

Identification, Characterization, and Intracellular Processing of ADAM-TS12, a Novel Human Disintegrin with a Complex Structural Organization Involving Multiple Thrombospondin-1 Repeats*

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We have identified and cloned a human fetal lung cDNA encoding a new protein of the ADAM-TS family (a disintegrin and metalloproteinase domain, with thrombospondin type-1 modules) that has been called ADAM-TS12. This protein exhibits a domain organization similar to the remaining family members including a propeptide and metalloproteinase-like, disintegrin-like, and cysteine-rich domains. However, the number and organization of the TS repeats is unique with respect to other human ADAM-TSs. A total of eight TS-1 repeats arranged in three groups are present in this novel ADAM-TS. Analysis of intracellular processing of ADAM-TS12 revealed that it is synthesized as a precursor molecule that is first activated by cleavage of the prodomain in a furin-mediated process and subsequently processed into two fragments of different size: a 120-kDa N-terminal proteolytically active fragment containing the metalloproteinase and disintegrin domains, and a 83-kDa C-terminal fragment containing most of the TS-1 repeats. Somatic cell hybrid and radiation hybrid mapping experiments showed that the human ADAM-TS12 gene maps to 5q35, a location that differs from all ADAM genes mapped to date. Northern blot analysis of RNAs from human adult and fetal tissues demonstrated that ADAM-TS12 transcripts are only detected at significant levels in fetal lung but not in any other analyzed tissues. In addition, ADAM-TS12 transcripts were detected in gastric carcinomas and in tumor cell lines from diverse sources, being induced by transforming growth factor- β in KMST human fibroblasts. These data suggest that ADAM-TS12 may play roles in pulmonary cells during fetal development or in tumor processes through its proteolytic activity or as a molecule potentially involved in regulation of cell adhesion.

tial for the development and maintenance of an organism. Likewise, proteolysis of the extracellular matrix is of vital importance for a series of tissue-remodeling processes occurring during both normal and pathological conditions, such as tissue morphogenesis, wound healing, inflammation, or tumor cell invasion and metastasis. These events are mediated by a variety of cell surface adhesion proteins and proteases, with different structural and functional characteristics (1). Among them, a group of recently described proteins called ADAMs (a disintegrin and metalloproteinase domain) have raised considerable interest because of their potential ability to perform both functions, adhesion and proteolysis (2, 3). ADAMs were first associated with reproductive processes like spermatogenesis and heterotypic sperm-egg binding and fusion (4). However, over the last few years, the spectrum of functional roles for ADAMs has considerably expanded to processes such as myogenesis (5), osteoblast differentiation (6), and host defense (7). Furthermore, some ADAM family members, including TACE (tumor necrosis factor- α -converting enzyme), ADAM-12 (meltrin α), and ADAM-23, have been suggested to play important roles in the development and progression of inflammatory and tumor processes (8–15).

The structural and functional complexity of the ADAM family of cellular disintegrins has considerably grown after the finding of a series of new members characterized by the presence of thrombospondin repeats in their amino acid sequence. The first member of this subfamily, ADAM-TS1, was identified as a consequence of its association with the development of cancer cachexia as well as with various inflammatory processes (16). Subsequently, the cloning of the cDNA for procollagen I amino-proteinase, whose deficiency cause Ehlers-Danlos syndrome type VIIC in humans, revealed a significant degree of structural similarities with ADAM-TS1, and it was called ADAM-TS2 (17). ADAM-TS4 and ADAM-TS5/TS11, other members of this subfamily of disintegrins containing thrombospondin motifs, have been characterized as aggrecanases responsible for the degradation of cartilage aggrecan in arthritic diseases (18, 19). ADAM-TS4 has been found to be responsible for brevicin cleavage in glioma cells, a proteolytic cleavage that has been proposed to be critical in mediating the invasiveness of these tumors (20). On the other hand, ADAM-TS8 (also called METH-2) and ADAM-TS1 have been characterized as proteins with angio-inhibitory activity (21). Finally, other family members identified in human tissues such as ADAM-TS3, ADAM-TS5, ADAM-TS6, ADAM-TS7, and ADAM-TS9 have been only characterized at the structural level, and their putative functional significance is still unclear (22, 23).

These recent findings have stimulated the search for new ADAMs potentially associated with some of the conditions in-

Cell-cell and cell-extracellular matrix interactions are essen-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ250725.

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volving cell-cell interactions or extracellular matrix degradation taking place during both normal or pathological conditions (1–3). In this work, we have examined the possibility that additional yet uncharacterized ADAMs could be produced by human tissues, with the finding of a novel family member, belonging to the ADAM-TS subfamily that we have called ADAM-TS12. We describe the molecular cloning and complete nucleotide sequence of a cDNA coding for this protein. We also report an analysis of the intracellular processing and enzymatic activity of ADAM-TS12. Finally, we describe the chromosomal location of the ADAM-TS12 gene and analyze its expression and regulation in normal and tumor tissues.

EXPERIMENTAL PROCEDURES

Materials—A human fetal lung cDNA library constructed in λ gt11, a human matched tumor/normal expression array, and Northern blots containing polyadenylated RNAs from different adult and fetal human tissues were from CLONTECH (Palo Alto, CA). Human tumors were obtained from patients who had undergone surgery for diverse malignancies at the Hospital Clínic, Barcelona, Spain (Banco de Tejidos y Tumores/Servicio Anatomía Patológica). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. All media and supplements for cell culture were obtained from Sigma, except for fetal calf serum, which was from Roche Molecular Biochemicals.

Isolation of a cDNA Clone for ADAM-TS12 from a Human Fetal Lung cDNA Library—A search of the GenBank™ data base of human ESTs¹ for sequences with homology to members of the ADAM family led us to identify a sequence (AI039653; WashU-Merck EST Project) derived from a fetal lung cDNA clone and showing significant similarity with sequences of previously described ADAMs. To obtain this DNA fragment, we performed PCR amplification of a human fetal lung cDNA (CLONTECH) with two specific primers 5'-CAACCCAGGAGGACATGTGA and 5'-TTCTCACGAGGAGAAGGACC, derived from the AI039653 sequence. The PCR reaction was carried out in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences for 40 cycles of denaturation (94 °C, 15 s), annealing (64 °C, 20 s), and extension (68 °C, 30 s). The 390-bp PCR product amplified from human fetal lung cDNA was cloned, and its identity was confirmed by nucleotide sequencing using the kit DR terminator *Taq* FS and the automatic DNA sequencer ABI-PRISM 310 (PerkinElmer Life Sciences). This cDNA was then excised from the vector, radiolabeled, and used to screen the same fetal lung cDNA library according to standard procedures.

3'-Extension of Isolated cDNAs—The 3'-ends of cloned cDNAs were extended by successive cycles of rapid amplification of cDNA ends (RACE) using RNA from human fetal lung and the Marathon™ cDNA amplification kit (CLONTECH), essentially as described by the manufacturer. Each cycle of RACE allowed the extension of ~100–200 bp of cDNA toward the 3'-end. Finally, the full-length cDNA was obtained by PCR amplification using the Expand Long PCR kit (Roche Molecular Biochemicals). The PCR reactions were performed for 35 cycles of denaturation (15 s at 94 °C), annealing (15 s at 64 °C), and extension (3 min at 68 °C), with primers 5'-ATGCCATGTGCCAGAGGAGCT and 5'-GGGCTTAGAGTTCTTTTGAC. Following gel purification, the amplification product was cloned and sequenced.

