

# 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 VIIIC and animal dermatosparaxis. In this study, we show that the aminopropeptide 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.

ADAMTS<sup>1</sup> (A Disintegrin and metalloprotease with thrombospondin type I repeats) is a novel family of metalloproteases

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<sup>1</sup> The abbreviations used are: ADAMTS, A disintegrin-like and metalloprotease with thrombospondin type I repeats; EDS, Ehlers-Danlos syndrome; RT, reverse transcription; FCS, 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.

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*<sup>−/−</sup> mice display abnormal growth, defective fertility, and altered organ morphology and function (7). A *C. elegans* *Adamts*, 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 VIIIC (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 VIIIC (EDSVIIC) patients or from dermatosparactic calves is NH<sub>2</sub>-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.

In this study, the specificity of the processing of the amino-propeptide of type I collagen in absence of ADAMTS-2 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<sup>-/-</sup>* mice (14), various organs and tissues were ground at liquid nitrogen temperature and extracted with 0.1 M acetic acid for 18 h at 4 °C. After centrifugation, the supernatants were neutralized and ammonium sulfate was added (40% saturation). After centrifugation (8000  $\times$  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 Laemmli 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 dermatosparactic calf tendon. Collagen was purified from 1 M NaCl extracts by sequential steps of precipitation and solubilization as described earlier (16). The collagen preparation was then treated or not with pyroglutamate aminopeptidase before electrophoresis on a pre-run 7.5% acrylamide/piperazine diacrylamide gel in 50 mM Tris borate buffer (pH 8.3) containing 0.1% SDS and 0.1 mM thioglycolic acid. After transfer on a polyvinylidene difluoride membrane (in 200 mM Tris borate, pH 9.5) and Coomassie Blue staining,  $\alpha$ 1I and  $\alpha$ 2I bands were collected and submitted to 6 cycles of Edman degradation amino acid sequencing.

**PCR Amplification and Sequencing of ADAMTS-14 cDNA**—Three large overlapping cDNA fragments covering the ADAMTS14 cDNA sequence that corresponds 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 *Taq* DNA polymerase (Takara) and three ADAMTS14 primer pairs (5'-CTATGGTGTGACAGTGGCCCTGCA-3' and 5'-GACGCTGCCAGGCTGGTCTCA-3'; 5'-GGCATGTGTCA-CCCCCTGAGGA-3' and 5'-TCCTTGTACAGCCGACAGGCACA-3'; 5'-GACGTGGTGTTCATGAACCAGGT-3' and 5'-GCCAGTGGGATGGCAGGGCACA-3'). PCR products were then gel purified and sequenced using the manufacturers recommended protocols (Thermo-sequenase radiolabeled terminator cycle sequencing kit, Amersham Biosciences Inc.). To amplify mouse *Adams14* cDNA, various human primer pairs were used. PCR conditions were: 2 cycles consisting of 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 1 min, followed by 35 cycles consisting of 94 °C for 20 s, 66 °C for 20 s, and 72 °C for 1 min. PCR products amplified by 5'-CTATGGTGTGACAGTGGCCCTGCA-3' and 5'-GACGCTGCCAGGCTGGTCTCA-3' or 5'-AGCCTGCCCTACAAGTACGTCA-T-3' and 5'-CTCTTCTGTGGTCACACAGGTGT-3' pairs were sequenced. The determination of partial mouse sequences allowed the design a mouse-specific primer pair that was used to amplify and sequence the central part of the murine cDNA.

For tissue distribution analysis, total RNA was purified from various normal mouse tissues (17). Duplicate samples from 3 dilutions of RNA from each tissue (10, 2, and 0.4 ng) were used for semi-quantitative RT-PCR amplification.

**Determination of the 5'-End of ADAMTS-14 cDNA**—The 5'-end of ADAMTS14 mRNA was amplified using the FirstChoice™ RLM-RACE Kit (Ambion) using the manufacturers recommended protocols. Briefly, mRNA from cultured fibroblasts was dephosphorylated and then treated with tobacco acid pyrophosphatase to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. A RNA adapter was then ligated to the decapped phosphorylated mRNA. After reverse transcription (BcaBest™ RNA PCR kit, Takara), the 5'-end of the ADAMTS14 cDNA was amplified using the sense "Outer Adapter primer" from the kit and the ADAMTS14 5'-CCAGACACCACGTGG-GAGAGGAA-3' antisense primer (30 cycles; 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min). One microliter of the outer amplification product was then re-amplified using the nested sense "Inner Adapter primer" and the 5'-CGTCCCCGAAAGTCTGTGCTGCA antisense primer (25 cycles; 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min). Resulting PCR products were then sequenced as described above.

**Northern Analysis**—PCR amplified products generated from the 5'-

or 3'-end of the ADAMTS14 cDNA (corresponding to amino acids 153–471 and 824–1078, respectively) were cloned using pCR4-TOPO cloning kit (Invitrogen) according to the manufacturers protocols. Antisense labeled riboprobes were synthesized from 500 ng of linearized plasmid (*Spe*I restriction site) using T7 RNA polymerase (Strip-EZ™ RNA kit, Ambion) and [<sup>32</sup>P]UTP (ICN).

