Cloning and characterization of ADAM28: evidence for autocatalytic pro-domain removal and for cell surface localization of mature ADAM28

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The metalloprotease disintegrins are a family of membrane-anchored glycoproteins with diverse functions in fertilization, myoblast fusion, neurogenesis and protein ectodomain shedding. Here we report a cDNA sequence, encoding a metalloprotease disintegrin, termed ADAM28 ('a disintegrin and metalloprotease 28'), which was cloned from mouse lung. From protein sequence comparisons, ADAM28 is more closely related to snake venom metalloproteases (SVMPs) than to other ADAMs, and hence may cleave similar substrates to SVMPs, perhaps including components of the extracellular matrix. Northern blot analysis of selected mouse tissues revealed that ADAM28 is expressed highly and in alternatively spliced forms in the epididymis, suggesting a possible role in sperm maturation, and at lower levels in lung. The intracellular maturation of ADAM28 expressed in COS-7 cells resembles that of other ADAMs, in that ADAM28 is made as a precursor and processed to a mature form in a late Golgi compartment of the secretory pathway. Most or all of the mature, and thus presumably catalytically active, form of ADAM28 in COS-7 cells is accessible to cell surface trypsinization, suggesting that ADAM28 functions mainly on the cell surface. A mutation converting the catalytic-site glutamate residue into alanine abolishes pro-domain removal, even though this mutant form of ADAM28 can be transported to the cell surface in a manner similar to the wild-type protein. This suggests that pro-domain removal and maturation of ADAM28 may be, at least in part, autocatalytic. This is in contrast with several other ADAMs, for which furin-like proprotein convertases are involved in pro-domain removal, and in which a glutamate-to-alanine mutation in the catalytic site does not alter pro-domain removal.

Key words: disintegrin, epididymis, MDC, metalloprotease.

INTRODUCTION

Metalloprotease disintegrin proteins have been implicated in several important biological processes, including neurogenesis, fertilization, muscle development and release of membrane-anchored proteins, such as tumour necrosis factor α, transforming growth factor α and L-selectin, from the plasma membrane (for recent reviews, see [1–4]). Metalloprotease disintegrins, which are also referred to as ADAMs (where ADAM stands for 'a disintegrin and metalloprotease') or MDC proteins (metalloprotease disintegrin cysteine-rich proteins), consist of an N-terminal signal sequence, followed by a pro-domain, a metalloprotease domain, a disintegrin-like domain and a cysteine-rich region. In most cases these proteins also include an epidermal growth factor (EGF) repeat, a transmembrane domain and a cytoplasmic tail. Of the 30 currently known ADAMs, 17 are predicted to be catalytically active, because they carry a catalytic site consensus sequence (HEXXH) followed by a Met-turn, a characteristic feature of the metzincin family of metalloproteases [5,6]. The remaining 13 ADAMs lack a zinc-binding catalytic site and are not predicted to be catalytically active, even though the sequence of the metalloprotease domain is otherwise clearly conserved [three frequently updated websites provide access to the GenBank accession numbers and sequence alignments of known ADAMs: (1) www.people.Virginia.EDU/~jag6n/adams.html; (2) www.uta.fi/~loiika/HADAMs.html; and (3) www.gene.ucl.ac.uk/users/hester/metallo.html].

Of the 17 ADAMs that have a catalytic site consensus sequence, to date four have been shown to have catalytic activity [7–14]. Little is known about the activity or functions of the other 13 ADAMs with a catalytic site consensus sequence, except that seven of them appear to be expressed predominantly in the testis, suggesting a role in sperm function [4,15–23]. ADAMs harbouring a catalytic site that are known to be expressed in somatic cells include MDC9 [24], ADAM10/KUZ [8,9,25], ADAM8/MS2 [26], ADAM12/meltrin α [27], MDC15 [28–30], ADAM17/TACE (tumour necrosis factor converting enzyme) [10,11] and ADAM19/meltrin β [31,32]. Here we report the cDNA cloning and initial biochemical characterization of the mouse protein ADAM28, a catalytic-site-containing ADAM that is expressed in at least two somatic tissues, mouse epididymis and lung. Our data suggest that at least two splice forms of ADAM28 exist in the epididymis and that, unlike several other ADAMs, ADAM28 is autocatalytically processed to its mature form.

EXPERIMENTAL

Reagents

Restriction endonucleases, T4 DNA ligase and *Taq* DNA polymerase were obtained from Roche Molecular Biochemicals. [^{32}P]dCTP was purchased from NEN Life Science Products. All reagents were obtained from Sigma, unless indicated otherwise.

Abbreviations used: ADAM, a disintegrin and metalloprotease; DTT, dithiothreitol; ECM, extracellular matrix; EGF, epidermal growth factor; GST, glutathione S-transferase; MDC, metalloprotease disintegrin cysteine-rich protein; pAb, polyclonal antibodies; SH3, Src homology 3; SVMP, snake venom metalloprotease; TACE, tumour necrosis factor converting enzyme.