Chromosomal Mapping—DNA from a panel of monochromosomal somatic cell hybrids containing a single human chromosome in a mouse or hamster cell line background was PCR screened for the presence of the genomic sequence flanked by the primers: 5'-TCCAGTCCGAGTAGATGCCAGTG and 5'-GTGCCACTGAGATGGCAGAGGGG. Amplification conditions were as follows: 35 cycles of denaturation (94 °C, 15 s), annealing (65 °C, 15 s), and extension (68 °C, 2 min) using the Expand Long PCR kit. Radiation hybrid mapping was carried out using the Genebridge 4 panel (Human Genome Mapping Resource Center, Cambridgeshire, UK). DNA samples from this panel (25 ng) were PCR screened for the presence of the genomic sequences flanked by the primers 5'-CTTGAGCTCAGGAGCTCATTCAT and 5'-GGGGAGGCTCTGATTTCTC AGCAA. Amplification conditions were as follows: 35 cycles of denaturation (94 °C, 15 s), annealing (68 °C, 15 s), and extension (72 °C, 1 min). PCR results were converted to a vector of 93 0 values (no amplification), 1 values (amplifi-

cation), and 2 values (blanks and uncertainties) and submitted to the mapping server of the Whitehead Institute/MIT Center for Genome Research, with a minimum LOD (log of the odds) score of 15.

Cell Culture—Human cancer cells from different sources were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂. Cells were subcultured weekly by incubation at 37 °C for 2 min with 0.0125% trypsin in 0.002% EDTA, followed by the addition of complete medium, washing, and resuspension in fresh medium. For most experiments, ~5 × 10⁵ cells/well were plated out in 100-mm dishes, transferred to serum-free Dulbecco's modified Eagle's medium for 24 h, and then exposed to the different cytokines and growth factors at the concentrations and for the times indicated. Extracellular matrix remaining on the dishes was extracted with Laemmli sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, and 10% glycerol) and analyzed by SDS-polyacrylamide gel electrophoresis.

Northern Blot Analysis—Nylon filters containing 2 μ g of poly(A)⁺ RNA of a wide variety of human tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 10× Denhardt's solution, 2% SDS, and 100 μ g/ml of denatured herring sperm DNA and then hybridized with a radiolabeled ADAM-TS12-specific 1.8-kb-long probe, corresponding to the 5'-region of the cDNA. Hybridization was performed for 20 h under the same conditions. Filters were washed with 0.1× SSC, 0.1% SDS for 2 h at 50 °C and exposed to autoradiography. RNA integrity and equal loading was assessed by hybridization with an actin probe.

Reverse Transcription-PCR Amplification—To assay the presence of ADAM-TS12 in human tumor specimens, total RNA was isolated from malignant tumors by guanidium thiocyanate-phenol-chloroform extraction and used for cDNA synthesis with the RNA PCR kit from PerkinElmer Life Sciences. After reverse transcription using 1 μ g of total RNA and random hexamers as primers according to the instructions of the manufacturer, the whole mixture was used for PCR with two ADAM-TS12-specific oligonucleotides (5'-AAGCATGCTCGGCGCATGCG and 5'-ACTGCGAATCCGCACTCCACC), as described above. The PCR products were analyzed in 1.5% agarose gels. cDNA quality was verified by performing control reactions with primers derived from the sequence of actin. Negative controls were also performed in all cases by omitting the template or reverse transcriptase.

Site-directed Mutagenesis—The H465Q and E466A mutations in the metalloproteinase domain of ADAM-TS12 were carried out by PCR-based methods. An oligonucleotide containing a *MumI* sequence and two mutations 5'-TTCACAATTGCCCAAGCGCTAGGACACAGC (with **A** and **C** indicating changes T to A and A to C in the original sequence) and a second oligonucleotide (5'-TTGCAGAGCCTCTCTGCGCTC) were first used to PCR amplify a DNA fragment with the following conditions: 94 °C for 2 min (1 cycle) and 94 °C for 0.1 s, 60 °C for 0.1 s, and 68 °C for 30 s (20 cycles). The PCR product of the expected size was digested with *MumI* and *BstEII* and cloned in pcDNA3. The presence of the mutations in the plasmid (pcDNA3-ADAM-TS12-MUT) was confirmed by nucleotide sequencing.

Construction of Eukaryotic Expression Vectors and Western Blot Analysis—A full-length cDNA encoding ADAM-TS12 was PCR amplified with oligonucleotides 5'-ATGCCATGTGCCAGAGGAGCT and 5'-GGGCTTAGAGTTCTTTTGAC and cloned in the *EcoRV* site of a modified pcDNA3 vector containing a 24-bp linker coding for the hemagglutinin (HA) epitope of human influenza virus. Thus, the resulting ADAM-TS12 protein was HA-tagged at the C terminus. Similarly, two oligonucleotides (5'-GTCACCTTGACTACAAGGACGACGATGACAAGGG and 5'-AACTGATGTTCTGCTGCTACT GTTCCCCAGTG) were used to introduce the FLAG epitope at the *BstEII* site at position 1642 of the ADAM-TS12 cDNA. COS-7, HT-1080, or LoVo cells were transfected with 1 μ g of plasmids pcDNA3-ADAM-TS12-HA, pcDNA3-ADAM-TS12-FLAG, pcDNA3-ADAM-TS12-MUT, or pcDNA3 alone, using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Transfected cells were used for preparing protein extracts, which were then analyzed by Western blot. Blots were visualized by ECL according to the manufacturer's instructions (Amersham Pharmacia Biotech).

In Vitro Transcription and Translation—One microgram of pcDNA3 containing the full-length ADAM-TS12 cDNA was transcribed and translated using the coupled reticulocyte TNT T7 kit (Promega) in the presence of [³⁵S]methionine (Amersham Pharmacia Biotech), following the manufacturer's instructions. Parallel experiments were conducted using empty pcDNA3 as control. Protein translation products were analyzed by SDS-polyacrylamide gel electrophoresis followed by overnight autoradiography.

¹ The abbreviations used are: EST, expressed sequence tag; bp, base pair(s); PCR, polymerase chain reaction; TS, thrombospondin; TGF, transforming growth factor; RACE, rapid amplification of cDNA ends; kb, kilobase(s); HA, hemagglutinin; IL, interleukin.

Enzymatic Assays—The proteolytic activity of ADAM-TS12 was determined using the α_2 -macroglobulin complex formation assay. Lysates from cells transfected with ADAM-TS12 were solubilized in 25 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycolate, 0.1% SDS, 100 mM NaCl, and 1% Triton X-100. The α_2 -macroglobulin substrate was added at a final concentration of 0.25 units/ml and incubated at 37 °C for 16 h in the presence or absence of 1 mM EDTA.

RESULTS

Identification and Characterization of ADAM-TS12—To identify putative novel members of the ADAM family expressed in human tissues, we used the BLAST algorithm to scan the GenBank™ data base of ESTs looking for sequences with significant similarity to previously described family members. This analysis allowed us to identify a 469-bp EST that, after conceptual translation, generated an open reading frame with significant amino acid sequence similarity to sequences found in disintegrins purified from snake venom. A cDNA containing part of this EST was obtained by PCR amplification of total λ -phage DNA prepared from a human fetal lung cDNA library. The 390-bp PCR amplified product was cloned, and its identity was confirmed by nucleotide sequence analysis. Then the cloned fragment was radiolabeled and used as a probe to screen the same fetal lung cDNA library employed for the previous PCR amplification experiment. Upon screening of $\sim 1 \times 10^6$ plaque forming units, a single positive clone was identified and characterized. DNA was isolated from this positive clone (called FL1), and its nucleotide sequence was determined by automatic DNA sequencing. This analysis revealed that the length of FL1 was 1.8 kb and contained the entire 469-bp sequence corresponding to the AI039653 EST. A comparative analysis of the identified sequence with that corresponding to other ADAMs suggested that it was incomplete at the 3'-end. To extend the partial cDNA sequence toward the 3'-end, we performed 3'-RACE experiments using a specific oligonucleotide deduced from the end of the FL1 clone and RNA from human fetal lung as a template. Successive rounds of 3'-RACE experiments performed in similar conditions led us finally to obtain a fragment containing an in frame stop codon. Nucleotide sequencing of several independently isolated subclones of the RACE reaction did not reveal any differences between them. Computer analysis of the obtained sequence (Fig. 1) revealed an open reading frame coding for a protein of 1,543 amino acids with a predicted molecular mass of 177.5 kDa (EMBL accession number AJ250725).