Messenger RNA purified from human skin fibroblasts in culture (PolyATtract™ mRNA Isolation System III, Promega) was separated by electrophoresis on a 0.9% agarose/formaldehyde gel and transferred and fixed to a nylon membrane (Hybond N, Amersham Biosciences, Inc.) by UV irradiation. The filters were then sequentially prehybridized for 1 h (at 65 °C in 0.2 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, 7% SDS, 20% formamide), hybridized with labeled probes for 18 h (same conditions as for prehybridization) and washed 3 times (at 65 °C in 40 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), 1 mM EDTA, 1% SDS) before autoradiographic exposure.

**Semi-quantitative RT-PCR Assay**—The determination of mRNA level by RT-PCR amplification requires the use of an internal standard allowing to monitor the efficiency of each step of the procedure in each sample. The construction of synthetic RNA standards that are used as controls for RT-PCR quantification of various mRNAs is described elsewhere (18). Briefly, we designed and generated synthetic RNAs that have two main characteristics. First, they can be RT-PCR amplified by using the same primer pairs used for RT-PCR amplification of the cellular mRNAs. Second, their amplification products are larger or smaller than those obtained from the cellular mRNAs, enabling their discrimination by electrophoresis when co-amplified in the same tube. Semi-quantification was obtained by calculating, for each sample, the ratio between the level of the product generated from the endogenous mRNA and from a defined copy number of the standard synthetic RNA. RT-PCR reactions were performed, in a 25- $\mu$ l reaction mixture, in an automated thermal cycler (GeneAmp PCR System 2400 or 9600, PerkinElmer Life Sciences, Norwalk, CT) using the GeneAmp Thermostable rTth 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). The various primer pairs allowed the amplification of human (5'-AGCCTGCCCTACAAGTACGTCA-3'/5'-CTCTTCTGTGGTCAC-ACAGGT-3') or mouse (5'-AGCCTGCCCTACAAGTACGTCA-3'/5'-CTCCCTCACAGGCTGTGCA-3') ADAMTS14 mRNA, human MMP1 mRNA (5'-GAGCAAACACATCTGAGGTACAGGA-3'/5'-TTGT-CCCGATGATCTCCCTGACA-3') and human (5'-GAACCATGAGGA-CGGCTCTCCT-3'/5'-GGCTGAGCGGGACCAGTGGAA-3') or mouse (5'-CAGGCGCACACATAGTACCATCCA-3'/5'-CAGCCGCTACCTGC-ATTCTATGA-3') ADAMTS2 mRNA. The RT step (70 °C for 15 s) was followed by denaturation of RNA/DNA duplexes (95 °C for 2 min) and by PCR amplification (adequate number of cycles consisting of 94 °C for 15 s, 66 °C for 20 s, and 72 °C for 10 s). RT-PCR products were resolved on a 10% polyacrylamide gel and analyzed (Fluor-S-MultiImager, Bio-Rad) after staining (Gelstar, FMC BioProducts).

**Effects of Cytokines and Growth Factors**—Human dermal fibroblasts, at passages 4 to 12, were plated at a density of  $8 \times 10^3$  cells/cm<sup>2</sup> and cultured for 1 or 2 days in Dulbecco's modified Eagle's medium (DMEM). The medium, supplemented with 10% dialyzed and decomplexed FCS, contained or not 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml), IL-1 $\beta$  (100 units/ml), tumor necrosis factor- $\alpha$  (5 ng/ml), epidermal growth factor (20 ng/ml), or transforming growth factor- $\beta$  (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<sup>-/-</sup>* mice were fixed for 60 min at room temperature in 2.5% glutaraldehyde in 0.1 Sörensen's 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 (Epon 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.

**Recombinant ADAMTS-14**—An expression vector containing the entire coding sequence of ADAMTS14 transcript A (see "Results" section) and part of the 5'-untranslated region was constructed. Briefly, 3 PCR products were amplified (*Pfu* Turbo™ DNA polymerase, Stratagene) using cDNA from human skin fibroblasts in culture and three different pairs of primers (1, 5'-CACACACAGCGGCCCTGCCCCAG-CCCGCGTCCCA-3' and 5'-TGAAGAATTGGTGTCTGTGCGGA-TGA-3'; 2, 5'-CACAGAATTCTCATTGAGCCTCTGGAGC-3' and 5'-CACAAAGCTTGAGAGCTCTGCTGCT-3'; 3, 5'-CACAAAGCTGTGAGATCCCAGCAGGTGCCA-3' and 5'-CTCTTCTAGATTAGCTAGTGTCAACCGGGGAGGCAGCAG-3'). By digestion with appropriate

restriction enzymes (1, *NotI/EcoRI*; 2, *EcoRI/HindIII*; 3, *HindIII/NheI*) 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/NheI* 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<sup>2</sup> 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 CaCl<sub>2</sub>, 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 dithiothreitol. Conditioned culture medium and the two cell layer-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 hygromycin B (100 µg/ml). In some cultures, dextran sulfate (average  $M_r$ , 500,000; 0.01%) or concanavalin A (10 or 50 µM) were added for the last 48 h of culture. Cells were then scraped and extracted as described above. Aliquots of cell extracts were then stored at 4 °C or incubated in presence of *p*-aminophenylmercuric acetate (0.3 mM for 15 min at 25 °C) or trypsin (100 µg/ml for 15 or 30 min at 37 °C) followed by an incubation with soybean trypsin inhibitor (500 µg/ml for 5 min at 37 °C). The various samples of cell extracts were then assayed for their aminoprocollagen peptidase activity using <sup>14</sup>C-labeled pNI collagen as described earlier (10). Values determined for the cell transfected with the empty vector were considered as background and subtracted from values obtained for cells expressing recombinant ADAMTS-2 or -14. Aminoprocollagen peptidase activity of ADAMTS-14 are reported in % of the ADAMTS-2 activity.