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The nucleotide sequence data reported (mouse ADAM28) will appear in the GenBank®, EMBL, GSDB and DDBJ Nucleotide Sequence Databases under accession no. AF153350.

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cDNA cloning and sequencing

A cDNA sequence corresponding to the disintegrin-like domain of ADAM 28 (sequence tag 16-3) [33] was used to probe a mouse lung λZap cDNA library under high-stringency conditions, as described in [24]. Seven cDNA clones were identified and plaque purified. *In vivo* excision of the cDNA insert was performed using the ExAssist/SOLR system according to the manufacturer's instructions (Stratagene), and clones were sequenced on both strands (The BioResource Center, Cornell University, Ithaca, NY, U.S.A.). The cDNA sequence was assembled using the SeqMan II program (DNASTAR).

Northern blot analysis

A mouse multiple-tissue Northern blot (Clontech) was probed under high-stringency conditions using a random-primed [^{32}P]dCTP-labelled probe corresponding to the coding region of ADAM28. Blots were prehybridized and hybridized using ExpressHyb (Clontech) following the manufacturer's protocol. To analyse ADAM28 expression, RNA was isolated from mouse lung and epididymis [34]. To look for alternative transcripts [35], duplicate RNA samples (25 μg for each tissue) were examined by Northern blotting using a probe corresponding to bases 1246–1613 (encoding part of the metalloprotease- and disintegrin-like domains) or bases 2065–2410 (the region encoding the transmembrane and cytoplasmic domains). A [^{32}P]dCTP-labelled human β-actin cDNA probe (Clontech) was used as a control for RNA loading.

Antibody production and Western blotting

A PCR product corresponding to the cytoplasmic tail of ADAM28 (amino acids 689–774) was generated using gene-specific primers designed to add a 5′ *Eco*RI site and a 3′ *Xho*I site. After digestion with *Eco*RI and *Xho*I, the PCR product was ligated into the corresponding sites of a pGEX4T-3 vector (Pharmacia) to allow production of glutathione S-transferase (GST) fusion proteins. The cDNA insert was sequenced prior to transformation of *Escherichia coli* BL21 to rule out the introduction of mutations by PCR. GST–(ADAM28 cytoplasmic domain) fusion proteins were isolated and used as antigens for production of rabbit polyclonal antibodies (pAb) as described previously [24,30]. As a control for the specificity of the anti-(cytoplasmic domain) antibodies, the pAb were depleted of antibodies recognizing GST alone (using GST–glutathione–Sepharose beads; referred to as ADAM28 pAb) or recognizing GST–(ADAM28 cytoplasmic domain) [using GST–(ADAM28 cytoplasmic domain)–glutathione–Sepharose beads; referred to as control pAb]. A polyclonal antiserum against the cytoplasmic domain of mouse MDC15 was described previously [30].

Mouse tissues were homogenized in lysis buffer [Tris-buffered saline containing 1 % (v/v) Nonidet P-40, 1 mM Ca^{2+}, 1 mM Mg^{2+} and protease inhibitors) [36] using a Polytron homogenizer (Kinematica). Lysates were centrifuged (14000 ***g***; 30 min) and supernatants were applied to concanavalin A–Sepharose beads (Amersham Pharmacia Biotech) for 2 h. Bound glycoproteins were eluted by heating to 95 °C for 5 min in SDS/PAGE loading buffer containing 10 mM dithiothreitol (DTT).

Mouse ADAM28 was excised from pBluescript using *Bam*HI and *Xho*I and ligated into pcDNA3 cut with the same enzymes. A site-directed mutant of ADAM28, in which codon 343 (GAA) encoding Glu was replaced by GCA encoding Ala, was prepared by a PCR-based site-directed mutagenesis procedure [37]. The resulting E343A mutant of ADAM28 was sequenced to rule out the introduction of any additional mutation. COS-7 cells (A. T. C. C.) were transfected using Lipofectamine (Gibco).

Metabolic labelling

COS-7 cells were transfected with full-length ADAM28 in pcDNA3 or with the pcDNA3 vector alone as a control. At 1 day post-transfection, cells were preincubated with growth medium lacking cysteine and methionine for 30 min, then incubated for 20 min with 210 μCi/ml Redivue Pro-mix *in vitro* cell labelling mix (Amersham Pharmacia Biotech). After removing the medium, the cells were either washed and lysed immediately, or chased in unlabelled complete medium for the indicated periods of time prior to lysis. Where indicated, the chase medium contained 5 μg/ml brefeldin A or 2 μg/ml monensin. Cells were lysed in lysis buffer and the cell lysate was cleared by centrifugation (13000 ***g***; 4 °C; 10 min). ADAM28 was immunoprecipitated using antibodies directed against its cytoplasmic tail, and the immune complex was captured on Protein A–Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech). After washing the beads with lysis buffer, bound proteins were eluted in SDS/PAGE sample buffer containing 10 mM DTT by heating to 95 °C for 5 min. The eluted proteins were diluted 25-fold in lysis buffer and then captured using concanavalin A–Sepharose beads. Beads were washed and bound proteins were eluted as above. After separation of the eluted proteins by SDS/PAGE, the gel was then fixed in 50 % (v/v) methanol/10 % (v/v) acetic acid, dried and exposed to X-ray film (Kodak).