Further structural analysis of the identified amino acid sequence showed a significant similarity with other human ADAMs, the maximum percentage of identities being with members of the ADAM-TS subfamily (57% with ADAM-TS7). An alignment of the deduced amino acid sequence confirmed that this protein possesses all characteristic domains of the ADAM-TS family members including signal sequence, propeptide, and metalloproteinase-like, disintegrin-like and cysteine-rich domains, as well as a series of TS-1 repeats (Fig. 1). However, the organization of the TS-1 repeats of the identified sequence is unique to this protein (Fig. 2). Thus, all previously described human family members exhibit a first TS repeat 52 amino acids in length that is separated from a C-terminal TS module through a cysteine-rich domain and a spacer sequence. This second module is composed of a single TS repeat in ADAM-TS5, ADAM-TS6, ADAM-TS7, and ADAM-TS8, two TS repeats in ADAM-TS1, and three TS repeats in ADAM-TS2, ADAM-TS3, and ADAM-TS9, whereas ADAM-TS4 lacks any additional repeat. The fetal lung ADAM-TS described herein contains three TS repeats in this second TS module, but, in addition, an extra C-terminal TS module can be identified in its sequence. This third TS module is separated from the second one by a spacer sequence (spacer-2) 319 amino acids long, and

it is formed by four TS repeats. This latter domain is followed by a C-terminal extension rich in cysteine residues that shows similarities with the C-terminal region of other ADAM-TSs. The additional domains present in ADAM-TS12 are the cause of the large size of this novel human disintegrin when compared with all remaining family members.

The alignment of the amino acid sequence deduced for ADAM-TS12 with that of other human ADAM-TSs also revealed the presence of additional structural hallmarks of the ADAM-TS family members (Figs. 1 and 2). Thus, the putative proregion contains two conserved Cys residues (at positions 139 and 160) as well as an additional one located in the sequence **PTCGLKD** (positions 206–212) that resembles the Cys switch motif (**PRCGXPD**) present in the matrix metalloproteinases and involved in maintaining enzyme latency. The prodomain ends in a dibasic motif that could correspond to a furin activation sequence for generation of the mature enzyme. The catalytic domain includes the consensus sequence **HEXX-HXXGXXHD** (at positions 392–403) involved in the coordination of the catalytic zinc atom at the active site of metalloproteinases and bearing the Asp residue that distinguishes reprotolysins from matrix metalloproteinases. This domain also contains the eight cysteine residues present in the catalytic region of all ADAM-TS family members as well as a conserved methionine residue located 16 amino acids C-terminal to the zinc-binding site that contributes to form the Met turn structure present in both reprotolysins and matrix metalloproteinases. Furthermore, the disintegrin-like domain is very similar in size (79 residues) to those found in most ADAM-TSs and contains the eight cysteines present in this region of ADAM-TS proteins with the exception of ADAM-TS6. Finally, the cysteine-rich domain shows a high percentage of sequence identity with the equivalent domain in other ADAM-TSs (59.6% with ADAM-TS7), including the 10 conserved cysteine residues present in all of them. Taking together all of these structural comparisons, it seems that the newly identified human protein is a member of the ADAM-TS family of disintegrins, albeit with a more complex organization of thrombospondin repeats. Following the nomenclature system for cellular disintegrins, the officially approved name for this enzyme is ADAM-TS12.

Chromosomal Mapping of the Human ADAM-TS12 Gene—To determine the chromosomal location of the human ADAM-TS12 gene, we used a PCR-based strategy to screen a panel of somatic cell hybrid lines containing a single human chromosome in a rodent background. The sequence-tagged site specific for the ADAM-TS12 gene was generated by using two specific oligonucleotides whose sequence was derived from a noncoding sequence flanking the second exon of the gene and from a coding sequence of this same exon. As can be seen in Fig. 3A, positive amplification results were only obtained in the hybrid containing the autosome number 5. Southern blot analysis confirmed that the amplified band corresponded to ADAM-TS12 (Fig. 3A). Because no amplification products of the expected size were observed in the hybrids containing the remaining human chromosomes, we can conclude that the ADAM-TS12 gene maps to chromosome 5. To further determine the chromosomal location of the human gene encoding ADAM-TS12, we used the same sequence-tagged site oligonucleotides to PCR screen a panel of radiation hybrids containing human chromosome fragments in a rodent background. Computer analysis of positive amplification results indicated that the ADAM-TS12 gene is located in chromosome 5q35 at 54.47 cR from marker WI-6737 (Fig. 3B and data not shown). Interestingly, ADAM-TS2 and ADAM-TS6 also lie on human chromosome 5, although they are not necessarily clustered. Thus, ADAM-TS2 is located at 5q23, and ADAM-TS6 is located at an

