FEBS Letters 498 (2001) 82–86 FEBS 24929

Catalytic activity of ADAM28

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Received 14 March 2001; revised 2 May 2001; accepted 2 May 2001

First published online 18 May 2001

Edited by Felix Wieland

Abstract ADAMs are membrane-anchored glycoproteins containing a disintegrin and metalloprotease domain that have important roles in fertilization, development, and diseases such as Alzheimer's dementia. Here we present the first evidence for catalytic activity of ADAM28, a protein that is highly expressed in the epididymis and lymphocytes. Recombinant ADAM28 cleaves myelin basic protein at two sites. The catalytic activity of ADAM28 is not sensitive to tissue inhibitors of metalloproteases 1 and 2, but can be abolished by a mutation in the catalytic site. Catalytically active ADAM28 will be valuable for further studies of its role in sperm maturation and lymphocyte function. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: ADAM; Metalloprotease disintegrin; Metalloproteinase

1. Introduction

ADAMs are a family of membrane-anchored glycoproteins with roles in fertilization, myogenesis, neurogenesis, diseases such as Alzheimer's disease, and in a process termed protein ectodomain shedding [1–3]. A typical ADAM consists of an amino-terminal signal sequence, followed by a pro-domain, a metalloprotease domain, a disintegrin domain and cysteine-rich region, an EGF repeat, transmembrane domain and cytoplasmic tail. Currently, 24 ADAMs are known to exist in the mouse (see the following websites for details: http://www.uta.fi/%7Eloiika/ADAMs/MMADAMs.htm, http://www.people.Virginia.EDU/~jag6n/Table_of_the_ADAMs.html). Of these, 14 have a catalytic site consensus sequence (HEXXH) in their metalloprotease domain, and are therefore predicted to have catalytic activity. The remaining 10 ADAMs do not possess a catalytic site consensus sequence in their otherwise conserved metalloprotease-like domain, and are therefore most likely not catalytically active.

Although 14 mouse ADAMs have a catalytic site, only a few of these ADAMs or their human orthologues have been shown to possess catalytic activity. The first reported assay for catalytic activity of an ADAM was cleavage of myelin basic protein (MBP) by ADAM10/MADM [4,5]. Subsequently, ADAM10 has been shown to be able to cleave peptides corresponding to the cleavage sites of tumor necrosis factor (TNF) α and APP [6,7]. The TNFα convertase/ADAM17 was first purified based on its ability to cleave TNFα [8,9] and has since been shown to cleave a variety of other peptides and protein substrates in vitro [10,11]. Recombinant ADAM9 cleaves the insulin B chain, a generic protease substrate, as well as several peptides derived from known cleavage sites of proteins that are shed from the plasma membrane [12]. Finally, catalytic activity of ADAM12 and ADAM19 has been demonstrated through their ability to cleave and bind the protease inhibitor α2 macroglobulin [13,14]. Here we use MBP as a substrate to demonstrate that ADAM28 is catalytically active, and to evaluate some of the properties of catalytically active ADAM28. ADAM28 is highly expressed in the epididymis and in lymphocytes [15–17], and establishing an assay for its catalytic activity is an important first step towards analyzing the potential function of ADAM28 in sperm maturation and in host defense.

2. Materials and methods

2.1. Reagents

Restriction endonucleases, T4 DNA ligase and Taq DNA polymerase were obtained from Roche Molecular Biochemicals. All reagents were obtained from Sigma unless indicated otherwise. Tissue inhibitors of metalloproteases (TIMPs) 1 and 2 were kindly provided by Dr. Gill Murphy (University of East Anglia, UK).

