Molecular cloning and expression analysis of a novel member of the Disintegrin and Metalloprotease-Domain (ADAM) family

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\textbf{Abstract}

We cloned and characterized the cDNA and the expression pattern of a novel member of the murine ‘Disintegrin and Metalloprotease-Domain Family’ (ADAM). The predicted protein sequence reveals highest homology to the testase-subgroup, composed of ADAM 24, ADAM 25 and ADAM 26 and is therefore called testase 4. Reverse transcription–polymerase chain reaction showed a strong expression of testase 4 in the adult testis, but not in any other organ or embryonic stage tested. Careful characterization by in situ hybridization confirmed specific expression of testase 4 in maturing sperm cells of 6-week-old mice, whereas no specific hybridization pattern was detectable in testes of 2.5-week-old mice. These data indicate a correlation between testase 4 expression and spermatogenesis and/or fertilization. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cell surface proteins, such as receptors and membrane-linked ligands are subject to consistent dynamic changes, which control their number and activity. Membrane anchored enzymes, receptors, ligands and adhesion molecules are targets of specific proteolytic modifications which determine the biological activity of these proteins. Typically, members of the ADAM (a disintegrin and metalloprotease domain) family play pivotal roles in mediating such specific adhesion or proteolytic processing events, often during development (Blobel, 1997; Schlondorff and Blobel, 1999). Important regulatory processes mediated by ADAMs include the shedding of extracellular domains of transmembrane proteins as well as the regulation of cell adhesion and fusion, e.g. during myogenesis (Yagami-Hiramas et al., 1995) or sperm-egg cell fusion (Myles et al., 1994). Besides in mammals, ADAMs have been identified in a wide range of organisms, including \textit{Drosophila}, \textit{Xenopus} and \textit{Caenorhabditis} (Rooke et al., 1996; Alfandari et al., 1997; Wen et al., 1997).

Structurally, ADAMs are transmembrane proteins whose extracellular domains bear strong homology with disintegrins and metalloproteases originally known as soluble components from snake venoms (Huang et al., 1987; Gould et al., 1990; Wolfsberg et al., 1995; McLane et al., 1998). Their conserved modular protein structure typically consists of a signal sequence at the N-terminus, followed by a pro-domain, a metalloprotease and disintegrin-domain and cysteine-rich region which is adjacent to a transmembrane and a cytoplasmic domain (Schlondorff and Blobel, 1999). ADAMs displaying proteolytic activity are characterized by a zinc-binding catalytic active consensus sequence (Bode et al., 1993; Rawlings and Barrett, 1995; Stocker et al., 1995). Well studied ADAMs bearing proteolytic activity, including Kuzbanian (ADAM10) and the TNF-\textalpha converting enzyme (ADAM17), are involved in regulation of Notch-receptor-dependent signaling and the shedding of tumor necrosis factor \textalpha, respectively (Black et al., 1997; Moss et al., 1997; Pan and Rubin, 1997; Qi et al., 1999). Although related by their overall domain structure, a considerable number of ADAMs can be distinguished by the absence of proteolytic activity and their precise functions need to be elucidated.

At least 15 ADAMs have been described being expressed
either predominantly or specifically in testicular cells. This includes the sperm surface proteins ADAM 1 and 2 (fertilin α and β) as well as ADAM 3 (cyritestin), which have been assigned to be important for mediating integrin dependent adhesion between sperm and egg membranes during fertilization (Primakoff et al., 1987; Blobel et al., 1992; Evans et al., 1997a,b; Yuan et al., 1997). Recently, another subgroup of testis-specific ADAMs, the so called testases 1–3 (ADAM 24–26) was identified (Zhu et al., 1999). At least for testase 1, which is present on mature sperm cells as a monomer, a role for the penetration of the zona pellucida or sperm-egg fusion was suggested (Zhu et al., 2001).