1	GAATTCCGGAGCGGGGGGGCTGGAGGGCCGGGGCCATCGGGAGGGCGGAGGGGGTGA	60	2641	TTCAATGACAGGAAAGAGCACTGAAAGAGCTGATGGCCACAGGTCCCAACAATGAGT	2700
61	COGGGTGGCTGGCCCAATCCACACCCGGGAAAGCGGACACTGTGAGCTGAATCACTCC	120	2701	QYDRKKGDLKLMATGPTNES	2760
121	CCTTTTAGGAGGAGGAGGGGAAAGGTTGCTAGCTAATTTCTGCTTAAAAAGCACAG	180	2761	CTGTGTGGATCCAGCTTCTATTCCAGGTGACTAACCTGCAATCAAGTATGAGTACACAA	2820
181	GAGATCGGGGGTCAAGTCTTGGAGTGGCTGCTCTCGGGGGTCAACATGCACCCCTGCAT	240	2762	VWILQLLFLQVTPNPGIKY EYTI	2880
241	CTTCTGCTGGGACAGGGGAGCGCTTATTTCTGGAGCTGAGGGCTAAAACCTTTTTCAC	300	2821	TCACAGAAAGTGGCCCTTGGCAATGATGTTGAGCAGATGTCTCTGCAAGTACGGCCACT	2940
301	CTTTTCTCTCCTCAACATCTGAATCATGCATGTGCCACAGGAGCTGGCTTGCACAAAC	360	2881	QKDGLDNDVVEQMYFWQYGHW	3000
	MPC A Q R S W L A N L		2941	GGACAGAGTGCAGTGTGACCTGGGGGAGGTATCCGGCCCAAACTGGCCATGCATAA	3060
361	TTTCOGTGGTGGCTCAGCTCTTAACCTTGGGGGGCTTGGCTATGGAGAGAGCTCAGC	420	2942	TECSVTCGTGCTGAGTGTATCCGGCCCAAACTGGCCATGCATAA	3120
	S V V A Q L L N F G A L C Y G R Q P Q P		2943	AGAAGGGGGGGGGTGGTGAAGCTACATCTGTGACCCGAAACACAAGCCCAATGGGA	3180
421	CAGGGCCGGTTCGCTTCCGGACAGAGGGCAAGAGCTTTTATCAAGGGCTGCCAAGAT	480	2944	KGRGMVKATFCDPETQPNGR	3240
	G P V R F P D R R Q E H F I K G L P E Y		3001	GACAGAAAGTGCATGAAAGGGCTTGTCCACCCAGTGGTGGGCAAGGGAGTGGGAAG	3300
481	ACCAAGTGGTGGTCCAGTCCAGTGCAGTGCAGTGGCAATTTTGTGATATGGCTTGC	540	3002	QKKCHEKACPPRWWA G E W E A	3360
	H V V G P V R V D A G S H F L S Y G L H		3061	CATGCTCGGGACATGGGGGGCCCAAGGGAGAAAGGAAAGCCGTGCTGTGCATCCAGA	3420
541	ACTATCCATCAGAGCAGCAGGAGGAGAGATTTGGATGGCTCAGAGGACTGGTGT	600	3062	CSATCTCGPHGEKKTRTLVCIQ T	3480
	Y P I T S S R R K R D L D G S E D W V Y		3121	CCATGCTCTGACAGCAGCTCTCCGGCCACAGACTGCCAGCCCTGCTGAAGGCCA	3540
601	ACTACAGAATTTCTCAGAGGAGGAGGCTGTTTTAACTTGAAGGCTCAATCAAGGAT	660	3181	MVSD EQALPPTDCQHLLKPK	3600
	Y R I S H E K E T F N L T V N Q G F		3182	AGACCTCTCTGCAAGCAGCATCTGCGCCCTGGACTGGACGTGGGCACT	3660
661	TTCTTTCCAATAGCTACATCATGAGAGAGATATGGGAAGCTCCCATGTTAAGATGA	720	3183	TLLS CNR D I L C P S D V T G N W	3720
	L S N S Y I M E K R Y G N L S H V K M M		3241	GGAGTGGTGTCTGTTCTCTGCTGGTGGTGGTGGGATGGGATGTCAGATGTGCCA	3780
721	TGGCTTCTCTCGGGCCCTGCTCCATCTCAGTGCAGCGTCTTCAAGCAGGGCCACAGAG	780	3242	SECSVSCGGGAGTIRSVTCAK	3840
	A S S A P L C H L S G T V L Q Q G T R V		3301	AGAACCATGATGAACTTGGATGTGACAAAGAAACCAACAGCCGCTGTGTGGCC	3900
781	TTGGGACGGGAGCCCTCAGTGGCTCAGTGGCTGATGATTTTCCAACATCCACATG	840	3302	NHDEPCDVTTRKPN S R A L C G L	3960
	G T A A L S A C H G L T G F F Q L P H G		3361	TCACCAATGGCTTCTAGGGGAGGTTCTGAAACCAAAAGGCACTATTTCCAAATG	4020
841	GAGACTTTTTTATTGACCCGTGAAGAGCATCAGCTGGTTGAGGGAGGGTACCACCCG	900	3362	Q Q C P S S R R V L K P N G I S N G	4080
	D F F I E P V K K H P L V E G G Y H P H		3421	GAAAAAGCAGCAACTAAAGCCCTCCCTCAGCTACATCCAGCCCAAGATGCTGA	4140
901	ACATCGTTTACAGAGGGCAGAGTTCAGAAAGCAGAGAGCCCAAGCTGTGGATTAAGG	960	3422	KNPPTLLKPVVPPTSRPRLMT	4200
	I V Y R R Q K V P E T K E P T C G L K D		3481	CCACCCCAAGGGGCTGAGTCTGAGGCAAGCAAGCTCCAGCAACTCAGCAGCCCTAGTC	4260
961	ACAGTGTAAACATCCCAAGAGCAGCTATGGCCGAGAGTGGGAGGGCCCAACAT	1020	3482	TPTGPESMSTSTPAI S S P S P	4320
	S V N I S Q K Q E L W R E K W E R H N L		3541	CTACACAGCTCCAAAGAGGAGCTGGTGGGAACAGTGGGAGGATGCTCAACCC	4380
1021	TGCAAGCAGAGAGCTCTCTCGGGTTCATCAGCAGAGAGAGTGGTGGGACACTGG	1080	3542	T T A S K E G D L G K Q W D S T O	4440
	P S R S L S R R S I S K E R W V E T L V		3601	AAGCTGAGCTGAGCTGCTGCTATCTCATTTCCACTGGAAAGCTTCCACGCCCTCCTCA	4500
1081	TGGTGGCCGACCAAGAGTATTGAATACCATGGAGTGGAGTGGAGTGGAGTCTACATCG	1140	3602	P E L S S R Y L I S T G S T S Q P I L T	4560
	V A D T K M I E Y H G S E N V E S Y I L		3661	CTTCCCAATCTTGAGCATTCAGCCAACTGAGGAAAGTGTTCAGTTCAGATCTGGTC	4620
1141	TCACCATCATGAACATGGTCACTGGTGTGTCATAAACCAAGCTGGCAATGCAATTC	1200	3662	S Q S L S I Q P S E E N V S S S D T G P	4680
	T I M N M V T G T F H N P S I G N A I H		3721	CTAAGTGGGAGGGGCTTGTAGCTACAACAAGTGGTCTGGCTTGTCACTTCC	4740
1201	ACATTGTTGGTGGCTGCTACTGCAAGAAAGAGCAAGGACTGAAAATAGTTC	1260	3722	T S E G G L V A T T S G S C T S S S R	4800
	I V V V R L I L L E E E E Q G L K I V H		3781	GCAACCTATCACTTGGCTGTGACTCCATTTACAATACCTTGACCAAGGTCAGAAA	4860
1261	ACCATGCAAAAAGCACTGTCTAGCTTCTGCAAGTCCAGAGAGTATCAATCCCAAGA	1320	3782	N P I T W P V T P F Y N T L T K G P E M	4920
	H A E K T L S S F C K W Q K S I N P K S		3841	TGGAGTTTCAAGTGGCTCAGGGGAGAGAAAGCAAGCAAGCTGGGCAAGAGTGAAGCA	4980
1321	GTGAACCTCAATCTGTTCATCCAGCGTGGCTGTCTTCCACAGAAAGGACATCTGTG	1380	3842	E I H S G S G E E R E Q P E D K D E S N	5040
	D L N P V H H D V A V L L L T R K D I C A		3901	ATCCTGTAATGGACCAAGTCAAGTACCTGGAATACCTCCAGTGGAAAGTACAG	5100
1381	CTGGTTCAATCGGGCTGGGAGCCCTGGGCTGTCTCACTTTGAGGAATGTGTGAGC	1440	3902	P V I W T K I R V P G N D A P V E S T E	5160