2.2. Expression constructs and generation of stable CHO cell lines

PCR was used to generate constructs corresponding to bases 60–2063 (EC-Fc) or 60–1292 (MP-Fc) of mouse ADAM28 with a 5′ *Kpn*I site and a 3′ *Bam*HI site. ADAM28 in pBluescript or a mutant ADAM28 (Glu343-Ala) were used as templates [15]. *Kpn*I/*Bam*HI-digested fragments were ligated into a pcDNA3 expression vector in frame with the cDNA for the human IgG-Fc domain [18]. All cDNA clones were sequenced on both strands (The BioResource Center, Cornell University, Ithaca, NY, USA). CHO cells (ATCC) were transfected using Lipofectamine (Gibco Life Technology). Cells were allowed to recover overnight and were then transferred into medium containing 500 μg/ml G418 (Life Technology). Medium was changed every 2 days for approximately 8 days. Single colonies were selected and expanded, and conditioned medium and cellular proteins were examined for the presence of Fc fusion proteins by Western blot analysis [18] using an anti-human IgG antibody (Promega).

2.3. Purification of secreted Fc-tagged fusion proteins and of ADAM10

Stably transfected CHO cell clones were grown in serum free OPTIMEM containing penicillin, streptomycin and glutamine for 4–5 days. Conditioned media from 30–40 150 mm plates were pooled. Cells and debris were removed by centrifugation (8000 rpm, Sorvall GS3 rotor, 15 min, 4°C) followed by filtration through a 0.2 μM filter

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0014-5793/01/$20.00
PII: S0014-5793(01)02506-6

(Nalgene). Fc fusion proteins were captured on a 1 ml Hi-Trap rProtein A column (Amersham Pharmacia Biotechnology), washed with phosphate-buffered saline containing 2 mM Zn^{2+} and eluted using 0.1 M glycine pH 3.2. 500 μl fractions were collected and immediately neutralized using 2 M Tris–HCl pH 8. Fractions containing the desired fusion protein were pooled. ADAM10 was purified from bovine brain as described previously [4,5].

2.4. Protease assays

Commercially available MBP (Sigma) was purified by electroelution from a 15% SDS–polyacrylamide gel (see [18] for a description of the electroelution procedure) in order to remove contaminating degradation products. Purified Fc-tagged ADAM28 (1 μg) was incubated with 2.7 μg of purified MBP at 37°C overnight in 0.1 mM Tris–HCl pH 7.4, 0.1 M glycine, 10 mM $CaCl_2$, and the resulting degradation products were separated by SDS–PAGE and stained with Coomassie brilliant blue R250. For N-terminal amino acid sequence determination, the digestion products were separated on a 15% Novex SDS–polyacrylamide gel, transferred to PVDF membrane, and analyzed by automated Edman degradation, using an Applied Biosystems 477A sequenator [19]. Cleavage of the insulin B chain was performed as described previously [12].

Wild-type and mutant forms of recombinant human ADAM28 were assessed for their ability to cleave a range of synthetic peptide substrates including MCA-Pro-Leu-Gly-Dap(Dnp)-Ala-Arg-NH_2 (8.3 μM), MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 (8.3 μM), MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 (5 μM) and 4′,5′-dimethoxyfluoresceinyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Cys (4-(3-succinimid-1-yl)) fluorescein-NH_2 (10 μM). All substrates were synthesized by Bachem. Full length recombinant human MMP13, 14 and 19 and TNFα convertase were used as standards. The MMPs were cloned, expressed and purified essentially as described in [20–22], while TNFα convertase was isolated from the membranes of THP-1 cells as described in [23]. The enzymes were tested at starting concentrations of 6 ng/assay (MMP14), 5.83 ng/assay (MMP13), 60 ng/assay (MMP19) and 0.0066 U/assay (TNFα convertase, where a unit is the amount of enzyme needed to cleave 1 μM of substrate in 2 h). ADAM28 wild-type and mutants were tested from 1 to 10 μl/assay (stock concentrations were between 35 μg/ml and 50 μg/ml) using. For all assays the buffer used was 0.1 M Tris–HCl pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Brij 35 and 0.02 mM ZnCl. Activity against the MCA containing synthetic substrates was determined after incubation at 37°C for 0.5, 1, 5.5 and >72 h using a Fluoroscan II (Labsystems) at settings λ_{ex} 328 nm, λ_{em} 393 nm, while activity against fluorescein containing substrate was determined after incubation at 25°C or 37°C for 18 h at settings λ_{ex} 490 nm, λ_{em} 530 nm.