Co-culture experiments were performed in DMEM supplemented with 10% FCS and ascorbic acid (50 µg/ml) in the absence of hygromycin B. Stably transfected 293 cells were plated with a similar number of skin fibroblasts isolated from normal or dermatosparactic calf. After 24 h, the culture medium was changed and L-[2,3-<sup>3</sup>H]proline (45 Ci/mmol, 10 µCi/ml) was added. After 24 h, the culture mediums and the cell layers were collected separately. The collagen polypeptides were recovered from the cell layer by extraction at 4 °C with 0.1 M acetic acid and from the culture medium by differential salt precipitation with ammonium sulfate as described earlier (16). The pattern of labeled collagen polypeptides was analyzed by 6.25% SDS-PAGE in nonreducing conditions and visualized after fluorography.

## RESULTS

**Procollagen Processing in Dermatosparaxis**—The level of type I aminoprocollagen (pNI) processing in the skin of wild type (WT) or *Adamts2*<sup>-/-</sup> mice was investigated by SDS-PAGE (Fig. 1A). Only  $\alpha 1$  and  $\alpha 2$  chains were observed in tissues of WT mice, illustrating the complete processing of the aminopropeptide of type I collagen. In *Adamts2*<sup>-/-</sup> mice, pN $\alpha$ 1I and pN $\alpha$ 2I chains were detected, as expected from an animal lacking *Adamts-2* activity. However, mature  $\alpha 1$  and  $\alpha 2$  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 tendon, it represents only 20 to 25%. When most of the pN-I collagen is not processed, such as in the skin of *Adamts2*<sup>-/-</sup> 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).

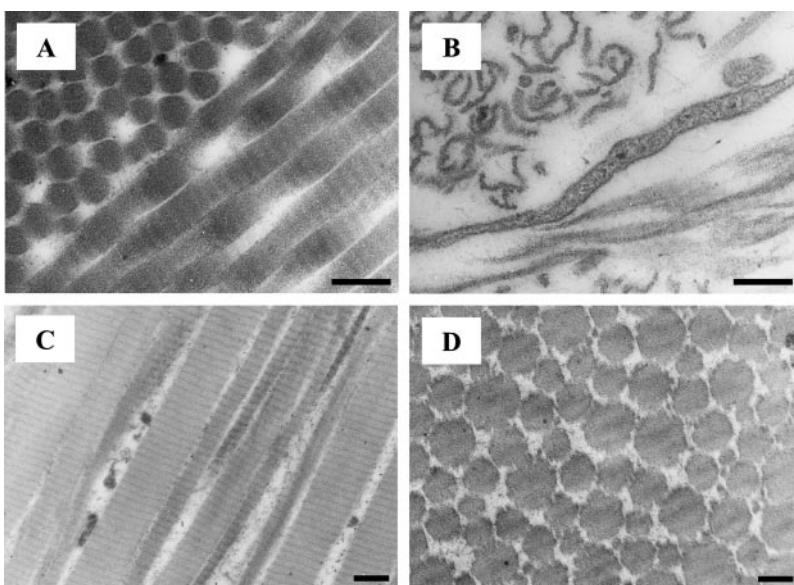
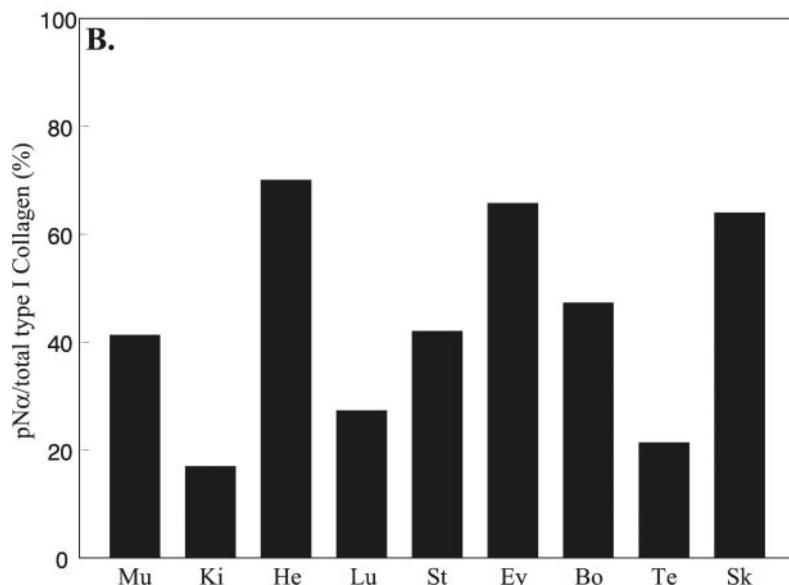
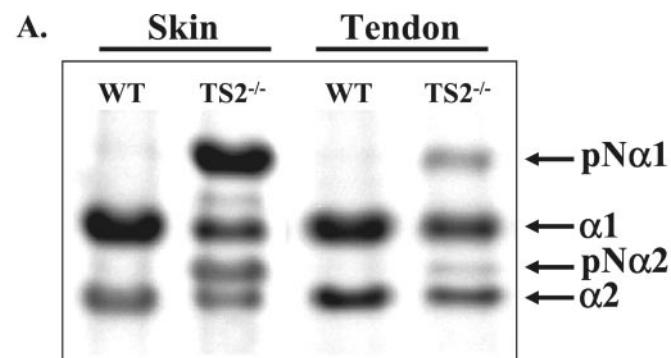
The specificity of the processing of pNI collagen in the absence of *Adamts-2* activity was assessed by amino acid sequencing of the NH<sub>2</sub> terminus of processed  $\alpha 1$  and  $\alpha 2$  chains isolated from dermatosparactic calf tendon. A first attempt failed to provide sequence information, suggesting that their amino terminus was blocked, possibly by modification of the glutamine residue which is the first expected amino acid of  $\alpha 1$  and  $\alpha 2$  when the processing occurs at the site normally cleaved by *Adamts-2*. After digestion with pyroglutamate aminopeptidase, a LSYGYD sequence was obtained for the  $\alpha 1$  chain and a FDAKGG sequence for the  $\alpha 2$  chain. In bovine type I collagen, these two sequences immediately follow the Gln residue in position 1 of correctly processed  $\alpha 1$  and  $\alpha 2$ . 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, and 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 I). 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 I). 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 I). Comparison with genomic sequences showed that the beginning of exon 1B (5'-TATTT) starts at a *tctgTATTT* potential Cap signal located 17 base pairs (bp) downstream a potential TATA-box (tgtatat) (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'

