Human tMDC III: a sperm protein with a potential role in oocyte recognition

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A number of MDC (metalloproteinase-like, disintegrin-like, cysteine-rich) proteins are exclusively or abundantly expressed on mammalian sperm from a range of species, with data from rodents supporting a role for some of these in oolemma binding. However, in the human, transcripts for three of the most likely candidates have been shown to contain deletions and in-frame termination codons, rendering them non-functional. In this paper we have addressed the expression, in humans, of an additional MDC protein, tMDC III (also known as ADAM 18), previously shown to be exclusively expressed on sperm from rodents and macaques. Using a PCR-based approach, we have determined the complete nucleotide sequence of human tMDC III cDNA which, unlike the three non-functional genes, contains an uninterrupted open reading frame encoding a full-length MDC protein. Furthermore, polyclonal antisera raised against human recombinant tMDC III demonstrated the presence of mature protein on human sperm. In common with orthologues from other species, human tMDC III contains a putative integrin-binding glu-cys-asp (ECD) motif, the relevance of which is discussed in the context of a possible role in oolemma binding.

Key words: disintegrin/fertilization/integrin/MDC protein/sperm–oocyte binding

Introduction

The molecular aspects of mammalian sperm–oocyte interactions at fertilization are poorly defined; indeed, the gamete ligands involved have yet to be conclusively identified. Although a number of sperm proteins have been implicated in gamete adhesion events (Frayne and Hall, 1999), much of the attention over the past decade has focused on a family of multidomain, integral membrane proteins called the MDC (metalloproteinase-like, disintegrin-like, cysteine-rich) or ADAM (a disintegrin and metallopeptide) family (see Table I). Many of these proteins are abundantly or exclusively expressed in the testis of species as diverse as Xenopus (Shilling et al., 1997), rodents (Wolfsberg et al., 1995; Frayne et al., 1997), primates (Frayne et al., 1998) and humans (Burkin et al., 1997).

As the name suggests, MDC proteins contain a number of distinct domains (pro-domain, metalloproteinase-like domain, disintegrin-like domain and cysteine-rich domain) reminiscent of their snake venom counterparts. Indeed it was by analogy to the integrin-binding activity of these venom proteins which first led to the disintegrin-like domain of cyritestin (tMDC I; ADAM 3) (Linder and Heinlein, 1997; Yuan et al., 1997) ‘knockout’ mice, whilst not precluding a role for these proteins in sperm–oolemma interactions, suggest that neither protein is essential. In both cases binding was reduced but not completely inhibited, implying that other sperm proteins may be involved in this process. Such a conclusion is supported by our recent data which show that, at least in vitro, MDC proteins other than fertilin β and cyritestin may be equally involved in sperm–oolemma binding (McLaughlin et al., 2001), and is in keeping with our proposed model of MDC functional redundancy and co-operativity (Hall and Frayne, 1999).

A number of additional MDC proteins have previously been reported to be abundantly expressed on sperm. One of these, fertilin α (ADAM 1) (Blobel et al., 1992; Perry et al., 1995; Wolfsberg et al., 1995; Hardy and Holland, 1996; Frayne et al., 1997; Waters and White, 1997), has been the subject of a number of in-vitro studies, some of which propose a role for this protein in sperm–oocyte binding and indicate that regions of this protein, in addition to the disintegrin-like domain, may participate in cell adhesion (Evans et al., 1997b; Yuan et al., 1997; Wong et al., 2001).

Two additional, less well characterized MDC proteins, tMDC II (ADAM 5) and tMDC III (ADAM 18), are exclusively expressed by spermatogenic cells in the rat and macaque (Frayne et al., 1997, 1998). Each contains a conserved ECD tripeptide in its disintegrin-like domain; a motif which is also conserved in all species orthologues of fertilin β and has been specifically implicated in integrin binding of both fertilin β and, interestingly, a number of snake venom disintegrins (Shimokawa et al., 1997). In the rat testis, both tMDC

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II and tMDC III are developmentally-regulated, with RNA transcripts first appearing in post-meiotic germ cells (Frayne et al., 1997). This temporal pattern of expression is further supported by our studies in the macaque, where protein was first detected on developing spermatids, both by immunofluorescence and on Western blots of testicular and epididymal sperm (Frayne et al., 1998). Furthermore, we have also demonstrated that macaque tMDC II and MDC III (as well as fertilin α, fertilin β and tMDC I) are proteolytically processed during epididymal transit to yield mature proteins on cauda epididymal sperm, which have lost both the pro- and metalloproteinase-like domains but have retained the disintegrin-like domain, emphasizing the importance of the latter for sperm function (Frayne et al., 1998).