3. Results and discussion

Three constructs for expression of ADAM28 fusion proteins in CHO cells were generated. One consisted of the entire ectodomain of ADAM28 including the EGF repeat fused to the Fc domain of human IgG1 (Fig. 1A, EC-Fc). A second consisted of the pro- and metalloprotease domains fused to the Fc domain (Fig. 1A, MP-Fc). Finally, as a control, a mutation that is known to abolish or greatly reduce the catalytic activity of Zn-dependent metalloproteases was introduced into the catalytic site of the EC-Fc construct (Fig. 1A, HEXXH-HAXXH, referred to as $E_{343}>A$ hereafter) [12,24]. CHO cells stably expressing each construct were selected, and the corresponding fusion proteins were isolated from supernatants on protein A Sepharose beads (Fig. 1B,C). Purified ADAM28 EC-Fc had an apparent mass of 122 kDa on an SDS gel. The apparent mass of mature ADAM28 MP-Fc was 85 kDa, whereas that of the less abundant precursor form of ADAM28 MP-Fc was 102 kDa (Fig. 1B). ADAM28 EC-Fc $E_{343}>A$ had an apparent mass of 134 kDa and thus migrated slower than wild-type ADAM28 EC-Fc (Fig. 1C), consistent with our previous observation that

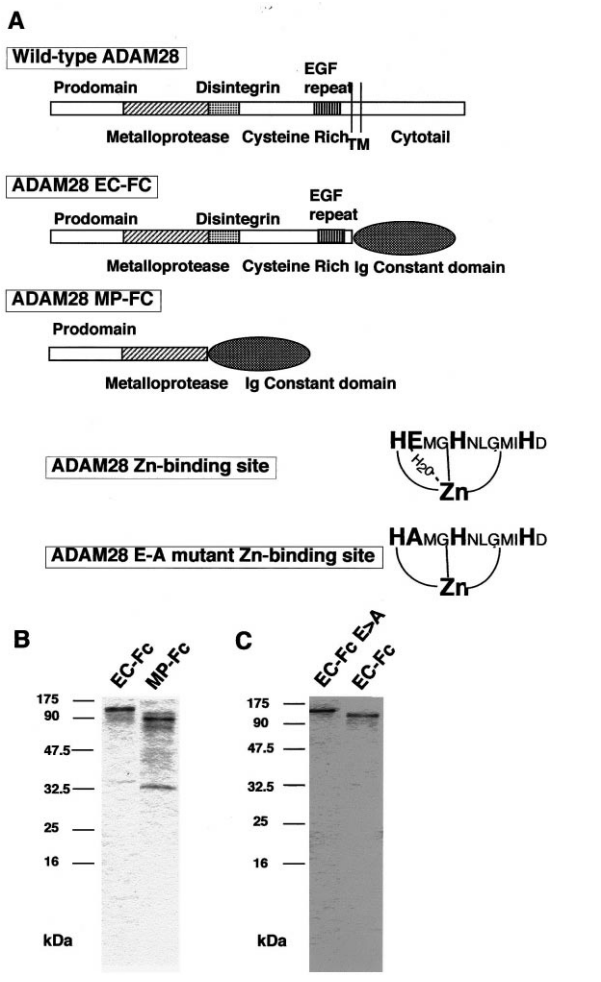

Fig. 1. Expression of ADAM28 EC-Fc and MP-Fc fusion proteins. Panel A presents the domain organization of ADAM28, which is also typical for other ADAMs, and a diagram of the ADAM28 EC-Fc and MP-Fc fusion proteins and of the wild-type and mutant catalytic site of ADAM28. A comparison of purified ADAM28 EC-Fc and MP-Fc is shown in B, and of the catalytic site mutant ADAM28 EC-Fc and wild-type ADAM28 EC-Fc is shown in C. In both B and C, the samples were separated by SDS–PAGE, and stained with Coomassie brilliant blue.

pro-domain removal of ADAM28 depends on the presence of the active site glutamate residue.