<sup>2</sup> A. Colige, C. M. Lapière, and B. V. Nusgens, manuscript in preparation.

**FIG. 1. Accumulation of p $\text{N}\alpha 1$  and p $\text{N}\alpha 2$  type I collagen in mouse tissues.** *A*, the pattern of type I collagen polypeptide purified from skin and tendon of "wild type" (WT) and *Adams2*<sup>-/-</sup> (TS2<sup>-/-</sup>) mice was determined after SDS-PAGE and Coomassie Blue staining. In WT, only  $\alpha 1$  and  $\alpha 2$  mature chains are visible. In TS2<sup>-/-</sup>, absence of *Adams2* activity results in the accumulation of p $\text{N}\alpha 1$  and p $\text{N}\alpha 2$  chains. Higher proportions of p $\text{N}\alpha$  chains were observed in skin. *B*, type I collagen was extracted from various tissues of TS2<sup>-/-</sup> mice. After electrophoresis on SDS-PAGE and staining, p $\text{N}\alpha 1$ , p $\text{N}\alpha 2$ ,  $\alpha 1$ , and  $\alpha 2$  type I collagen bands were quantified. For each tissue, the proportion of p $\text{N}\alpha$  chains is expressed as a percentage of total type I collagen. *Mu*, muscle; *Ki*, 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 *Adams2*<sup>-/-</sup> (TS2<sup>-/-</sup>) mice were observed by electron microscopy. In TS2<sup>-/-</sup>, skin collagen fibrils are strongly disorganized while they display an almost normal shape in tendon. Bars represent 0.3  $\mu\text{m}$ . *Top panels*, collagen fibrils (longitudinal and cross-section) in skin from a WT mouse (*A*) or a TS2<sup>-/-</sup> mouse (*B*). *Bottom panels*, collagen fibrils, in longitudinal (*C*) or cross (*D*) section, in tendon from a TS2<sup>-/-</sup> mouse.

end of the transcript had not been cloned. A tcag 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

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TABLE I  
ADAMTS14 gene structure

Exon partial sequences are in capital letters. The nucleotide consensus sequences of the 5' and 3' splice junctions of the introns are shown in boldface letters.

Exons			Introns		
No.	Size	Position <sup>a</sup>	No.	Size	Splice sites
	bp			bp	5' Donor 3' Acceptor
1A <sup>b</sup>	135	-53 to 82	1A	1668	CCCAAG/ <b>gtgcgt</b>
1B <sup>b</sup>	156	— <sup>c</sup>	1B	468	TCTAG/ <b>gtgtgt</b>
1C <sup>b</sup>	1064	— <sup>c</sup>	2	>27000	<b>GATTG/gtaagg</b>
2	440	83 to 522	2	>27000	<b>GATTG/gtaagg</b>
3	157	523 to 679	3	6119	TGAAG/ <b>gttagc</b>
4	191	680 to 870	4	20549	ATACT/ <b>gtgagt</b>
5	84	871 to 954	5	724	GACAG/ <b>gttaac</b>
6 <sup>d</sup>	157	955 to 1111	6 <sup>d</sup>	1995	GCAAG/ <b>gtactg</b>
7	106	1112 to 1217	7	1525	CACGT/ <b>gttaat</b>
8	144	1218 to 1361	8	1141	CTCCC/ <b>gttagt</b>
9	133	1362 to 1494	9	1378	TGGCA/ <b>gttaat</b>
10	114	1495 to 1608	10	2046	GCAAG/ <b>gtacct</b>
11	149	1609 to 1757	11	2146	CCCTC/ <b>gtgtgt</b>
12	176	1758 to 1933	12	2385	CGATG/ <b>gtgagt</b>
13	134	1934 to 2067	13	384	GTGTG/ <b>gtgggt</b>
14	124	2068 to 2191	14	1036	GGCAG/ <b>gtgagc</b>
15	81	2192 to 2272	15	4515	CATTG/ <b>gtgagt</b>
16	164	2273 to 2436	16	1492	TCCTG/ <b>gtgagc</b>
17	169	2437 to 2605	17	448	TGGAG/ <b>gtaccc</b>
18	133	2606 to 2738	18	1572	CCTGT/ <b>gtgagt</b>
19	208	2739 to 2946	19	3955	CCCAG/ <b>gtgact</b>
20	130	2947 to 3076	20	83	TGGAG/ <b>gtgagc</b>
21	111	3077 to 3187	21	2075	ATCAA/ <b>gttaat</b>
22	>519	3188 to			