In a PCR-based screening for novel members of the ADAM family, we identified and cloned a novel mouse ADAM cDNA bearing closest homology with testase 3 (ADAM26) of the testate. Therefore we propose the name testase 4. Further PCR analysis revealed presence of testase 4 RNA in testes during puberty with highest levels in 7-week-old mice. In situ analysis confirmed the presence of testase 4-specific signals in stage I–VIII spermatids of 6-week-old mice but not in testes of 2.5-week-old animals. Therefore, we suppose a role of testase 4 during spermatogenesis or, possibly, for fertilization.

2. Materials and methods

2.1. Identification of ADAM cDNA fragments

To identify novel putative ADAM cDNA-sequences, degenerate oligonucleotides corresponding to highly conserved ADAM-domains we have employed a PCR-based cloning protocol. We have isolated total RNAs from various mouse tissues using the Trizol reagent (Life Technologies) according to the manufacturer’s instructions, followed by reverse transcription with Superscript II polymerase (Gibco BRL). For PCR analysis, we have employed the cDNAs together with the degenerate primers SK-1 (5’-GAG TCT GGC AGC GAT TA-3’) corresponding to the HELHTLG protein-sequence of the ADAM-Metalloprotease site) and SK-2 (5’-GWI CCR CAR TCR CAY TKY TC-3’ corresponding to the QCDCGT consensus-sequence within the disintegrin-domain: I indicates an inosine residue). An approximately 250 bp PCR-fragment encoding for an ADAM-related sequence (clone K12-II) was specifically amplified and sequenced. A mouse testis cDNA library was screened by the Resource Center/Primary Database (RZPD) service with K12-II as the probe. Sequencing of five positive clones revealed two clones containing a K12-II-related, partial sequence (clones DKFZp411I16127 Q2 and DKFZp411N17196Q2). Both clones contained a poly(A) tail.

2.2. RACE–PCR

To identify the missing 5’-sequence, we have employed a 5’-RACE strategy using the GeneRacer-Kit (Invitrogen, Carlsbad) with the specific primer 5’-ATG ATG AAT GCC ATA TCT GAT AAC GA-3’ on adult mouse testis RNAs (mouse strain C57BL/6). Separation of the PCR products on agarose gels revealed a strong specific band of approximately 1300 bp. Subcloning and sequencing of the RACE-product revealed a putative ATG start codon, a long open reading frame as well as an 100% identical overlap with the RZPD-clones.

2.3. RT–PCR

For detailed RT–PCR analysis, we used specific primers (5’-GAA CCA CCG GAC TGT CAA CT-3’ and 5’-TTT GAG TCT GGC AGC GAT TA-3’), binding within the 3’-untranslated region of testase 4. We utilized total RNAs derived from various adult C57BL/6 mouse tissues and embryonic stages as well as total RNAs from various cell lines. Control PCRs were performed using primers specific for the house-keeping gene hypoxanthine–guanine phosphoribosyl transferase (HPRT).

2.4. In situ hybridization

Testes were isolated from 2.5- and 6-week-old C57BL/6 mice, snap frozen in Tissue Tek (Sakura) and microtome sections were cut at 5–10 μm. In situ hybridizations were performed using 35S-labeled mRNA riboprobes as described previously (Sorokin et al., 1997). The sense and antisense probes correspond to nucleotides 1914–2542 of testase 4, cloned into the pCR II-TOPO vector (Invitrogen). Tissue sections were exposed for 4 weeks to Kodak NTB-1 emulsion and counterstained with toluidine blue.

2.5. DNA sequencing and in silico analysis

All sequencing reactions were performed using the BigDye DNA sequencing kit (Applied Biosystems) and appropriate primers using an ABI 310 DNA sequencer (Perkin Elmer, Foster City). Sequence analysis was performed using the Chromas software program (Technelysium Ltd, Helensvale, Australia) and sequence comparisons were done using BLAST.

For transmembrane analysis, we employed the program TMHMM (Sonnhammer et al., 1998) to identify possible transmembrane domains of testase 4. Signal peptide analysis was performed using the program SignalP (Nielsen et al., 1997).

