped and extracted for 2 h at 4 °C in a Tris buffer containing 0.5 M NaCl. An aliquot of the extract was migrated on SDS-PAGE and analyzed by Western blotting using an anti-FLAG antibody.
were performed to verify this hypothesis (not shown). ADAMTS3 is expressed only at low levels in many organs. In addition, no correlation was observed between the proportion of ADAMTS3 in skin fibroblasts from dermatosparactic (DF) or normal (NF) calf were cultivated, in DMEM supplemented with 10% FCS and ascorbic acid (50 μg/ml), alone (lanes 1 and 6), or with a similar number of 293 cells containing the empty vector (cont, lane 2), an ADAMTS-2 expression vector (TS-2, lane 3), or an ADAMTS-14 expression vector (cell populations resulting from two separate transfections, lanes 4 and 5). After a 24-h labeling with l-[2,3-3H]proline, the pattern of type I collagen polypeptides secreted in the culture medium was visualized after SDS-PAGE and autoradiography. pNα1 and pNα2, α1 and α2 collagen chains that contain the amino-terminal propeptide; α1 and α2, fully matured type I collagen polypeptides.

were verified to perform this hypothesis (not shown). ADAMTS3 is expressed only at low levels in many organs. In addition, no correlation was observed between the proportion of processed type I collagen in the tissues of Adamts2−/− mice and the relative level of Adamts3. These data suggested that an enzyme other than Adamts-2 or -3 could display aminoprolag collagen peptidase activity.

ADAMTS14 Cloning—By scanning data bases, homology was found between ADAMTS2 cDNA and sequences located on chromosome 10 (q21.3) that could represent exons of a new ADAMTS gene. After confirmation, by RT-PCR, that this potential novel gene can be expressed as mRNA, the complete sequence of human ADAMTS14 cDNA (name assessed in agreement with Human Gene Nomenclature Committee) and also part of the ADAMTS14 mouse ortholog were determined by sequencing overlapping RT-PCR fragments and 5′-RACE products. Different transcripts are expressed from the gene. An alternative splicing mechanism occurring at the end of exon 6 leads to an in-frame deletion of 9 bases (Table I). Only the 9-base skipped form is found in mouse, while in ADAMTS2 the corresponding 9 bases are always present, suggesting that this alternative splicing does not have a major biological significance. The presence of three different exons 1, named 1A, 1B, and 1C (Table I, Fig. 3), was also determined. Exon 1A on one hand and exon 1B or 1C on the other hand resulted from the alternative use of two different signals of initiation of transcription (Fig. 3). The difference between transcripts B and C (Fig. 3) is the presence in transcript C of a sequence that is spliced (intron 1B) during the maturation of transcript B. As a result, exon 1C is a large exon (1064 bp) consisting of sequences that correspond on transcript B to exon 1B, intron 1B, and exon 2. Another example of such a large first exon is found in the ADAMTS1 gene, where exon 1 is 1136 bp long (25). The existence of 2 sites of initiation of transcription, in two different promoter contexts, suggests that transcripts resulting from these two sites can be differently regulated. Preliminary data support this hypothesis, indicating that transcript A is expressed at relatively high level in skin, placenta, and human fibroblasts in culture while transcripts B and C are found at significant levels in fibroblasts only. Northern analysis showed a 4.5-kilobase pair major product. This size is similar to the size of other ADAMTS mRNA and is consistent with the cDNA sequence that was determined. The larger product of about 5.7 kilobase pairs probably results from the alternative use of a more 3′ polyadenylation signal. The origin of the three smaller transcripts, identified with the 3′-specific probe only, is still unknown. Determination of their mechanism of generation will require fine mapping of gene products with a set of smaller probes. Existence of transcripts lacking either 5′ or 3′ sequences is not unique in the ADAMTS family and have been determined for ADAMTS2 (11). The physiological significance of these truncated transcripts remains to be elucidated. A puzzling observation is the presence, on Northern blot, of only one band in the 4.5-kilobase pair region while, from RT-PCR data, we would expect to see two bands: one from transcript C and a smaller for transcript A (489 bases shorter), transcript B being expressed at a too low level to be seen on Northern blot. Two hypotheses could explain these apparently contradictory obser-