To test for catalytic activity of ADAM28, the purified Fc fusion proteins were incubated with MBP, a substrate that has previously been shown to be cleaved by ADAM10 (Fig. 2) [4,5], and with the insulin B chain, which was previously shown to be cleaved by ADAM9 and TNFα convertase. ADAM28 was unable to cleave the insulin B chain protein (data not shown). However, when MBP was incubated with ADAM28 EC-Fc, two major cleavage products of around 4 and 6 kDa were generated. When an equal amount of ADAM28 EC-Fc $E_{343}>A$ was incubated with MBP, no cleavage products were seen. The catalytic activity did not depend on the presence of the disintegrin domain, cysteine-rich region and EGF repeat, as the MP-Fc construct generated cleavage products from MBP that co-migrated with those generated by the EC-Fc fusion protein. These results provide the first direct evidence for catalytic activity of ADAM28.

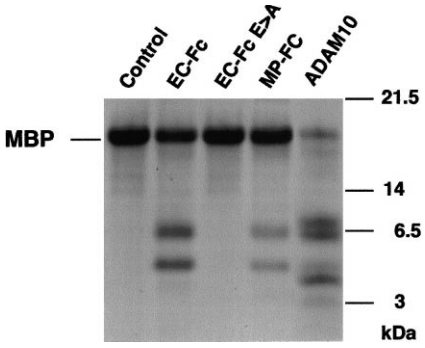

Fig. 2. Cleavage of MBP by ADAM28 EC-Fc, EC-Fc E > A, MP-Fc, and ADAM10. Cleavage products generated by incubation of different ADAM28 Fc fusion proteins or ADAM10 with bovine MBP were separated by SDS–PAGE and stained with Coomassie brilliant blue (A).

An unresolved question regarding the catalytic activity of different ADAMs is whether these enzymes have similar or distinct substrate specificities and cleavage site preferences. ADAM9 has previously been shown to have a different in vitro cleavage specificity for peptide substrates compared to ADAM17/TNFα convertase [12]. On the other hand, TNFα convertase and ADAM10 cleave certain substrates at the same site, such as peptides mimicking the cleavage site of TNFα [6,8,9] and APP [7,25]. To address this issue, we compared the MBP cleavage products produced by ADAM28 to those produced by ADAM10 (Fig. 2). Different-sized cleavage products are generated when MBP is incubated with ADAM28 versus ADAM10. Microsequencing of ADAM28 and ADAM10 cleavage products generated in a separate experiment nevertheless revealed two cleavage sites that are used by ADAM10 and ADAM28 (SLP_{72}/QKA and ASA_{17}/STM, see Fig. 3). In addition, one cleavage site was only observed after cleavage with ADAM28 (KGR_{107}/GLS), and another only after cleavage with ADAM10 (HAA_{63}/RTT). These findings suggest that the cleavage site specificity of ADAM28 in vitro is similar, but not identical to ADAM10.

In an attempt to identify other substrates of ADAM28, wild-type and mutant forms of recombinant human ADAM28 were assessed for their ability to cleave a range of synthetic peptide substrates. No cleavage by wild-type or mutant ADAM28 was detected using a general matrix metalloproteinase-like substrate (MCA-Pro-Leu-Gly-Dap(Dnp)-Ala-Arg-NH_2), a collagenase-like substrate (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2), a stromelysin-like substrate (MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2) or a TNFα convertase-like substrate (4′,5′-dimethoxyfluoresceinyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Cys (4-(3-succinimid-1-yl)) fluorescein-NH_2) despite the standards being active in these assays at concentrations of <2 ng/well (data not shown). The results with the TNFα convertase-like substrate further support the observation that ADAM28 has a different cleavage site specificity than ADAM10 and ADAM17/TNFα convertase.

ADAM28 is more closely related to soluble snake venom metalloproteases than other ADAMs with a catalytic site. Since snake venom metalloproteases are able to cleave a variety of extracellular matrix proteins, we tested whether ADAM28 EC-Fc can cleave collagen types I, II, IV, and V, laminin, or fibronectin. However, under conditions where cleavage of MBP was readily observed, we did not detect any cleavage of the matrix proteins tested here (data not shown).