	G F N R P C E T L G L S H L S G M C Q P		3961	AAATGGCACTTGCACCTCACTAACCCAGATCTCAGCAGGAGTCTGGTGGCCACCT	5220
1441	CTCACCGAGTGTAAACATCAATGGAGTTCGGGACTCCCTCTGGCTTTCACAAATGGCC	1500	3962	M P L A P P L T P D L S R E S W W P P F	5280
	H R S C N I N E D S G L P L A F T I A H		4021	TCAGCAGGTAATGGAAAGTCTCCCGCCAAAGCCCACTACTTCCGAACTGGGA	5340
1501	ATGAGCTAGGACACAGCTTCGGCATCCAGCATGATGGGAAAGAAAGTCACTGGAGCTG	1560	4022	S T V M E G L L P S Q R P T S E T G T	5400
	E . L . G . H . S . F . G . : . Q . H . D . G . K . E . N . D . C . E . P . V		4081	CACCGAGTGGAGGGATGGTACTGAAAGCCAGCAAGCTGCTCCCTCCCTGGAG	5460
1561	TGGGCAGACATCCGTAACATCATGTCCCGCCAGCTCCAGTACCATCCCACTCCGCTGACAT	1620	4082	P R V E G M V T E K P A N T L L P L G G	5520
	G R H P Y I M S R Q L Q Y D P T P L T W		4141	GAGAACCCAGCAGAACCTCAGGAAAGCCGCAAAACCTGAAACTCCAA	5580
1621	GGTCCAAAGTGCAGGAGGAGTACATCAACCGCTCTTGGACCGAGCTGGGGTCTGTCTG	1680	4142	D H Q P E P S G K T A N R N H L K L P N	5640
	S K C S E E Y I T R F L D R G W G F C L		4201	ACAACATGAACCAAAAGAGTCTGAACAGTCTGCTCAGCAGGATGCACCAAGTC	5700
1681	TTGATGACATACCTAAAAGCAAGCTTGAAGTCCAAAGTCTTGGCTCCCGGAGTGTCTG	1740	4202	M N Q T K S S E P V L T E A D A T S L	5760
	D D I P K K K G L K S K V I A P G V I Y		4261	TGATTACTGAGGGCTTTTGTAAATGGCTCCAAATACAGCAGCTCACAAGCCGCAAG	5820
1741	ATGATGTTCCACCAAGTGGCAAGTACAATAGGACCAATGCTACCTCTGCCAGGAAG	1800	4262	I T E G F L L N A S N Y K O L T N G H G	5880
	D V H H Q C Q L Q Y G P N A T F C Q E V		4321	GCTCTGCACACTGGATCGTGGAACTGGAGGAGTCTCCAGCCAGTGTGGCTGGGGG	5940
1801	TAGAAAACGTCTGCCAGCACTGTGGTGGTCCGGTGAAGGGCTTTTGGCTCTAAGCTGG	1860	4322	S A H W I V G N W S E C S T T C G L G A	6000
	E N V C Q T L W C S V L K G F C R S K L D		4381	CCCTACTGGAAAAGGGTGGAGTCCACCCAGGATGGATGCTGACTGTGGGGCATCCAGA	6060
1861	ACGCTGCTGCAGATGCAACTCAATGTGGTGAAGAGAGTGGTGTATGGCAGGCAAGTCCA	1920	4382	Y W K R V E C T T Q M D S D C A I Q R	6120
	A A A D G T Q C G E K K W C M A G K C I		4441	GACCTGACCTGCAAAAAGTGGCACTCCGCTCCCTGTGCTGGCTGGAAAGTGGGAACT	6180
1921	TCACAGTGGGAGAAACAGAGAGCATCTCCGAGGCTGGGGCCCTGGTCAACCTGGT	1980	4442	P D P A K R C H L R P C A G A G W K V G N W	6240
	T V G K K P E S I P G G W G R W S P W S		4501	GGAGCAAGTGGTCCAGAACTGCACTGGGGCTTCAAGATCCGGAGTTCAGTGGTGGT	6300
1981	CCCACTGTCCAGCACTGTGGGGCTGGAGTCCAGAGCGCAGAGGGCTCTGCAACAACC	2040	4502	S K C S R N C S G G F K I R E I Q C V D	6360
	H C S R T C G A G V Q S A E R L C N N P		4561	ACAGGGGAGCAGCCGAGCTGAGGGCAATTCAGTGGCAGTCTGTGGGGCCATCTCTC	6420
2041	CCSAGCCAAAGTTTGGAGGAAATATGCACTGGAGAAAGAAAGCTATCCCTGTGCA	2100	4562	S R D H R N L R P F C H Q F L A G I P P	6480
	E P K F G G K Y C T I G E R K R Y R L C N		4621	CCCAATGAGCATGAGCTGTAAACCCAGGCTGTGAGGGTGGCAGTGGAGCTTGG	6540
2101	ACGTCACCCCTGTGGCTCAGAGCCCAACATTTGGCAGATGCAGTGGATGAATTTG	2160	4622	P L S M S C N P E P C E A W Q V E P W S	6600
	V H P C R S E A P T F R O M Q C S E F D		4681	GGCAGTCTCCAGCTGTGGAGTGGTGTGAGAGTGGTGTGAGAGAGGAGTGTCTGTCCAGG	6660
2161	ACACTGTTCCATACAAGTGAAGCTCCACCTGGTTCACCAATTTTAAACCAAGCAGCAT	2220	4682	Q C S R S C G G V G V E R G V F C P G G	6720
	T V P Y K N E L Y H W F P I F N P A H P		4741	GGCTCTGTGATTGGACAAAAGCCACATCCACATGTCTGATGAGCAGCTGTGGT	6780
2221	CTTGTGAGCTACTGGCCACCATAGATGGCCAGTTTCTGAGAAAATGCTGGATGCTG	2280	4742	L C D W T K R P T S T M S C N E H L C C	6840
	C E L Y C R P I D G Q F S E K M L D A V		4801	GTCACTGGGGCACTGGGACTGGGCACTGTGTCCACTCTGTGCTGCTGCTGCTGCTG	6900
2281	TCATGATGGTACCGCTTGGTTGAGGGGGCAAGCAGCAAGATGCTGTATTAATGGCA	2340	4802	AGAGGATGTTCAATGTGTGGCTCAGAGGCAATAAACTGAAAGCAAGCAACTGTC	6960
	I D G T P C F E G G N S R N V C I N G I		4861	R I V Q C V P S E G N K A A T D D Q D C L	7020
2341	TATGTAAAGTGGTGGCTGTGAGATGAGATCCAAATGCCAAGAGGATGGTGGC	2400	4862	TATGTGATCACAACCCAGCTCCAGAAATCAAAAATGCAACCAAGCCGCTGCAAGA	7080
	C K M V G C D Y E I D S N A T E D R C G		4921	C D H K P R P P E F K K C N Q G A C K K	7140
2401	GTGTGGCTGGGAGTCCCTCTCCGCAAGTGTGAGAAAGATGTTAAAGCAGAAAG	2460	4922	AAAGTGGGATTTACTTTGCACTAAGGCAAACTGTGAGCAGTCTGAGCAGTCTGCA	7200
	V C L G D G S S C Q T V R K M F K Q K E		4981	S A D L L C T K D K L S A S F C Q T L K	7260
2461	AAGGATGCTGGTATGTGACATGGCTCAITCCAAAAGGAGCAAGGAGATCAAGAGTGA	2520	4982	AAGCATGAAGAAATGTTCTGTCCCAAGCTGAGTGGCTGCTGCTGCTGCTGCTG	7320
	G S G Y V D I G T P K G A R D I R V M		5041	A M K K C S V P T V R A E C C F S C P Q	7380
2521	TGGAAATGAGGGAGCTGGAACTCTGGCACTCAGGAGTGAAGATCTGMAAATATT	2580	5042	AGACACACATCACACACCAAAAGCAAGAGGCAAGGCAAGGCTGCTCCAAAGTCAAAAG	7440
	E I E G A G N F L A I R S E D P E K Y Y		5101	T H I T H T Q R Q R R Q R L L Q K S K E	7500
2581	ACCTGAATGGAGGTTTATTTCCAGTGGAGCGGCAACTAAGCTGGCAGGAGCTGTCT	2640	5102	ACTCTAAGCCAAA 5115	
	L N G G F I I Q W N G N Y K L A G T V F				