<sup>a</sup> A of the ATG start codon in exon 1A is considered as +1.

<sup>b</sup> 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.

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

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

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 ( $\pm$ 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 NH<sub>2</sub>-terminal domain (amino acid 52 to 76) that could serve as a signal peptide (Fig. 4A). Low homology was found between part of this sequence and the NH<sub>2</sub> 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 Met<sup>68</sup> of the sequence reported for transcript A (Fig. 4A) and does not contain an obvious signal peptide. Besides this difference, the predicted proteins trans-

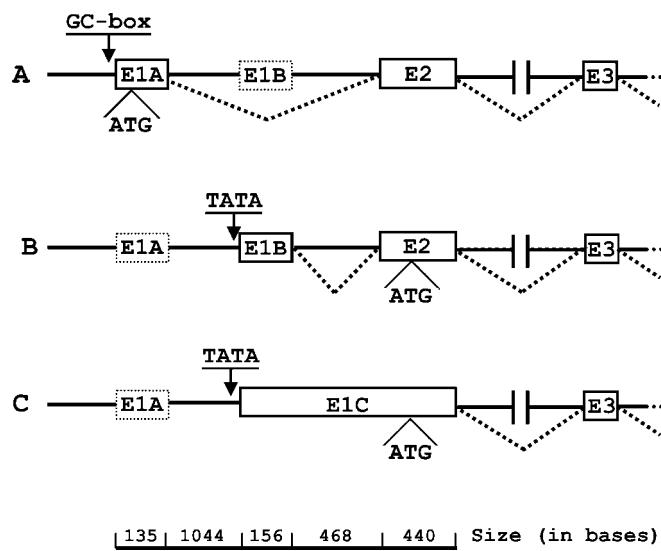
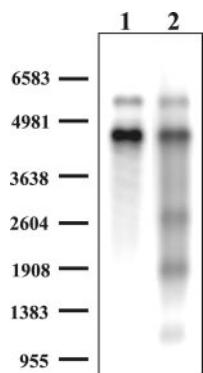


FIG. 3. Mechanisms of generation of alternative exon 1A (A), 1B (B), and 1C (C). Open rectangles representing exons and lines representing promoter or intronic sequences are not at scale. Dotted lines illustrate splicing mechanisms. For transcript A, exon 1A is joined to exon 2 by splicing of intron 1A. A potential regulatory GC-box is located upstream 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 17 bp downstream a potential TATA-box. Intron 1B is spliced during the maturation of transcript B while it is conserved in transcript C. The first potential 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 the cDNA sequence determined by sequencing of 5'-RACE products. The use of a more upstream CAP signal would lead to a larger E1A.

A

**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 (\*).



**Fig. 5. Northern analysis of ADAMTS14 transcripts.** The size of the RNA markers is shown on the left. mRNA from skin fibroblasts in culture was separated on an agarose/formaldehyde gel and transferred onto a nylon membrane. Blots were revealed using labeled cRNA probes specific either for the 5'-end (lane 1) or the 3'-end (lane 2) of the ADAMTS14 mRNA.

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).

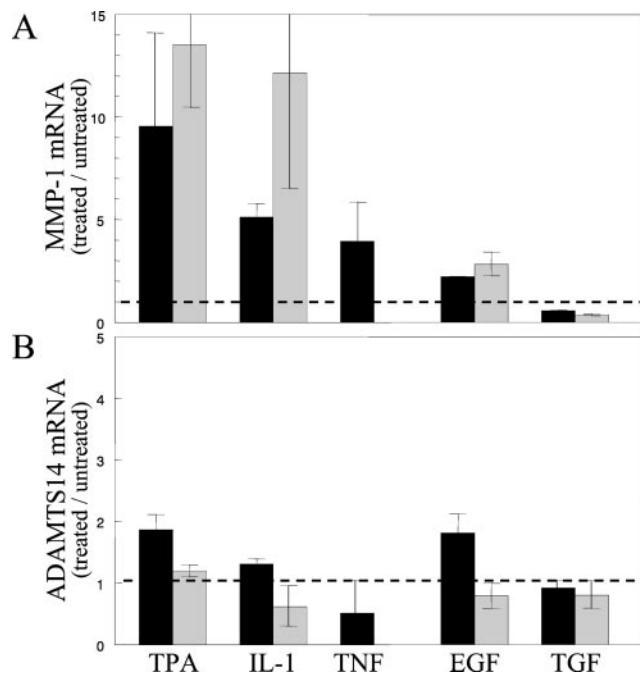
**Tissue Distribution and Regulation of Expression**—Tissue-specific expression was evaluated by RT-PCR. *Adamts14* is expressed in all examined tissues, with highest levels in type I collagen-rich tissues and in fibroblasts in culture (Fig. 6). Lower levels were observed in liver, stomach, brain, and 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 BF906335), 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 investi-