To further characterize the catalytic activity of ADAM28, we assessed the effects of known inhibitors of metalloproteases on MBP cleavage using ADAM28 EC-Fc (Fig. 4). Identical results were obtained in a similar experiment using ADAM28 MP-Fc instead of EC-Fc (data not shown). As expected for a Zn^{2+}-dependent metalloprotease, ADAM28 cleavage of MBP can be inhibited by 5 mM EDTA, and by 1 mM of the Zn^{2+} chelator 1:10 phenanthroline. TIMPs have emerged as very useful tools for the characterization of metalloprotease activities in cells and in vitro [26–31]. Cleavage of MBP by ADAM28 was not inhibited by 76 nM TIMP1, and

ADAM28 EC-Fc, MP-Fc and ADAM 10 cleavage sites on MBP

EC-Fc	MP-Fc	ADAM10
QKAQGHR	QKAQGHR	QKAQGHR RTXHYGS
STMDHAR GLSLSRF	STMDHAR GLSLSRF	STMDHAR

Bovine Myelin Basic Protein Sequence

AAQKRPSQRSKYLASA/**STMDHAR**HGFLPRH
RDTGILDSLGRFFGSDRGAPKRGSGKDGHHA
A/**RTTHYGS**LP/**QKAQGHR**PQDENPVVHFFKN
IVTPRTPPPSQGKGR/**GLSLSRF**SWGAEGQK
PGFGYGGRASDYKSAHKGLKGHDAQGTLSKI
FKLGGRDSRSGSPMARR

Fig. 3. Amino-terminal sequence of the MBP cleavage products generated by ADAM28 or ADAM10. The cleavage sites for ADAM28 EC-Fc, ADAM28 MP-Fc, and ADAM10 are shown in the table (upper panel) and are highlighted in bold font in the complete sequence of bovine MBP (lower panel, GenBank accession number P02687).

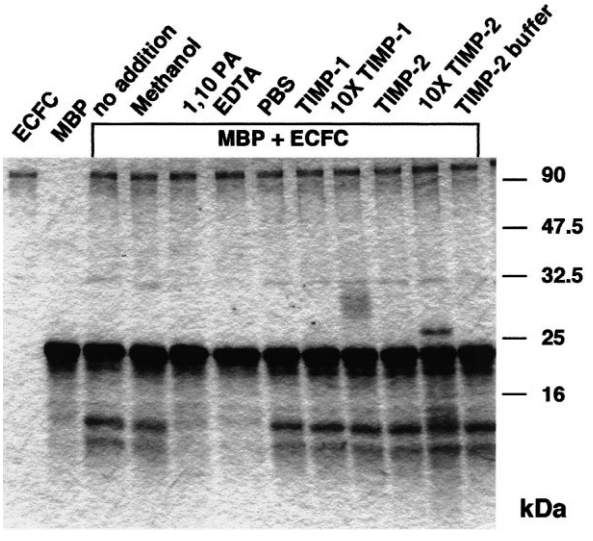

Fig. 4. Evaluation of the effect of inhibitors of metalloproteases on MBP cleavage by ADAM28 EC-Fc. ADAM28 EC-Fc and MBP were co-incubated in the presence of known inhibitors of metalloproteases, or in the appropriate control buffers. The first two lanes show either ADAM28 EC-Fc or MBP incubated under identical conditions.

by up to 178 nM TIMP2. Thus, ADAM28 is not inhibited by TIMPs 1 and 2 at concentrations which potently inhibit known matrix metalloproteases [32]. In this respect, ADAM28 resembles ADAM17/TNFα convertase and ADAM19 [14,28]. In contrast, ADAM10/KUZ is inhibited by TIMP1, but not by TIMP2 [27]. ADAM17/TNFα convertase, ADAM10/KUZ, and ADAM12 are also inhibited by TIMP3 [27,28,30]. In this study we were unable to obtain sufficient quantities of TIMP3 to test the sensitivity of ADAM28 to this inhibitor.