Cell surface biotinylation and trypsin treatment

The generation of a construct encoding mouse MDC15 in pcDNA3 was described previously [30]. COS-7 cells were transiently transfected with full-length mouse ADAM28 or MDC15 cDNA in pcDNA3. At 2 days post-transfection, cells were washed three times with PBS at room temperature, then once with PBS at 4 °C. After incubating the cells with the non-membrane-permeable biotinylation reagent NHS-LC-biotin (Pierce) (0.5 mg/ml; 45 min; 4 °C), the reaction was quenched by washing with 0.1 M glycine in Tris-buffered saline. The cells were then incubated with cold PBS with or without bovine pancreas trypsin (500 μg/ml; 30 min; 4 °C), and subsequently washed using 500 μg/ml soybean trypsin inhibitor in PBS. Detached cells were pelleted (500 ***g***; 5 min; 4 °C) and lysed in cell lysis buffer containing protease inhibitors. The cell lysate was cleared by centrifugation (13000 ***g***; 20 min; 4 °C), and ADAM 28 or MDC15 was immunoprecipitated using antibodies directed against their cytoplasmic tails and Protein A–Sepharose as described above.

RESULTS AND DISCUSSION

cDNA sequence and expression pattern of mouse ADAM28

A 140 bp sequence tag, corresponding to a fragment of the disintegrin-like domain of ADAM28, was first identified in a PCR-based screen to identify novel metalloprotease disintegrins (referred to as sequence tag 16-3) [33]. In order to isolate a cDNA encoding the entire open reading frame of ADAM28, a mouse lung cDNA library was screened, and seven cDNA clones were isolated. The longest cDNA clone thus isolated comprised 2941 bp, and included an open reading frame of 774 amino acid residues. BLAST searches against sequence databases indicated that this protein is the mouse orthologue of human MDC-L and

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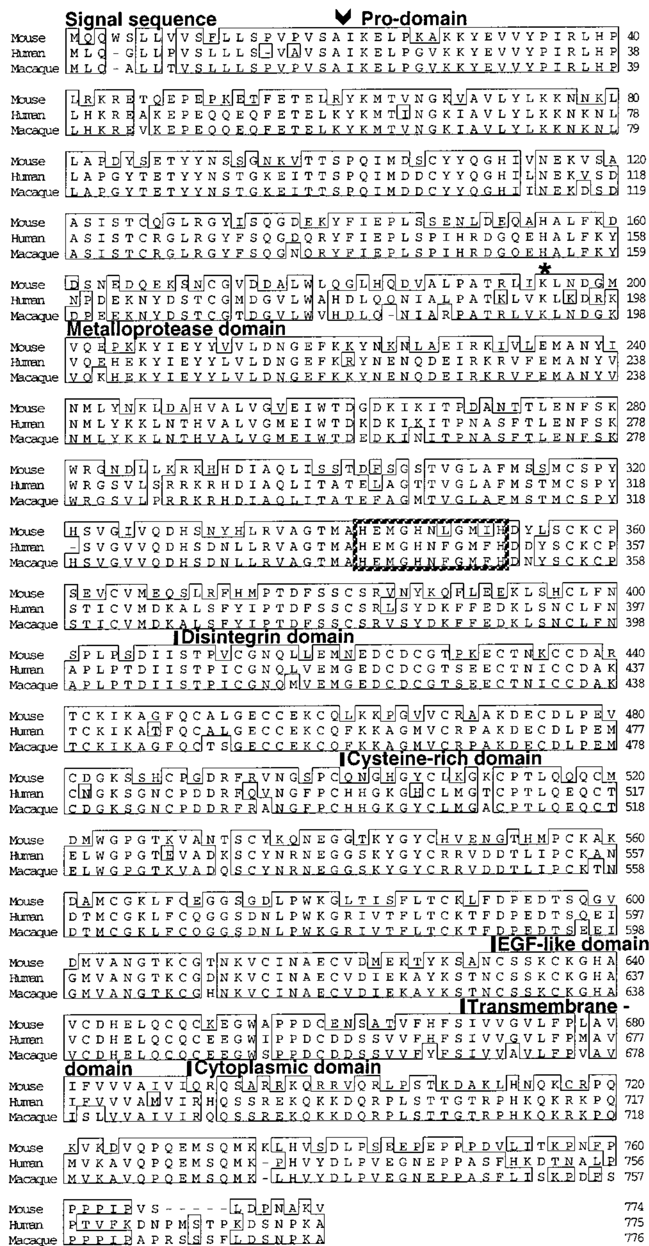

Figure 1 Alignment of the protein sequences of full-length ADAM28 from mouse, human and macaque

The approximate boundaries between domains are marked by bold vertical lines above the sequence. An arrowhead indicates the predicted signal sequence cleavage site [56]. The approximate position of the proprotein convertase cleavage site present in other ADAMs (but absent from ADAM28) is marked by an asterisk. The catalytic zinc-binding site required for metalloprotease activity is marked with a hatched box. The GenBank accession numbers for the sequences used in this alignment are: mouse ADAM28, AF153350; membrane-anchored form of human ADAM28, AF137334/AJ242015; macaque ADAM28, AJ242014. The sequence alignment was generated with DNASTAR Megalign.