gated 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 (p $\text{N}\alpha$  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 p $\text{N}\alpha$  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 p $\text{N}\alpha$  chains into  $\alpha$  chains was observed (Fig. 10, lanes 4 and 5). A quantification of the polypeptides pattern indicated that the relative level of  $\alpha$  chains rose from 20 to 52% in the presence of ADAMTS-14, evidencing its aminoprocollagen peptidase activity.

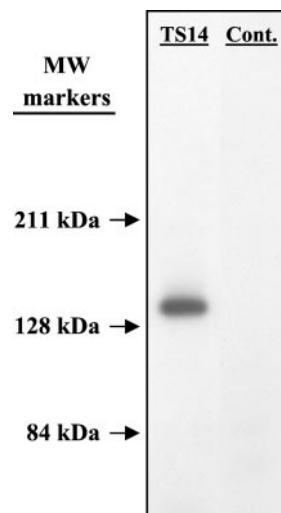
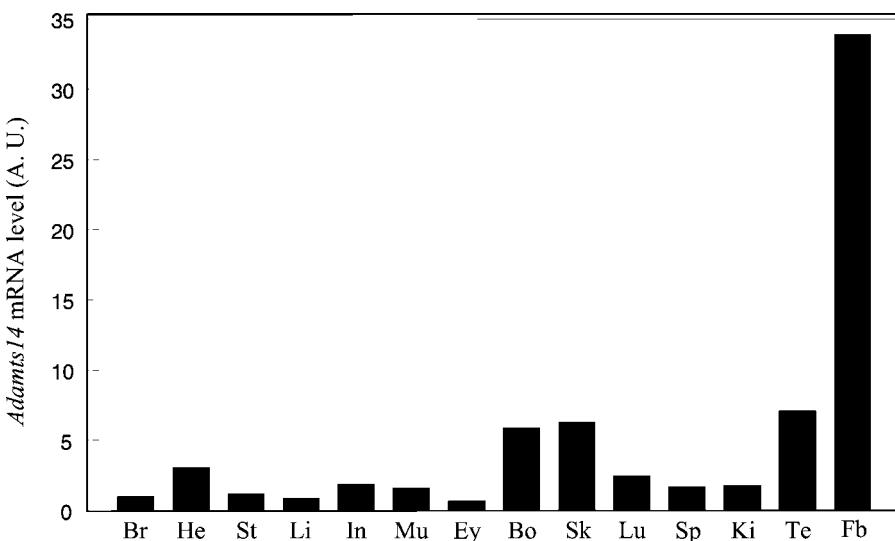
**FIG. 6. Distribution pattern of *Adams14* in mouse tissues.** Duplicate samples containing 10, 2, or 0.4 ng of total RNA purified from various tissues were RT-PCR amplified using specific primers for *Adams14* and 28 S RNA. After electrophoresis on 10% polyacrylamide gel and staining, the amplified cDNA products were quantified. Values obtained with 28 S primers were equivalent in all tissues (not shown). Values measured for products amplified from *Adams14* 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; *Sk*, 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-1 $\beta$  (IL-1, 100 units/ml), tumor necrosis factor- $\alpha$  (TNF, 10 ng/ml), epidermal growth factor (20 ng/ml), or transforming growth factor  $\beta$  (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.

#### DISCUSSION

An intriguing observation in Ehlers-Danlos type VIIC (EDS-VIIC) 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice, tendon has a normal mechanical resistance and contains almost normal collagen fibers while the skin is highly fragile and contains the abnormal heteroglyptic collagen polymers seen in dermatosparactic calf and EDSVIIC patients (12, 13). Studies investigating the processing of pN-I collagen in AD-



**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.

AMTS-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  $\alpha 1$  and  $\alpha 2$  chains extracted from dermatosparactic calf tendon demonstrated that pN-I cleavage occurred at the bonds that are cleaved by ADAMTS-2 (Pro<sup>142</sup>-Glu<sup>143</sup> for  $\alpha 1$  and Ala<sup>79</sup>-Glu<sup>80</sup> for  $\alpha 2$ ). Processing of pN-I collagen requires a complex three-dimensional structure of the substrate in which the 3 propeptides (two  $\alpha 1$  and one  $\alpha 2$ ) 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  $\alpha 1$  and  $\alpha 2$  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