3. Results and discussion

3.1. cDNA cloning and sequence analysis of mouse testase 4

Members of the ADAM (A Disintegrin and Metalloprotease domain)-gene family encode for transmembrane proteins involved in the regulation of many biological processes such as cell adhesion, fertilization and myogenesis. At least some ADAMs are capable of processing recep-
Fig. 1. Nucleotide and predicted amino acid sequence of testase 4 (GenBank accession number AF373288). The first methionine indicates the potential start codon of the suggested longest open reading frame.
tors and membrane linked ligands and are thought to play an essential role in Notch- and TNF-α signaling (Schlondorff and Blobel, 1999; Primakoff and Myles, 2000). Using degenerate primers in a PCR-based strategy to identify novel members of the ADAM family, we cloned and identified a novel putative mouse ADAM cDNA-sequence, whose 2608 base pairs contain an open reading frame, encoding a predicted protein of 714 amino acids (Fig. 1). The protein sequence shows 49% identity with testase 1 (ADAM 24), 52% identity with testase 2 (ADAM 25) and 82% identity with testase 3 in mouse, respectively. Since the sequence (GenBank accession number AF373288) bears closest homology to testase 3 (ADAM 26) (Zhu et al., 1999), we propose the name testase 4 (Fig. 2).

3.2. Amino acid sequence and structure of testase 4

The testase 4 cDNA displays all important features characteristic for a catalytic active member of the ADAM family: A signal sequence is adjacent to a pro-domain and a metalloprotease domain containing the putative conserved zinc-dependent metalloprotease sequence HEMGHNLGMMHD corresponding to the consensus sequence HEXGHNLGXX HD described by Rawlings and Barrett (1995). The disintegrin domain is followed by the cysteine-rich domain, the EGF-like domain and by a short intracellular domain adjacent to the transmembrane sequence (Fig. 2). Interestingly, compared with ADAM 26 (testase 3), the putative testase 4 protein contains a longer signal peptide sequence with the start codon located 36 bp upstream of the translation start site of ADAM 26 (Figs. 2 and 3).

Possible proteolytic activity of the predicted testase 4 protein is reflected by comparison with ADAM 9, ADAM 10 and ADAM 17, proteins which act as sheddases for specific membrane-anchored proteins (Schlondorff and Blobel, 1999). In detail, ADAM 17 appears to be capable to cleave Notch receptors (Brou et al., 2000) as well as tumor necrosis factor α, or transforming growth factor α (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998). The minimal consensus sequence for a zinc-binding proteolytic site is the HEXXH motif found in active zinc peptidases of the metzincin superfamily (Jongeneel et al., 1989; Bode et al., 1993).
Although many ADAMs nevertheless do not contain proteolytic activity, the HEMGHNLMGMHD motif of testase 4, indicative for catalytic activity of zinc-dependent metalloproteases, resembles the HELGHNFGAEHD sequence of ADAM 17 and the HEIGHNLGMSHD motif of ADAM 24, both active metalloproteases (Rawlings and Barrett, 1995).

As is the case for ADAM 24, testase 4 does not contain a RX(K/R)R furin cleavage site between the pro-domain and the metalloprotease domain, which is found in most other ADAMs (Zhu et al., 2001). Furin-like convertases are responsible for cleaving the pro-domains of ADAMs which contain a furin cleavage motif. This happens typically during the intracellular transport to the cellular surface, in order to activate their metalloprotease activity. Nevertheless, since it has been shown for ADAM 24 that the removal of the pro-domain occurs at the cell surface and not intracellularly, we suggest a similar mechanism for the activation of testase 4. In this context, it has been speculated whether ADAM 24 modifies other sperm surface proteins during epididymal maturation or during fertilization (Zhu et al., 2001). This may be a possible function for testase 4 as well, perhaps with a different specificity in regard of the modified proteins.