FIG. 1. Nucleotide and amino acid sequences of ADAM-TS12 from human fetal lung. The zinc-binding site characteristic of metalloproteinases and the eight thrombospondin domains present in the amino acid sequence are shaded. The putative N-glycosylation sites are indicated with asterisks.

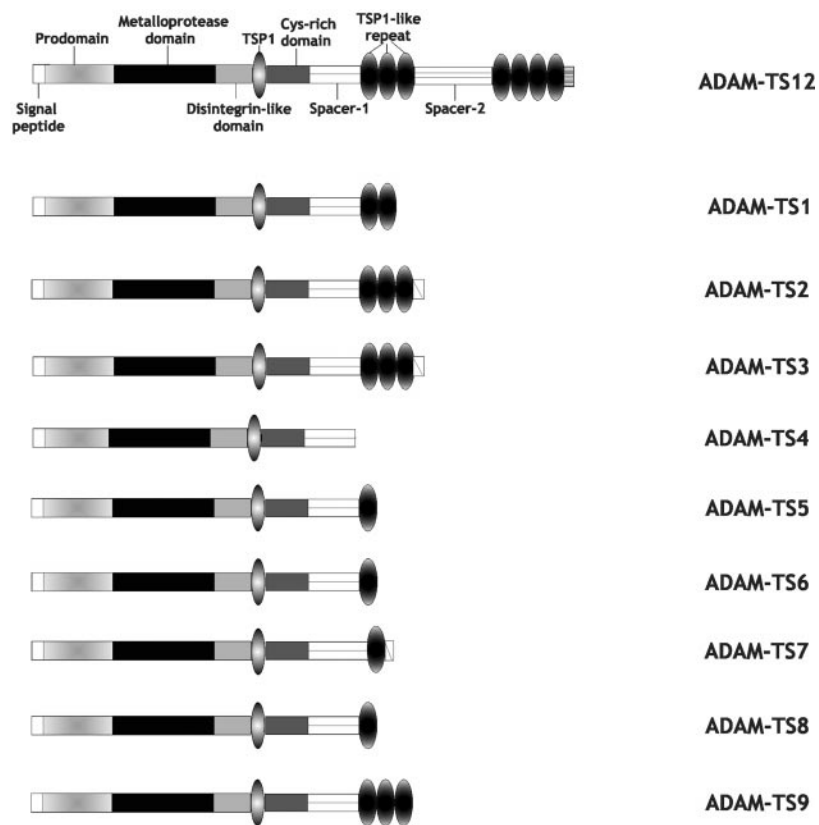
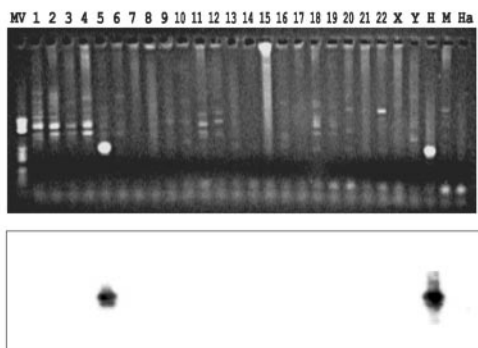


FIG. 2. Domain organization of ADAM-TS12. The domain structures of the remaining members of the human ADAM-TS subfamily of disintegrins are also shown. The structure of ADAM-TS10 has not been reported yet, whereas ADAM-TS11 is identical to ADAM-TS5.

A



B

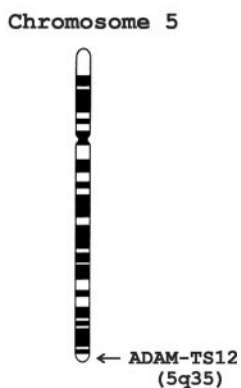


FIG. 3. Chromosomal location of the human ADAM-TS12 gene. A, 100 ng of total DNA from the 24 monochromosomal somatic cell lines was PCR amplified with primers described under "Experimental Procedures." λ DNA digested with EcoRI and HindIII (Marker III, Roche Molecular Biochemicals) was used as a size marker. The lower panel shows a Southern blot of the material amplified above hybridized with an ADAM-TS12 specific probe. B, cytogenetic ideogram of human chromosome 5. The position of ADAM-TS12 gene at 5q35 is indicated.

undefined locus at this chromosome. However, all of the remaining ADAM-TS genes are dispersed in the human genome. Thus, ADAM-TS1 and ADAM-TS5/TS11 are linked on 21q21-q22, ADAM-TS3 maps to 4q21, ADAM-TS4 maps to 1q31, ADAM-TS8 (METH-2) maps to 11q25, ADAM-TS9 maps to 3p14, and ADAM-TS7 maps to an undefined locus at chromosome 15 (22, 23).

Intracellular Processing and Enzymatic Activity of ADAM-TS12—Members of the ADAM family of metalloproteinases are synthesized as precursor molecules that are subjected to proteolytic processing-mediated events to generate the final active molecules. ADAM-TS12 contains putative Cys switch and furin cleavage motifs that could be involved in the activation mechanism of this enzyme by removal of the inhibitory prodomain. To analyze the intracellular processing of ADAM-TS12, we first prepared a pcDNA3 expression vector (ADAM-TS12-HA) containing the full-length cDNA for ADAM-TS12 with an HA epitope at the 3'-terminal end. Western blot analysis of COS-7 cell extracts transiently transfected with this construct re-

vealed the presence of a band of about 83 kDa, immunoreactive against anti-HA and absent in cells transfected with an empty vector (Fig. 4A). This size is considerably lower than that of 175 kDa, which was derived for ADAM-TS12 after *in vitro* transcription and translation experiments (Fig. 4B). According to these results, it seems that ADAM-TS12 is extensively processed at the N-terminal end leading to the removal of a considerable part of this region. Amino acid sequence analysis of ADAM-TS12 allowed us to estimate that the putative processing site to generate a protein of about 83 kDa would be necessarily located after the cysteine-rich domain of the protein and around its first TS-1 domain. To further analyze this question, we prepared an additional construct (pcDNA3-ADAM-TS12-FLAG) also containing the HA epitope at the C-terminal end but including a FLAG epitope at the beginning of the first TS-1 domain of ADAM-TS12 (position 548 of the protein). After transfection of COS-7 cells with this double-labeled construct followed by Western blot analysis, we first confirmed that the size of the HA-immunoreactive band was identical (83 kDa) to

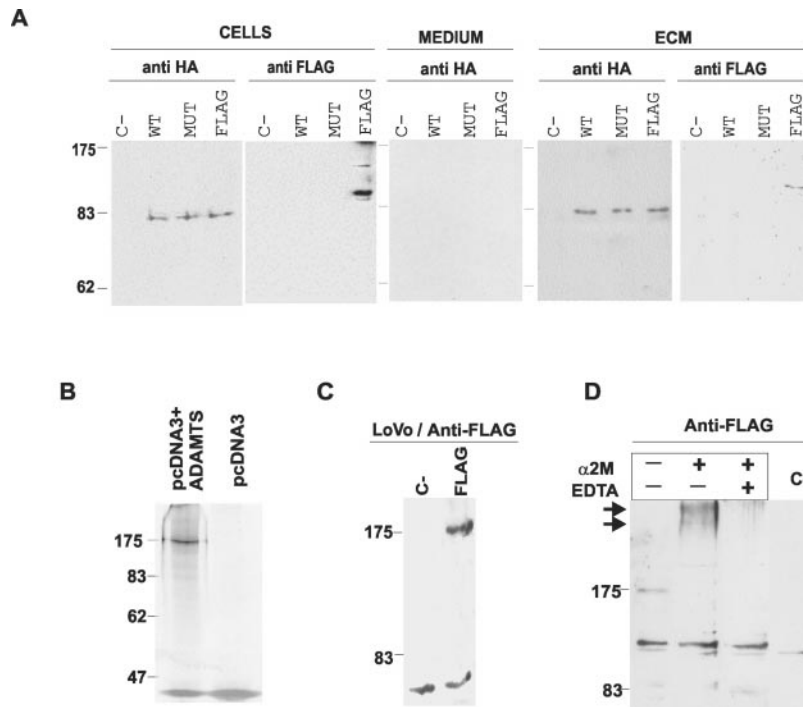


FIG. 4. Intracellular processing and enzyme activity of ADAM-TS12. *A*, Western blot analysis of COS-7 cells, conditioned medium, and extracellular matrix derived from these cells transfected with the indicated constructs. *HA*, pcDNA3-ADAM-TS12-HA; *FLAG*, pcDNA3-ADAM-TS12-FLAG; *MUT*, pcDNA3-ADAM-TS12-MUT; *C-*, pcDNA3 alone; *WT*, wild type. *MUT* indicates the mutations carried out in the metalloprotease domain: H392Q and E393A. *B*, SDS-polyacrylamide gel electrophoresis analysis of ADAM-TS12 cDNA after *in vitro* transcription and translation. *C*, Western blot analysis of furin-deficient LoVo colorectal carcinoma cells transfected with the pcDNA3-ADAM-TS12-FLAG construct. *D*, enzymatic activity of ADAM-TS12 using α_2 -macroglobulin as substrate. Extracts from COS-7 cells transfected with pcDNA3-ADAM-TS12-FLAG were incubated with purified α_2 -macroglobulin and analyzed by Western blot with anti-FLAG antiserum. The high molecular weight species generated by covalent binding between ADAM-TS12 and α_2 -macroglobulin are indicated by arrows. The binding was abolished in the presence of 1 mM EDTA. In all cases, the sizes of the molecular weight markers are shown to the left.

that observed in the single labeled construct (Fig. 4A). However, when the blot was incubated with the anti-FLAG antiserum, we detected a major band of about 120 kDa that was only present in the double-labeled ADAM-TS12 construct. A minor band of about 150 kDa was also detected in the same cell extract (Fig. 4A). This product could correspond to an incompletely processed protein. Similar processing events were obtained when transfections were performed using HT-1080 instead of COS-7 cells (data not shown), indicating that the observed maturation events are not exclusive of a single cell line. According to these results, we can conclude that upon synthesis, ADAM-TS12 is subjected to an intracellular maturation process leading to the generation of a fragment containing the N-terminal region of the molecule including the metalloproteinase, disintegrin-like, Cys-rich, and TS-1 domains and a C-terminal fragment containing the spacer-2 and the four additional TS-1 domains characteristic of ADAM-TS12.