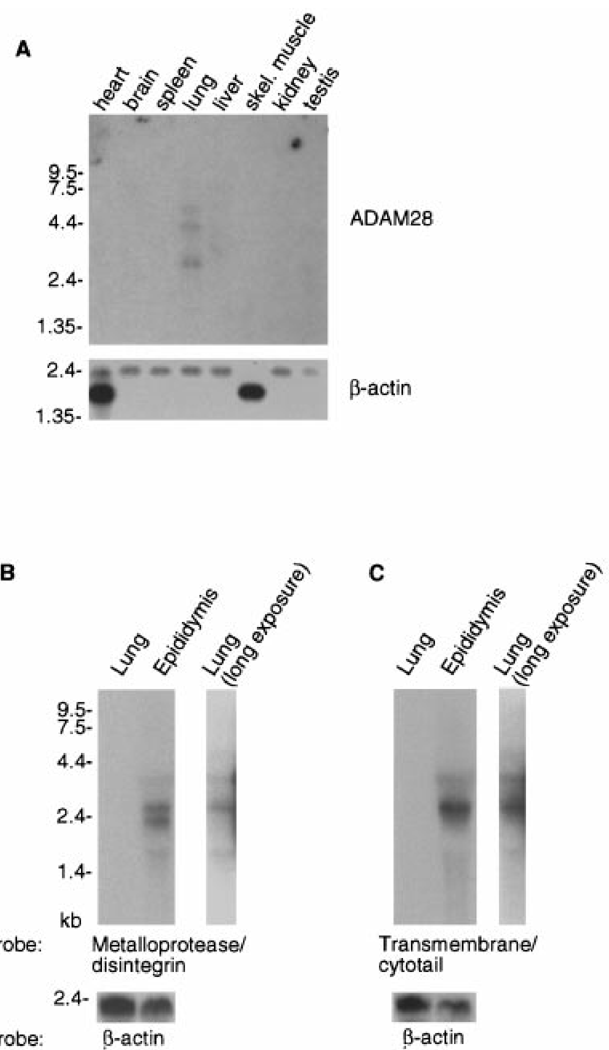

Figure 2 ADAM28 expression in mouse tissues

(**A**) A mouse multiple-tissue Northern blot (Clontech) was probed under stringent conditions with a ^{32}P-labelled probe corresponding to the entire ADAM28 protein coding region. In (**B**) and (**C**), total RNA from mouse lung or epididymis (25 μg/lane) was probed with (**B**) a ^{32}P-labelled cDNA probe corresponding to ADAM28 bases 1246–1613 (encoding a portion of the metalloprotease and disintegrin-like domains), or (**C**) a ^{32}P-labelled cDNA probe corresponding to bases 2065–2410 [the region encoding the transmembrane and cytoplasmic ('cytotail') domains]. Panels (**B**) and (**C**) show a short exposure of the Northern blot (8 h), and a long exposure of the lane containing lung RNA (80 h) to visualize the ADAM28 transcripts in lung and allow comparison with epididymal transcripts. Human β-actin was used as a control to compare mRNA loading. The migration of RNA standards is indicated.

macaque eMDC II (Figure 1) and a polypeptide encoded by a rat EST (expressed sequence tag) (AI030215) [35,38]. This protein is designated ADAM28. The amino acid sequences of the human and macaque proteins are 91 % identical to each another, whereas the amino acid sequence of the mouse protein is 70 % identical with that of the human protein, and 73 % identical with the macaque ADAM28 (see Figure 1).

Like other metalloprotease disintegrins, mouse ADAM28 consists of several domains: a signal sequence followed by a pro-domain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich region, an EGF-like repeat, a membrane-spanning sequence and a cytoplasmic tail (Figure 1). ADAM28 possesses a catalytic zinc-binding consensus sequence (His-Glu-Xaa-Xaa-His; HEXXH) followed by a Met-turn, a characteristic feature of metalloproteases belonging to the metzincin family [5,6]. These features suggest that ADAM28 is a catalytically active metalloprotease. Like many other ADAM family members, e.g. MDC9 [24], ADAM12 [27], ADAM13 [39], MDC15 [28–30], TACE [10,11] and ADAM19 [31,32], the cytoplasmic tail of ADAM28 contains proline-rich regions which may serve as