Several ADAMs expressed on the cellular surface of sperm cells are capable to mediate adhesion with mouse eggs. Various experiments suggest that ADAM 2, expressed on the cellular surface of mouse sperm, may be a major binding molecule of α6β1 integrin present on the egg (Almeida et al., 1995; Chen and Sampson, 1999; Chen et al., 1999). The notion that ADAMs expressed in the testis may be important for fertilization has been studied in detail for ADAMs 1–3. Instantly, male ADAM 2 or 3 knock-out mice are infertile due to defective sperm cells incapable to bind to the zona-pellucida (Cho et al., 1998, 2000; Shamsa-din et al., 1999). As a putative transmembrane protein with a
RGD consensus sequence, testase 4 has the potential to mediate integrin dependent cell adhesion via this motif. Nevertheless, of the three testases cloned before, only ADAM 24 contains an RGD-motif, but not the closer testase 4 homologs ADAM 26 or ADAM 25 which contain a QDE-motif instead. Regarding the various testases, this opens the possibility of their binding either to different integrins or to different integrin domains.

3.3. RT–PCR

Using total RNA from embryonic stages and various adult murine tissues, RT–PCR analysis with testase 4-specific primers revealed a significant and specific amplification product only in testis (Fig. 4A). More thorough analysis, where we used total testis RNAs from different time points after birth, confirmed this finding. We identified low levels
of the testase 4 PCR-product in all tested stages except of testes derived from 7-week-old mice (Fig. 4B) where high testase 4 levels were present.

Testase 4 does not seem to participate in myoblast differentiation or fusion, since no RT-PCR-signals above background could be detected using RNAs from differentiating C2C12 cells. Neither is the testase 4 gene expressed in a variety of tested primary cells or lines from the mouse B- or T-cell lineage representing different developmental stages of maturation (data not shown).

3.4 In situ analysis
When we used specific antisense probes from the 3′-end of testase 4 for in situ hybridization analysis, we found significant amounts of testase 4 mRNA within the seminiferous tubules of 6-week-old mice (Fig. 5D–I), whereas no specific signals were observable in testis of 2.5-week-old mice (Fig. 5A–C). The expression was limited to areas of round stage I–VIII spermatids (Russell et al., 1990) and no expression was detectable in areas of seminiferous tubules where spermatogonia and spermatocytes can be found. Applying the corresponding sense probe in control experiments, we were unable to observe significant staining (Fig. 5J–L). These results show a correlation of testase 4 expression with the maturation of spermatids in the adult testis.

Most mammalian ADAMs show a wide tissue distribution, but at least 12 members of the family can be assigned to an expression predominantly in the testis, including ADAM 1–3 and ADAM 24–26. The distribution of testase 4 mRNAs within the seminiferous tubules, as shown by the in situ analysis (Fig. 5), may indicate a role for the development of early spermatids during stages I–VIII. Nevertheless, as was described in the case of the acrosomal protein cyritestin (ADAM 3), translation of testase 4 mRNA in the testis may be initiated only several days after the first appearance of the RNA (Linder et al., 1995). Therefore, a role for the testase 4 protein for later sperm cell function, rather than for germ cell maturation within the seminiferous tubules, is very well conceivable. This possible function could include a participation in integrin-dependent adhesion of the sperm cell with the egg or, in case proteolytic activity retains, a role in penetrating the zona pellucida during fertilization.

It is obvious that sperm cells express various ADAMs on their surface which potentially can interact with several integrins on the egg. In future, careful binding studies and knock-out experiments will help to reveal possible binding partners of testase 4 and elucidate its function for sperm development and/or fertilization.

3.5 Conclusions
1. We have cloned and sequenced the cDNA for mouse testase 4, a novel ADAM family member. The cDNA consists of 2608 bp and the predicted open reading frame encodes for 714 amino acids.
2. Expression of testase 4 mRNA was found only in mouse testis where it could be localized to around stage I–VIII spermatids.

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