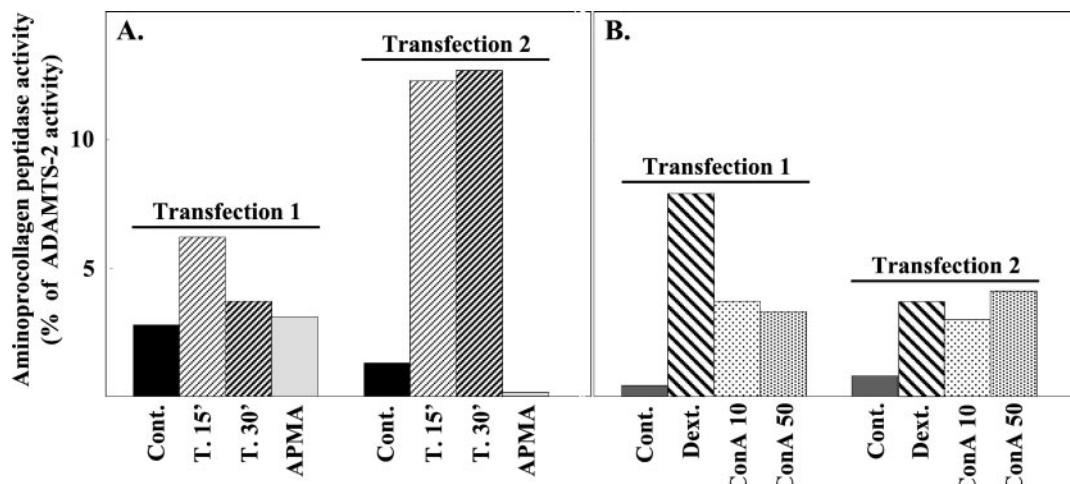


FIG. 9. Aminoprocollagen peptidase activity of recombinant ADAMTS-14 *in vitro*. *A*, cells stably transfected with the empty vector, with an ADAMTS-2 expression vector or an ADAMTS-14 expression vector (two independent transfections) were grown to confluence in DMEM supplemented with 10% FCS. Cell layers 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 aminoprocollagen 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 confluence, culture medium was removed and replaced by fresh medium containing 10% FCS only (■), 10% FCS and dextran sulfate at 0.01% (▨), or 10% FCS and concanavalin A at 10 (▨) or 50 (▨)  $\mu$ M. Cell extracts were not treated and immediately investigated for their aminoprocollagen peptidase activity as described in *A*.

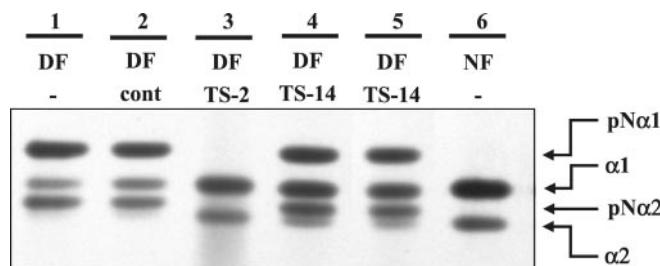


FIG. 10. Processing of aminoprocollagen type I in co-culture. Skin fibroblasts from dermatosparasitic (DF) or normal (NF) calf were cultivated, in DMEM supplemented with 10% FCS and ascorbic acid (50  $\mu$ g/ml), alone (lanes 1 and 6), or with a similar number of 293 cells containing the empty vector (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-<sup>3</sup>H]proline, the pattern of type I collagen polypeptides secreted in the culture medium was visualized after SDS-PAGE and autoradiography. pNo<sub>1</sub> and pNo<sub>2</sub>,  $\alpha$ 1 and  $\alpha$ 2 collagen chains that contain the amino-terminal propeptide;  $\alpha$ 1 and  $\alpha$ 2, fully matured type I collagen polypeptides.

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 processed type I collagen in the tissues of *Adamts2*<sup>-/-</sup> mice and the relative level of *Adamts3*. These data suggested that an enzyme other than Adamts-2 or -3 could display aminoprocollagen peptidase activity.

**ADAMTS14 cDNA 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<sup>3</sup> (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-

<sup>3</sup> A. Colige, personal observation.

vations. Since quantification of transcripts A and C does not use the same primer pair, we cannot exclude that differences in the efficiency of the two assays would have led to biased results, a transcript being actually relatively much more expressed, compared with the other, than expected from RT-PCR quantification. According to this hypothesis, only this transcript would be visible by Northern analysis, the two others being undistinguishable from the background. Another explanation is that exon 1A is longer than the 135 bp reported on Table I. Evaluation of its size is based upon the presence in genomic DNA of a suitable CAP signal situated 27 bp upstream the *ADAMTS14* cDNA sequence determined by 5'-RACE. If, instead, exon 1A extends a few hundred bases upstream, the size of transcripts A and C would be too close to be discriminated on Northern blot.

*Adamts14 Regulation of Expression*—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,<sup>3</sup> *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  $\beta$  (2) and of aggrecanases (*ADAMTS4* and -5) by IL-1, IL-6, and tumor necrosis factor- $\alpha$  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 NH<sub>2</sub> 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 Met<sup>68</sup> 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 NH<sub>2</sub> 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 subfamily 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 dermatosparactic calves, *Adamts2*<sup>-/-</sup> mice, and EDSVIIC 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*<sup>-/-</sup> 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 (pN $\alpha$ 1 and pN $\alpha$ 2 chains) synthesized by dermatosparactic calf skin fibroblasts were significantly processed into mature  $\alpha$  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 dermatosparactic 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 dermatosparactic 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*<sup>-/-</sup> mice, and eventually their breeding with *Adamts2*<sup>-/-</sup> 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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## REFERENCES

1. Tang, B. L. (2001) *Int. J. Biochem. Cell Biol.* **33**, 33–44
2. Cal, S., Argüelles, J. M., Fernandez, P. L., and López-Otin, C. (2001) *J. Biol. Chem.* **276**, 17932–17940
3. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., Rockwell, A.,