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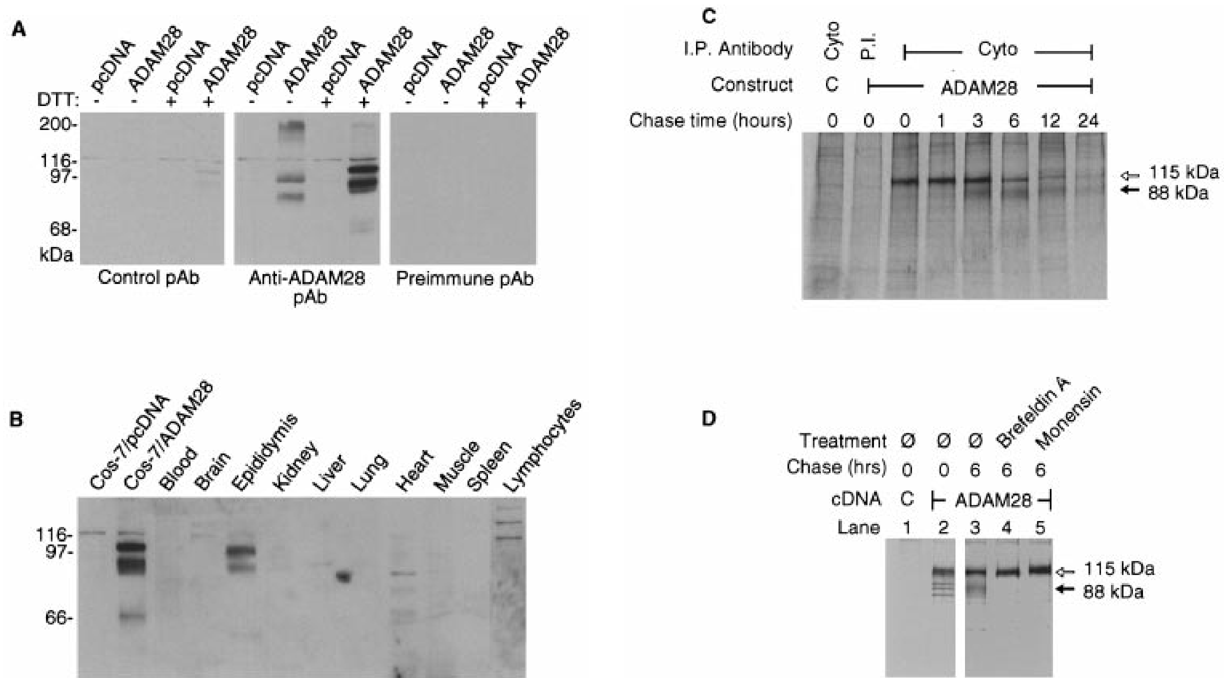

Figure 3 Characterization of antibodies against ADAM28, ADAM28 expression and analysis of the maturation of ADAM28 in COS-7 cells

COS-7 cells were transiently transfected with full-length ADAM28 in pcDNA3 or with a pcDNA3 control, as indicated. (**A**) Western blot analysis. Transfected cells were lysed in lysis buffer, boiled with sample loading buffer with or without DTT, and separated on 7.5% (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose and immunoblotted with an antiserum against the cytoplasmic tail of ADAM28. As a control for specificity, identical blots were probed either with preimmune serum or with an antiserum that was depleted of ADAM28-specific antibodies by incubation with GST–(ADAM28 cytoplasmic tail) beads (control pAb). The ADAM28 pAb sample was mock-depleted on GST beads. (**B**) Western blot of ADAM28 tissue distribution. A portion of 3.5 μg of transfected COS-7 lysate or concanavalin A-binding glycoproteins isolated from a 1.4 mg protein tissue homogenate or 2×10^7 mouse lymphocytes were immunoblotted using ADAM28 pAb. (**C**) Pulse–chase analysis of ADAM28 maturation. COS-7 cells transfected with ADAM 28 in pcDNA3 were labelled for 20 min with [^{35}S]methionine/cysteine and chased with complete medium for the indicated periods of time. Cells were lysed at each time point and ADAM28 was immunoprecipitated (I.P.) with the anti-(ADAM28 cytoplasmic domain) antiserum, or with a preimmune control (P.I.). Samples were reduced prior to electrophoresis on a 7.5% (w/v) polyacrylamide gel. Following electrophoresis, the gel was dried and exposed to Kodak X-AR autoradiography film. 'Cyto' denotes the anti-ADAM28 cytoplasmic domain pAb. (**D**) COS-7 cells transfected with ADAM28 (lanes 2–5) or with a pcDNA3 control vector (lane 1) were labelled as above and chased for 6 h in the presence or absence of 5 μg/ml brefeldin A or 2 μg/ml monensin. ADAM28 was immunoprecipitated as above. In (**C**) and (**D**), the putative pro-form of ADAM28 (under reducing conditions) is indicated by an open arrow, and the putative mature form is marked by a solid arrow.

ligands for proteins containing SH3 (Src homology 3) domains. The first evidence that ADAMs may indeed interact with SH3-domain-containing proteins came from a recent study in which two SH3-domain-containing proteins, endophilin I and SH3PX1, were found to interact with proline-rich sequences in the MDC9 and MDC15 cytoplasmic domains [40]. In principle, interaction of the cytoplasmic domain of an ADAM with other proteins may play a role in signalling, intracellular protein maturation, or localization of the ADAM to sites of activity.