In a previous study, we found that ADAM28 pro-domain removal can be abolished by the $E_{343} > A$ mutation ([15], see also Fig. 1C). This suggests that ADAM28 pro-domain removal is, at least in part, autocatalytic. In contrast, the pro-domains of most catalytically active ADAMs are apparently removed by furin or a related pro-protein convertase [12,18,33,34]. In order to identify the sites of ADAM28 pro-domain cleavage, we subjected the EC-Fc and MP-Fc fusion proteins to N-terminal sequencing. This revealed four cleavage sites which were identical for both fusion proteins (see Fig. 5). One of the cleavage sites (VSA_{20}/IKE) is the predicted signal sequence cleavage site [15], whereas the other three are in the pro-domain of ADAM28. This suggests that ADAM28 pro-domain removal involves several steps. In comparison, the pro-domains of ADAM15 and ADAM17 are also cleaved at least three times after processing by a pro-protein convertase [18,35]. The pro-domains of all ADAMs with a catalytic site consensus sequence contain an additional cysteine residue that is thought to inhibit the protease via a cysteine switch mechanism (see for example [12,18,34]). Evidently the pro-domain of ADAM28 does not prevent the autocatalytic pro-domain cleavage, although it is not clear whether the pro-domain must be removed for ADAM28 to be able to cleave other proteins.

Taken together, these studies provide the first direct evidence for catalytic activity of ADAM28. The finding that ADAM28 is catalytically active in vitro further supports the notion that the catalytic activity of this molecule will be relevant for its function in vivo. The relatively high expression of ADAM28 in the epididymis and in lymphocytes suggests a role for this ADAM in sperm maturation and in lymphocyte function. Further studies, including a targeted deletion of ADAM28 in mice, will be required to learn more about the physiological role of this protein in reproduction, development, host defense, and in adult homeostasis.

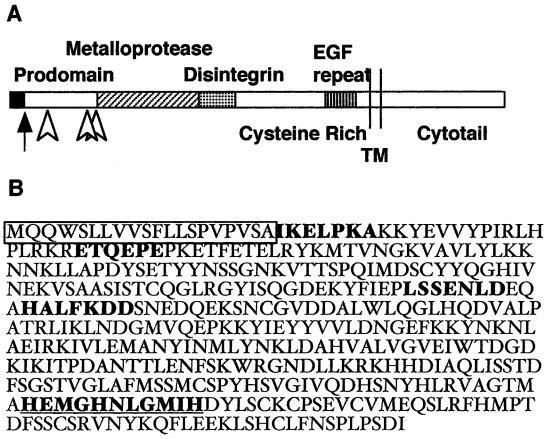

Fig. 5. Amino-terminal sequence determination of ADAM28 EC-Fc and MP-Fc fusion proteins. Panel A shows a diagram of ADAM28 indicating the approximate positions of four cleavage sites that were identified in both catalytically active Fc fusion proteins of ADAM28 (EC-Fc and MP-Fc). The first cleavage site corresponds to the predicted signal sequence cleavage site [15]. The remaining cleavages occur within the pro-domain of ADAM28. The amino acid sequences that were identified by N-terminal sequencing (see A) are highlighted in bold font in the sequence of ADAM28 in B (only the amino acid sequence of the pre-, pro-, and metalloprotease domains of ADAM28 is shown). The amino acid sequence of the catalytic site (HEGMHNLGMIH) is shown in underlined bold font.

Acknowledgements: We are grateful to Dr. G. Murphy for donating recombinant TIMP1 and TIMP2, and to Drs. H. Erdjument-Bromage and P. Tempst, and L. Lacomis for N-terminal sequence determination. This work was supported by the AstraZeneca Strategic Research Fund (as part of a grant to C.P.B.), by the Samuel and May Rudin Foundation and the DeWitt Wallace Fund, and by the Memorial Sloan-Kettering Cancer Center Support Grant NCI-P30-CA-08748.

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