To address the possibility that this proteolytic cleavage could be an autocatalytic process mediated by the metalloproteinase domain of ADAM-TS12, we next prepared an expression vector containing two point mutations in the cDNA for this protein (pcDNA3-ADAM-TS12-MUT). These mutations would lead to the production of a protein with changes in His and Glu residues essential for the catalytic activity of metalloproteinases. However, after transfection of COS-7 cells with this mutant construct and subsequent Western blot analysis, we did not observe any difference in the 83-kDa anti-HA immunoreactive band (Fig. 4A). Therefore, we can conclude that the proteolytic event leading to ADAM-TS12 processing is mediated by a cellular protease distinct from ADAM-TS12 itself. A preliminary analysis of the ability of a series of proteinase inhibitors to block this processing event revealed that only BB-94 was able to partially inhibit the ADAM-TS12 maturation, suggesting

that the protease involved in this step is a metalloprotease (data not shown). Finally, we tried to evaluate the location of the two fragments generated after ADAM-TS12 processing. Western blot analysis of conditioned medium from COS-7 cells transiently transfected with the different constructs (HA, FLAG, MUT) failed to detect any HA- or FLAG-immunoreactive bands (Fig. 4A and not shown, respectively), indicating that none of the ADAM-TS12-derived fragments was secreted into the cell culture medium. By contrast, extracellular matrix preparations from COS-7 cells transfected with these constructs and analyzed by Western blot with anti-HA antiserum revealed in all cases the presence of the 83-kDa immunoreactive band previously detected in total cell extracts (Fig. 4A). Similarly, when the same blot was hybridized with an anti-FLAG antibody, a 120-kDa immunoreactive band corresponding to the N-terminal region of the processed ADAM-TS12 was detected in the construct containing this epitope (Fig. 4A). According to these results, we can conclude that both fragments generated during ADAM-TS12 maturation are anchored to the extracellular matrix.

We next tried to determine whether furin, a widely expressed proprotein convertase, was involved in the first step of the ADAM-TS12 maturation. To do that, we examined the processing of this protein in furin-deficient LoVo cells. As shown in Fig. 4C, these cells were unable to efficiently process ADAM-TS12, indicating that furin was actually involved in the release of the ADAM-TS12 prodomain. Finally, we evaluated the possibility that ADAM-TS12 could be an active metalloprotease. To this purpose, we used the α_2 -macroglobulin trapping assay previously employed for demonstrating the proteolytic activity of other ADAMs (24, 25). To do that, extracts from COS-7 cells transfected with ADAM-TS12 tagged with the FLAG epitope were incubated with purified α_2 -macroglobulin and then ana-

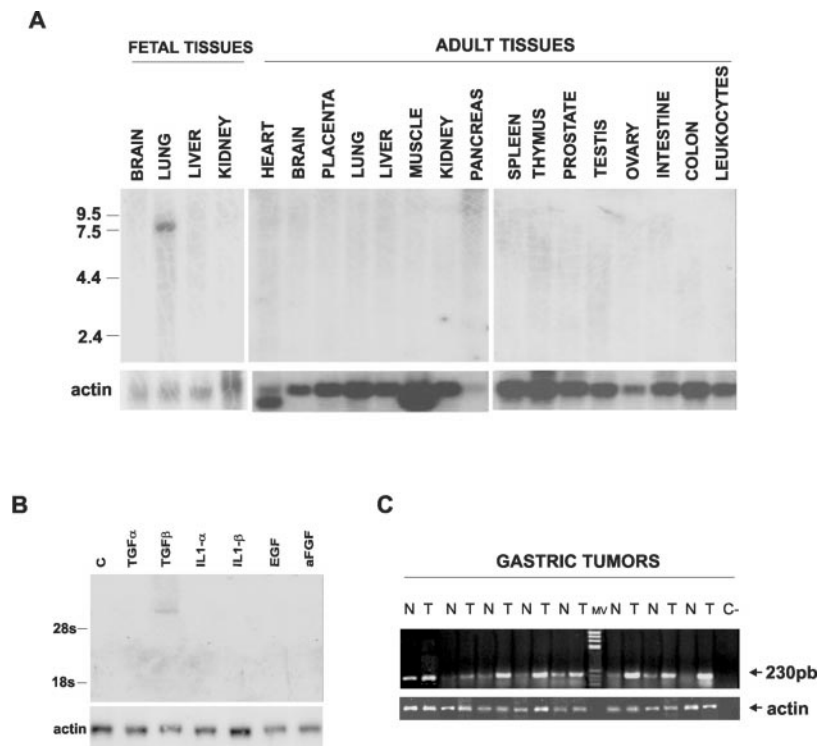


FIG. 5. Analysis of ADAM-TS12 expression and regulation in human tissues. *A*, Northern blot analysis of ADAM-TS12 in a panel of fetal and adult human tissues. 2 μ g of poly(A)⁺ RNA prepared from the indicated tissues was analyzed by hybridization with a 1.8-kb probe corresponding to the 5'-end of the cDNA for human ADAM-TS12. The positions of RNA size markers are shown. *B*, effect of TGF- β 1 and other cytokines and growth factors on ADAM-TS12 expression in KMST human fibroblasts. Northern blot analysis was performed using 10 μ g of total RNA from KMST cells incubated for 24 h in the presence of TGF- β 1 (5 ng/ml), TGF- α (50 ng/ml), IL-1 α and IL-1 β (5 ng/ml), epidermal growth factor (EGF; 10 ng/ml), and acidic fibroblast growth factor (aFGF; 10 ng/ml). Filters shown in *A* and *B* were subsequently hybridized with a β -actin probe to ascertain equal RNA loading for the different samples. *C*, reverse transcription-PCR analysis of ADAM-TS12 expression in paired normal gastric mucosa and gastric tumors. A 230-bp fragment corresponding to a segment of ADAM-TS12 was amplified with primers indicated under "Experimental Procedures" in a volume of 50 μ l, and 10 μ l of the reaction were separated on a 1.5% agarose gel run in Tris borate-EDTA buffer. Amplification of β -actin was used to ascertain RNA integrity and equal loading. C- indicates negative control. Marker V (MV; Roche Molecular Biochemicals) was used as a size marker.

lyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4D, at least two high molecular weight species immunoreactive against the FLAG antiserum were detected in cells containing ADAM-TS12 but not in control cells nor in cells incubated in presence of EDTA, an inhibitor of metalloproteinases. Furthermore, these high molecular weight complexes reacted with an anti- α_2 -macroglobulin antibody (data not shown), confirming that they resulted from the covalent binding of the ADAM-TS12 protein to α_2 -macroglobulin.