The protein sequence of the extracellular portion of mouse ADAM28 is more closely related to snake venom metalloproteases (SVMPs) than to most currently known ADAMs, particularly in its pro- and metalloprotease domains. Since the SVMP jararhagin can cleave components of the extracellular matrix (ECM), such as fibrinogen and von Willebrand Factor, and also $\alpha 2\beta 1$ integrin [41–43], a potentially interesting implication of the sequence relationship between ADAM28 and SVMPs is that substrates of ADAM28 may also include components of the ECM. The only other ADAM which has been shown to cleave an ECM component is ADAM10, which can apparently cleave native collagen [44].

Northern blot analysis revealed that ADAM28 is expressed at high levels in mouse epididymis and at lower levels in lung, whereas expression was not detectable in the other tissues analysed here (heart, brain, spleen, liver, skeletal muscle, kidney and testis; Figure 2). This expression pattern is similar to that observed for the macaque orthologue, eMDC II [38]. A probe corresponding to bases 1246–1613 of ADAM28, i.e. encoding portions of the metalloprotease and disintegrin-like domains, hybridizes with transcripts of 4.3, 2.9 and 2.3 kb in epididymal RNA, but only hybridizes with transcripts of 4.3 and 2.9 kb in lung RNA (see long exposure in Figure 2B). We note the presence of a 5.3 kb band which could be detected in the poly(A)$^+$ RNA (Figure 2A), but not in the total RNA (Figures 2B and 2C). These transcripts either represent alternatively spliced versions of ADAM28 or very closely related gene products.

To evaluate potential splicing of ADAM28 RNA, a duplicate of the Northern blot of lung and epididymal RNA shown in Figure 2(B) was probed with a cDNA probe corresponding to bases 2065–2410, i.e. coding for the transmembrane and cytoplasmic domains of ADAM28 (Figure 2C). Expression of ADAM28 in the epididymis was very high compared with that in lung, where expression could only be detected after a longer exposure of the Northern blot. For epididymal RNA, the probe 2065–2410 recognized an abundant 2.9 kb transcript and a lower level of a 4.3 kb transcript, but not the 2.3 kb transcript recognized by the probe 1246–1613 (Figure 2C). These data suggest

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that mouse ADAM28 RNA exists in at least two splice forms in the epididymis, and thus resembles the two splice forms of human ADAM28 in lymphocytes, MDC-Lm and MDC-Ls [35]. The 2.9 kb and 4.3 kb transcripts are predicted to encode membrane-anchored forms of ADAM28, whereas the 2.3 kb transcript, which is not detected in the lung, presumably encodes a secreted form lacking the membrane-spanning and cytoplasmic domains.

ADAM28 immunoblotting and processing in the secretory pathway

Antibodies were raised against a GST–(ADAM28 cytoplasmic domain) fusion protein and used to characterize the ADAM28 protein by Western blot analysis (Figure 3A). As a control for specificity, the polyclonal antiserum was depleted of antibodies which recognize either GST (referred to as ADAM28 pAb) or the GST–(ADAM28 cytoplasmic domain) fusion protein (referred to as control pAb). On Western blots of ADAM28-expressing COS-7 cells, ADAM28 pAb recognized proteins of 78 kDa and 100 kDa in samples run under non-reducing conditions, and proteins of 88 kDa and 115 kDa in samples run under reducing conditions. We conclude that these proteins correspond to ADAM28, since they were not recognized by the pre-immune antiserum and their intensity was significantly reduced for the immunodepleted control pAb. Furthermore, these proteins were also not present in an extract of COS-7 cells transfected with the pcDNA3 vector as a control (Figure 3A). The shift in apparent molecular mass upon reduction is a characteristic feature of ADAM proteins, due to the number of disulphide bonds present in the disintegrin-like and cysteine-rich domains [24,45]. A band of ≈ 200 kDa which was not present in the control cell lysate was also recognized by the ADAM28 antibodies. This band may represent an SDS-resistant multimer of ADAM28, or ADAM28 in a complex with one or more other yet to be identified proteins.

To examine further the expression of ADAM28 in different tissues, glycoproteins were isolated from a number of mouse tissues and examined by immunoblotting (Figure 3B). The mouse ADAM28 protein, like the macaque orthologue, was highly expressed in epididymis, but was not detected in other tissues examined under the conditions used in this study. The bands seen here in heart and lymphocytes are non-specific, as they were also recognized by control pAb (results not shown).