**Fig. 9. Aminoprolag collagen peptidase activity of recombinant ADAMTS-14 in vitro.** A, cells stably transfected with the empty vector, with an ADAMTS-2 expression vector or an ADAMTS-14 expression vector (two independent transfections) were grown to confluence in DMEM supplemented with 10% FCS. Cell layers extracts (performed in a buffer containing 0.2 M NaCl) were left untreated (□), or were incubated with trypsin for 15 (△) or 30 min (○) or with p-aminophenylmercuric acetate (APMA) for 15 min (□) before determination of the aminoprolag collagen peptidase activity. Values obtained with cells containing the empty vector were considered as background and subtracted from the other measurements. Activities of ADAMTS-14 are reported in % of activities determined for ADAMTS-2 under identical assay conditions. B, the same cells as in A were grown in DMEM supplemented with 10% FCS. 48 h before confluency, culture medium was removed and replaced by fresh medium containing 10% FCS only (□), 10% FCS and dextran sulfate at 0.01% (○), 10% FCS and concanavalin A at 10 (△) or 50 (○) μM. Cell extracts were not treated and immediately investigated for their aminoprolag collagen peptidase activity as described in A.

**Fig. 10. Processing of aminoprolag collagen type I in co-culture.** Skin fibroblasts from dermatosparactic (DF) or normal (NF) calf were cultivated, in DMEM supplemented with 10% FCS and ascorbic acid (50 μg/ml), alone (lanes 1 and 6), or with a similar number of 293 cells containing the empty vector (cont, lane 2), an ADAMTS-2 expression vector (TS-2, lane 3), or an ADAMTS-14 expression vector (cell population resulting from two separate transfections, lanes 4 and 5). After a 24-h labeling with l-[2,3-3H]proline, the pattern of type I collagen polypeptides secreted in the culture medium was visualized after SDS-PAGE and autoradiography. pNα1 and pNα2, α1 and α2 collagen chains that contain the amino-terminal propeptide; α1 and α2, fully matured type I collagen polypeptides.
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The highest levels of ADAMTS14 were found in collagen-rich tissue, supporting its role as an aminoprocollagen peptidase. However, significant levels were also detected in all tissues investigated, such as brain, spleen, and liver, indicating that ADAMTS-14 may have other functions. Similar findings have been reported for ADAMTS-2 (14). As ADAMTS2 and -3, ADAMTS14 is not transcriptionally regulated in fibroblasts by various soluble factors. At the opposite, ADAMTS1 is an inflammatory associated gene (25) that can be induced by IL-1 (26). Up-regulation of the expression of ADAMTS12 by transforming growth factor β (2) and of aggrecanases (ADAMTS4 and -5) by IL-1, IL-6, and tumor necrosis factor-α is also reported (27).

ADAMTS-14 Primary Structure—Determination of the ADAMTS-14 primary structure requires the determination of the translation start site. For transcript A, the most 5’ in-frame ATG in a suitable Kozak consensus sequence is found in exon 1A (Fig. 3). This ATG codon would result in the synthesis of a polypeptide displaying a moderately hydrophobic sequence at its NH2 terminus, which could serve as a signal peptide (Fig. 4A). Homology existing between this sequence (amino acids 42 to 61) and the sequence derived from exon 1 of ADAMTS2 or from mouse ADAMts14 suggests the actual use of this ATG as a start of translation. For transcripts B and C, the first suitable ATG corresponds to Met68 of the protein synthesized from transcript A (Figs. 3 and 4A). As a result, ADAMTS-14 polypeptides from transcripts B and C do not contain an obvious signal peptide. It remains to be determined whether these ADAMTS-14 variants are secreted, as all the ADAMTS described so far, or sequestered in the cytoplasm.