ADAM-TS12 Expression and Regulation in Human Tissues—To examine the expression of ADAM-TS12 in human tissues, Northern blots containing poly(A)⁺ RNAs prepared from a variety of adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes) and fetal tissues (brain, lung, liver, and kidney) were hybridized with a 1.8-kb cDNA specific for ADAM-TS12. As shown in Fig. 5A, a transcript of about 8 kb was exclusively detected in fetal lung. The predominant expression of ADAM-TS12 in human fetal tissues suggests that this novel enzyme could participate in some of the tissue remodeling processes taking place in this tissue during physiological conditions. In an effort to identify factors with the ability to up-regulate ADAM-TS12 expression, human KMST fetal fibroblasts were incubated for 24 h in the presence of a series of cytokines and growth factors, and total cellular RNAs were purified and analyzed by Northern blot using a specific ADAM-TS12 radiolabeled probe. As shown in Fig. 5B, TGF- β clearly induced the expression of this gene, whereas other growth factors and cytokines, including TGF- α , IL-1 α , IL-1 β , acidic fibroblast growth factor, and epidermal

growth factor, did not have any significant effect on ADAM-TS12 expression by KMST human fetal fibroblasts.

To examine the possibility that ADAM-TS12 was produced by human tumors, we performed a preliminary survey of the expression of this gene in a commercially available matched tumor/normal expression array. These analyses indicated that ADAM-TS12 was widely expressed in gastrointestinal carcinomas but not in the paired normal tissues (data not shown). To further extend this observation, we performed reverse transcription-PCR amplification with RNAs obtained from a panel of paired primary gastric carcinomas and adjacent normal mucosa. As illustrated in Fig. 5C, which shows some representative cases, ADAM-TS12 was overexpressed in a significant number of gastric carcinomas (9 of 12) when compared with the low or undetectable levels observed in the paired adjacent normal tissues, thus confirming and extending the previous results obtained after hybridization with the tumor/normal expression array. Finally, preliminary analysis of ADAM-TS12 expression in other tumor samples and cancer cell lines from different sources revealed that some colorectal, renal, and pancreatic carcinomas, as well as HeLa, A549 lung carcinoma, and Burkitt's lymphoma (Daudi) cells, also have the ability to overexpress this gene (data not shown).

DISCUSSION

In this work we describe the finding of ADAM-TS12, a novel member of the ADAM-TS subfamily characterized by its complex pattern of thrombospondin domains and its restricted expression in normal human tissues. The strategy followed to identify ADAM-TS12 was first based on an extensive scanning

of the EST data bases, looking for sequences with similarity to previously characterized ADAM family members. A sequence presumably encoding the disintegrin region of a new ADAM was identified, PCR amplified from human fetal lung cDNA, and used to screen a cDNA library from the same source. After screening of this library and further RACE-3' experiments, a full-length cDNA coding for ADAM-TS12 was finally isolated and cloned. Structural analysis of the identified sequence for ADAM-TS12 shows that it exhibits a series of protein domains characteristic of ADAM-TS proteins, including a prodomain, a catalytic domain, and metalloproteinase-like, disintegrin-like, and cysteine-rich domains, as well as a series of TS repeats. However, the number and organization of these repeats are unique to ADAM-TS12. Thus, it contains a total of eight TS repeats organized in three modules, whereas all remaining human ADAM-TS proteins contain from one to four repeats, organized in one or two modules (Fig. 2 and Refs.16–23). An additional distinctive feature of the structure determined for ADAM-TS derives from the presence of an additional domain called the spacer-2 region, with an absence of overall sequence similarity to proteins present in the data bases. The pattern of ADAM-TS expression in human tissues is also somewhat unusual. Thus, in this work we have provided evidence that expression of this gene is highly restricted in normal human tissues. The observation that ADAM-TS12 is predominantly expressed in fetal lung suggests that it could be involved in extracellular matrix remodeling processes occurring within the fetal lung during development. The restricted expression of ADAM-TS12 in normal tissues also suggests that this gene is highly regulated. A survey of factors that could control ADAM-TS12 expression has shown that TGF- β is able to up-regulate ADAM-TS12 in human fibroblast cells from fetal origin, whereas other factors such as TGF- α , IL-1 α , IL-1 β , acidic fibroblast growth factor, and epidermal growth factor do not show any apparent up-regulatory effect on the transcription of this gene. The finding that TGF- β , widely assumed to be inhibitory for matrix metalloproteinases expression, is able to induce ADAM-TS12 is not unprecedented because recent studies have shown that this growth factor up-regulates expression of potent metalloproteases associated with tumor progression such as gelatinase A and collagenase-3 (26, 27). Nevertheless, recent works have also shown that the activity of ADAM-TS proteins can be regulated at other levels, including activation through the putative Cys switch or through furin cleavage during secretion (25, 28). Therefore, in this work we have examined the intracellular processing mechanisms of ADAM-TS12. Studies with cells transfected with the full-length ADAM-TS12 cDNA have led us to conclude that this protein is synthesized as a precursor molecule that is first activated by cleavage of the prodomain in a furin-mediated process and subsequently processed into two fragments of different size. One of these fragments corresponds to the N-terminal region of the molecule and contains the metalloproteinase and disintegrin domains of ADAM-TS12. This fragment is proteolytically active as assessed by its ability to interact with α_2 -macroglobulin. The second fragment generated during the intracellular processing of ADAM-TS12 corresponds to the C-terminal region of the protein and contains most of the TS-1 repeats. To date, the functional significance of this processing mechanism in the context of putative normal or pathological roles of ADAM-TS12 is unclear. In this regard, we are interested in the recent observation that ADAM-TS1 also undergoes a complex processing mechanism, leading to the formation of two distinct active forms (29). Preliminary attempts to identify substrates for the proteolytically active fragment of ADAM-TS12 have not provided positive results. Nevertheless, the peculiarities of

ADAM-TS12 in terms of structural organization and tissue distribution may imply the possibility that their substrates may be distinct from those hydrolyzed by other proteases of this family. Similarly, the possibility that the released C-terminal fragment containing a number of TS-1 repeats could regulate angiogenic processes will require further exploration.

In contrast with its restricted expression in normal human tissues, ADAM-TS12 is widely expressed in gastric carcinomas and in cancer cells of diverse origin. These findings are suggestive of a role for this enzyme in the progression of these tumors as proposed for ADAM-TS4 in the case of human gliomas (20). Recent studies have shown that other metalloproteases like MT1-matrix metalloproteinase are overexpressed in gastric carcinomas and that their levels correlate with the grade of vascular invasion (30). Further studies will be required to evaluate the clinical significance of the expression of ADAM-TS12 in gastric carcinomas as well as to extend the preliminary observations indicating that this protease can be also overproduced by other malignant tumors such as colorectal, renal, and pancreatic carcinomas. Finally, we have determined the chromosomal location of the ADAM-TS12 gene in an attempt to explore further associations between this gene and tumor processes. According to our mapping studies, the ADAM-TS12 gene is located at the telomeric region of the long arm of chromosome 5. It is of interest that this region (5q35) has been found to be a recurrent site of translocations in hematological malignancies (31, 32). In addition, a putative tumor suppressor gene involved in the development of hepatocellular carcinomas without liver cirrhosis has been mapped to 5q35-qter (33). Furthermore, it is also remarkable in the context of ADAM-TS12 overexpression in gastric carcinomas that minisatellite probes corresponding to 5q35 have detected loss of heterozygosity or new mutant alleles in gastrointestinal cancers (34). The localization of ADAM-TS12 in this region will be useful for future studies aimed at exploring the possibility that this gene can be directly involved in any of these 5q35 alterations associated with tumor processes and especially with those of the gastrointestinal tract.

In summary, our results indicated that ADAM-TS12 is a novel member of the disintegrin family of metalloproteases that contains a complex array of thrombospondin domains. Most of these repeats are released after a series of sequential proteolytic processing events that may be of relevance for the *in vivo* function of this protein. Further studies, now in progress, will be required to determine whether this intracellular processing of ADAM-TS12 contributes to the regulation of its activity in processes involving tissue remodeling and cell adhesion and migration, as recently demonstrated for other members of this growing family of proteases (21, 29, 35).

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