Several ADAMs are processed by proprotein convertases in the secretory pathway, leading to removal of an N-terminal pro-domain [12,30,46]. In order to examine the processing of ADAM28 in the secretory pathway, pulse–chase experiments were performed on COS-7 cells transiently transfected with full-length ADAM28 (Figure 3C). After a 6 h chase, about one-third to one-half of the 115 kDa pro-ADAM28 was converted into a protein of 88 kDa. Since the 88 kDa and 115 kDa proteins were both immunoprecipitated by an antibody against the ADAM28 cytoplasmic domain, the 88 kDa protein most likely represents ADAM28 lacking its N-terminal pro-domain. The pro-domain contains a sequence which resembles a cysteine-switch motif, a motif known to maintain metalloproteases in an inactive form during biosynthesis [47]. Removal of the pro-domain is thought to be a prerequisite for the protease to become active [12,47].

In order to identify the subcellular compartment in which pro-domain removal occurs, ADAM28-transfected COS-7 cells were subjected to pulse–chase analysis in the presence of brefeldin A (which blocks trafficking to the Golgi from the endoplasmic reticulum [48–50]) or monensin (which interferes with transport to late Golgi compartments [51,52]) (Figure 3D). Treatment with

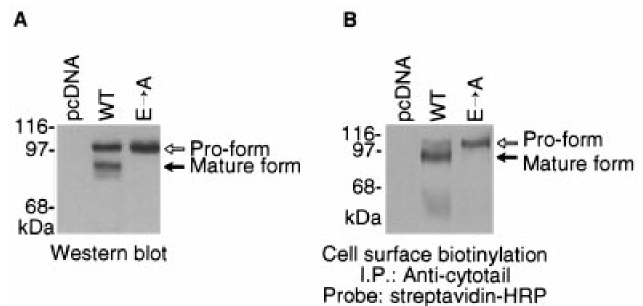

Figure 4 A catalytically inactive form of ADAM28 is not converted from the pro- form into the mature form

(**A**) Western blot of lysates from COS-7 cells transfected with a pcDNA control, wild-type ADAM28 (WT) or ADAM28 E343A (E → A). All samples were reduced with DTT and separated on a 7.5% (w/v) polyacrylamide gel. Proteins were transferred to nitrocellulose and immunoblotted with anti-(ADAM28 cytoplasmic domain) antiserum. (**B**) Cell surface biotinylated material from COS-7 cells transfected with a pcDNA control, wild-type ADAM28 (WT) or ADAM28 E343A (E → A) was immunoprecipitated (I.P.) with an anti-(ADAM28 cytoplasmic domain) antiserum. The immunoprecipitated material was separated on a 10% (w/v) polyacrylamide gel and transferred to nitrocellulose, and biotinylated material was visualized using streptavidin–horseradish peroxidase (HRP) in combination with the enhanced chemiluminescence detection system.

both brefeldin A and monensin prevented removal of the ADAM28 pro-domain. This suggests that, like other ADAMs, such as MDC9 [12], ADAM12 [46] and MDC15 [30], ADAM28 is processed after passage through the medial Golgi network.

Whereas many ADAM proteins possess a typical cleavage site for proprotein convertases (Arg-Xaa-Lys/Arg-Arg [53]; see Figure 1), ADAM28 lacks such a clear consensus site. This raises the possibility that ADAM28 may be processed by an enzyme other than a proprotein convertase. To address whether ADAM28 pro-domain removal may require ADAM28 to be catalytically active, Glu-343 in the catalytic site (HEXXH) was replaced by Ala (ADAM28 E343A). Thus a mutation was introduced into ADAM28 that has been shown to abolish metalloprotease activity in other metalloproteases [12,54]. Western blot analysis revealed that COS-7 cells transfected with ADAM28 E343A produced only the 115 kDa pro-form of ADAM28, but yielded no detectable 88 kDa mature ADAM28 (Figure 4A). This is in contrast with MDC9 [12], ADAM12 [14] and MDC15 (L. Lum, unpublished work), in which similar Glu-to-Ala mutations in the catalytic site have no effect on pro-domain removal. Surface biotinylation followed by immunoprecipitation of ADAM28 from COS-7 cells expressing either wild-type ADAM28 or the E343A mutant demonstrated that both reached the cell surface. This indicates that the Glu-to-Ala mutation, which abolishes pro-domain removal, does not alter the productive folding or transport of ADAM28 to the cell surface (Figure 4B). Taken together, these data suggest that the catalytic activity of ADAM28 plays a role in its own maturation, most probably by autocatalytic removal of the pro-domain. This may be the first experimental evidence that ADAM28 is indeed an active metalloprotease. However, these results do not rule out the possibility that the Glu-to-Ala mutation renders ADAM28 insensitive to the enzyme that normally processes its pro-domain.