Besides differences at the NH2 terminus, ADAMTS-14 variants are identical and display very high homology with ADAMTS-2 and -3, in terms of length of polypeptide chain, primary structure, and domains organization. The highest similarity was observed around the catalytic site and the lowest, as expected, in the pro-domain and the COOH-terminal tail (Fig. 4). These results indicate that ADAMTS-2, -3, and -14 are three members of a structurally and functionally distinct sub-family of ADAMTS proteases. It has recently been demonstrated that ADAMTS-3, as ADAMTS-2, can process pN-II collagen (28). Because of the high homology between these three enzymes, it was conceivable that ADAMTS-14 may also display aminoprocollagen peptidase activity and may be the enzyme responsible for pN-I collagen maturation in tissues of dermatsparactic calves, Adamts2−/− mice, and EDSV1C patients. Preliminary data were in favor of this hypothesis. For example, highest ADAMTS14 mRNA levels were detected in collagen-rich tissues. In addition, the mRNA levels of ADAMTS14 and ADAMTS2 were similar, suggesting that the amount of ADAMTS-14 enzyme is high enough to allow maturation of significant amounts of pN-I collagen. Finally, the eye of Adamts2−/− mice, where Adamts14 is barely expressed, contained very low levels of processed type I collagen. The suspected aminoprocollagen peptidase activity of ADAMTS-14 was evaluated using recombinant enzyme. When produced by 293 cells grown in culture medium supplemented with fetal calf serum alone, recombinant ADAMTS-14 did not display any activity. However, upon limited digestion of the latent enzyme with trypsin or when recombinant ADAMTS-14 was extracted from cells cultivated in conditions known to promote metalloprotease activation (29, 30), significant aminoprocollagen peptidase activity was evidenced. These results were further confirmed in a co-culture model. In these experiments, aminoprocollagen type I (pNα1 and pNα2 chains) synthesized by dermatsparactic calf skin fibroblasts were significantly processed into mature chains in the presence of cells producing ADAMTS-14. Together, these results suggest that ADAMTS-14 could be the enzyme responsible for the aminoprocollagen type I processing activity in the absence of ADAMTS-2. They indicate also that ADAMTS-14 is synthesized as a latent enzyme that requires activation to display aminoprocollagen peptidase activity. Generation of active ADAMTS-14 in co-culture demonstrates that this activation can occur extracellularly and probably results from enzymatic activity associated with fibroblasts. This activation is not performed in 293 cells cultivated in basal culture conditions suggesting a specific, possibly cell type-regulated, mechanism. Such a tissue-regulated mechanism of activation is also suspected for ADAMTS-2 since this enzyme is expressed at high levels in some tissues (heart, kidney, and muscle) that are poor in type I collagen and from which no significant aminoprocollagen peptidase activity can be extracted (21). The existence of a cell type-regulated activation of ADAMTS-14 would also explain why dermatsparactic skin and tendon, which express similar levels of ADAMTS-14, contain a different proportion of fully processed type I collagen. According to this hypothesis, ADAMTS-14 produced in fibroblasts would be only poorly processed into an active aminoprocollagen peptidase while the activation process would be much more efficient in tendon. This will be evaluated, in further studies, by expressing recombinant ADAMTS-14 in fibroblasts from dermatsparactic skin and tendon using an adenoviral vector, the only one procedure allowing significant synthesis of recombinant protein in almost every primary fibroblasts.

In summary, the gene structure and the primary structure of mouse and human ADAMTS-14 have been determined. The tissue distribution of this novel ADAMTS, its homology with ADAMTS-2 and -3, and its aminoprocollagen peptidase activity, which was demonstrated in vitro using fibroblasts lacking ADAMTS-2 activity, suggest its participation in procollagen processing in vivo. Various transcripts have been identified. They result from the use of two different promoters and transcription start sites and lead to the synthesis of ADAMTS-14 isoforms that differ by their amino terminus. These observations suggest complex mechanisms of regulation of gene expression and enzyme function. Creation of Adamts14−/− mice, and eventually their breeding with Adamts2−/− mice, is planned to unravel other functions of ADAMTS-14 and to evaluate potential overlaps in the role of these two closely related enzymes.

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Cloning and Characterization of ADAMTS-14, a Novel ADAMTS Displaying High Homology with ADAMTS-2 and ADAMTS-3

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