Yang, F., Duke, J. L., Solomon, K., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, H., Wiswall, B. H., Murphy, K., Hillman, M. C., Jr., Hollis, G. F., Newton, R. C., Magolda, R. L., Trazaskos, J. M., and Arner, E. (1999) *Science* **284**, 1664–1666

4. Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, W. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M., Wynn, R., Duke, J. L., George, H. J., Hillman, M. C., Jr., Murphy, K., Wiswall, B. H., Copeland, R. A., Decicco, C. P., Bruckner, R., Nagase, H., Itoh, Y., Newton, R. C., Magolda, R. L., Trazaskos, J. M., Hollis, G. F., Arner, E., and Burn, T. C. (1999) *J. Biol. Chem.* **274**, 23443–23450

5. Kuno, K., Okada, Y., Kawashima, H., Nakamura, H., Miyazaki, M., Ohno, H., and Matsushima, K. (2000) *FEBS Lett.* **478**, 241–245

6. Vázquez, F., Hastings, G., Ortega, M.-A., Lane, T. F., Oikemus, S., Lombardo, M., and Iruela-Arispe, M. L. (1999) *J. Biol. Chem.* **274**, 23349–23357

7. Shindo, T., Kurihara, H., Kuno, K., Yokoyama, H., Wada, T., Kurihara, Y., Imai, T., Wang, Y., Ogata, M., Nishimatsu, H., Moriyama, N., Oh-hashi, Y., Morita, H., Ishikawa, T., Nagai, R., Yazaki, Y., and Matsushima, K. (2000) *J. Clin. Invest.* **105**, 1345–1351

8. Blelloch, R., and Kimble, J. (1999) *Nature* **399**, 586–590

9. Lapière, C. M., Lenaers, A., and Kohn, L. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68**, 3054–3058

10. Colige, A., Beschin, A., Samyn, B., Goebels, Y., Van Beeumen, J., Nusgens, B. V., and Lapière, C. M. (1995) *J. Biol. Chem.* **270**, 16724–16730

11. Colige, A., Sieron, A. L., Li, S.-W., Schwarze, U., Petty, E., Wertelecki, W., Wilcox, W., Krakow, D., Cohn, D. H., Reardon, W., Byers, P. H., Lapière, C. M., Prockop, D. J., and Nusgens, B. V. (1999) *Am. J. Hum. Genet.* **63**, 308–317

12. Nusgens, B. V., Verellen-Dumoulin, G., Hermans-Le, T., De Paepe, A., Nytinck, L., Piérard, G. E., and Lapière, C. M. (1992) *Nat. Genet.* **1**, 214–217

13. Piérard, G. E., and Lapière, C. M. (1976) *J. Invest. Dermatol.* **66**, 2–7

14. Li, S.-W., Arita, M., Fertala, A., Bao, Y., Kopen, G. C., Långsjö, T. K., Hyttinen, M. M., Helminen, H. D., and Prockop, D. J. (2001) *Biochem. J.* **355**, 271–278

15. Prockop, D. J., Sieron, A. L., and Li, S.-W. (1998) *Matrix Biol.* **16**, 399–408

16. Lapière, C. M., Nusgens, B. V., and Piérard, G. E. (1977) *Connect. Tissue Res.* **5**, 21–29

17. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299

18. Lambert, C. A., Colige, A., Lapière, C. M., and Nusgens, B. V. (2001) *Eur. J. Cell Biol.* **80**, 479–485

19. Bucher, P. (1990) *J. Mol. Biol.* **212**, 563–578

20. Kozak, M. (1986) *Cell* **44**, 283–292

21. Colige, A., Li, S.-W., Sieron, A. L., Nusgens, B. V., Prockop, D. J., and Lapière, C. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2374–2379

22. Lambert, C. A., Lapière, C. M., and Nusgens, B. V. (1998) *J. Biol. Chem.* **273**, 23143–23149

23. Colige, A., Lambert, C. A., Nusgens, B. V., and Lapière, C. M. (1992) *Biochem. J.* **285**, 215–221

24. Krane, S. M., Byrne, M. H., Lemaitre, V., Henriet, P., Jeffrey, J. J., Witter, J. P., Liu, X., Wu, H., Jaenish, R., and Eeckhout, Y. (1996) *J. Biol. Chem.* **271**, 28509–28515

25. Kuno, K., Lizasa, H., Ohno, S., and Matsushima, K. (1997) *Genomics* **46**, 466–471

26. Sasaki, M., Seo-Kiryu, S., Kato, R., Kita, S., Kiyama, H. (2001) *Mol. Brain Res.* **89**, 158–163

27. Flannery, C. R., Little, C. B., Hughes, C. E., Curtis, C. L., Caterson, B., and Jones, S. A. (2000) *Matrix Biol.* **19**, 549–553

28. 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**, 31502–31509

29. Cowell, S., Knauper, V., Stewart M. L., D'Ortho, M. P., Stanton, H., Hembry, R. M., Lopez-Otin, C., Reynolds, J. J., and Murphy, G. (1998) *Biochem. J.* **331**, 453–458

30. Bateman, J. F., and Golub, S. B. (1990) *Biochem. J.* **267**, 573–577