Western blots of COS-7 cells transiently transfected with wild-type ADAM28 showed that the pro- and mature forms were detected at approximately equal levels (see Figures 3A, 4A and 5A). However, immunoprecipitation of ADAM28 from trans-

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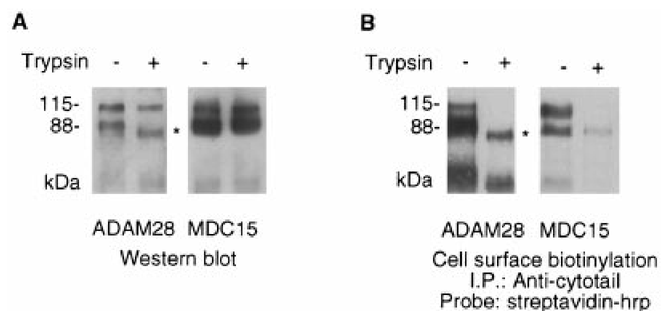

Figure 5 ADAM28 is present on the surface of transfected COS-7 cells, as determined by cell surface trypsinization and cell surface biotinylation

Surface-biotinylated COS-7 cells expressing either ADAM28 or MDC15 were treated with 500 μg/ml trypsin in PBS (+Trypsin), or in PBS on ice for 30 min (−Trypsin), and subsequently washed in a buffer containing 0.5 mg/ml soybean trypsin inhibitor. (**A**) Western blot of COS-7 cells transfected with ADAM28 or mouse MDC15, probed with antibodies against the cytoplasmic domains of the appropriate protein, as indicated. (**B**) ADAM 28 or MDC15 was immunoprecipitated (I.P.) from cell-surface-biotinylated cells and transferred on to a nitrocellulose membrane, and biotinylated material was detected using streptavidin–horseradish peroxidase (hrp). Note that the mature form of ADAM28 (marked by an asterisk) migrates faster after cell surface trypsinization, whereas the pro-form of ADAM28 is not detectably affected.

fected cells labelled with a non-membrane-permeable biotinylation reagent revealed that mainly a processed 88 kDa form of ADAM28 is present on the cell surface, as well as a weaker band of 50 kDa (Figures 4B and 5B). The 88 kDa protein is recognized by antibodies against the ADAM28 cytoplasmic tail; therefore it most probably represents ADAM28 lacking its N-terminal prodomain. Since the 50 kDa protein is not clearly detected by Western blot analysis, it may represent a minor form of ADAM28 lacking both the pro-domain and the metalloprotease domain which is found mainly on the cell surface. The 50 kDa band is not seen on immunoprecipitation of surface biotinylated material from COS-7 cells expressing the ADAM28 E343A mutant, supporting the notion that it may also be a product of ADAM28 autocatalysis. Another possibility is that the 50 kDa protein is only associated with wild-type ADAM28 on the cell surface.

To evaluate how much of the total processed form of ADAM28 resides on the cell surface, COS-7 cells expressing ADAM28 were trypsinized on ice (to prevent endocytosis), and the cell lysate was subsequently analysed by Western blot analysis. While the pro-form of ADAM28 was not affected by cell surface trypsinization, all detectable mature ADAM28 (88 kDa) was converted into a faster migrating form of approx. 82 kDa by trypsinization (Figure 5). This trypsin sensitivity indicates that most or all of the mature ADAM28 expressed in COS-7 cells resides on the cell surface. The observation that trypsin treatment only removes a small portion of ADAM28 (corresponding to $\sim$ 6 kDa) and does not completely degrade ADAM28 is consistent with the protease-resistance of some cysteine-rich extracellular protein domains, such as the ADAMs fertilin α and β [36,55]. To compare the surface accessibility of ADAM28 to trypsin with that of another ADAM, MDC15-expressing COS-7 cells were trypsinized under identical conditions. As shown previously [30], most of the mature form of MDC15 that is detectable by Western blot analysis was protected from trypsinization (Figure 5A), suggesting that the majority of MDC15 is not present on the cell surface. However, all cell surface biotinylated MDC15 can be degraded when exposed to trypsin, demonstrating that MDC15 is in principle susceptible to proteolysis (Figure 5B). Although it remains to be shown that ADAM28 and MDC15 have different subcellular localizations in non-transfected cells, the different behaviour of these two proteins in COS-7 cells suggests that ADAM28 may function mainly on the cell surface, whereas MDC15, as well as TACE, may function mainly intracellularly [30,45].

In summary, the present study describes the cloning and characterization of ADAM28, a disintegrin metalloprotease which is more closely related to SVMPs than to presently known ADAMs. By Northern blot analysis, ADAM28 transcripts were detected in epididymis and lung, but not in other tissues examined here. Transiently transfected COS-7 cells produce a 115 kDa form of ADAM28 which is processed late in the secretory pathway, most probably autocatalytically, to give an 88 kDa mature form of the protein. Because the mature and presumably catalytically active form of ADAM28 resides mainly on the cell surface, it is likely also to function predominantly on the cell surface. The high level of ADAM28 expression in the epididymis suggests a potential role in sperm maturation. The close relationship with SVMPs raises the intriguing possibility that the substrates of ADAM28 may be similar to those of SVMPs, and may thus include components of the ECM in addition to membrane-anchored cell surface molecules.

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