As MDC proteins have been strongly implicated in fertilization in animal model systems, we have recently addressed their expression in the human. Rather unexpectedly, we found that all transcripts for human fertilin α (Jury et al., 1997), tMDC I (Frayne and Hall, 1998) and tMDC II (Frayne et al., 1999) contained a variety of deletions, insertions and in-frame termination codons, rendering them non-functional. To further corroborate this finding, we used antisera raised against regions conserved between the macaque and human orthologues. Whilst each antisera readily detected a specific protein of the expected size on Western blots of macaque sperm, no equivalent protein bands were detected in any of the human sperm samples tested, confirming the absence of these proteins in the human. Our finding that human tMDC I is non-functional has been further confirmed by Grzmił and colleagues (Grzmił et al., 2001).

Hence, of the MDC proteins implicated in sperm–oocyte binding in rodents, humans are lacking fertilin α, tMDC I and tMDC II. In contrast, transcripts for fertilin β in the human are functional (Burkin et al., 1997) and fertilin β protein has been demonstrated on human sperm (Frayne et al., 1999). Nevertheless, although apparently involved in fertilization, the fertilin β knockout data suggest it is not essential.

In light of the above, we wanted to characterize the human orthologue of tMDC III, the remaining MDC protein which we have shown to be exclusively expressed on macaque sperm, with a view to determining its possible involvement in human fertilization. Here we show that human tMDC III transcripts are indeed functional and that tMDC III protein is expressed on human sperm. Furthermore, we argue that tMDC III shares much in common with fertilin β, suggesting a related function.

### Materials and methods

#### RT–PCR

A pool of human testis total RNA was obtained from Clontech Labs (Palo Alto, CA, USA). Total RNA (5 µg) was then used as a template for oligo(dT)12-18-primed Expand™ (Roche, Lewes, East Sussex, UK) reverse transcriptase-directed cDNA synthesis using buffer and conditions supplied by the manufacturer. Subsequent PCR amplification of tMDC III sequences (1 min at 94°C, 2 min at 58°C, 1 min at 72°C; 30 cycles) used 10% of this cDNA and Expand High Fidelity PCR system (Roche). The resulting PCR products were resolved on low melting temperature agarose gels and appropriate bands were excised, purified and directly sequenced on both stands using an ABI 377 automated DNA sequencer.

For tissue distribution studies, PCR reactions were carried out using tMDC III-specific primers (5′-GACTAAATGCGCTCAGAAGCT-3′ and 5′-AAAGCACATTGTGCTTTCAGTTA-3′) and the same conditions as described above with cDNAs from a panel of human tissues (brain, heart, kidney, liver, lung, pancreas, placenta, skeletal muscle) obtained from Clontech Laboratories. Control reactions were performed on all cDNA preparations using GAPDH-specific primers (5′-TGAAGTCCGGAGTAACCGATTG-3′ and 5′-CATGTGCGGCATAGGTCTCACAC-3′) to check the integrity and concentration of each cDNA.

### Expression of recombinant human tMDC III and generation of specific antisera

Primers (5′-GAACTCCATAGAATGTGAAGCT-3′ and 5′-GTGGTATGGTGCTTCAGTTAAGCT-3′) were designed to amplify the disintegrin plus cysteine-rich domain of human tMDC III (bases 1208 to 2064) for in-frame insertion within the N-terminal thioredoxin leader and C-terminal V5 and polyhistidine region of the pBAD/TOPO Thiofusion expression vector (Invitrogen, Groningen, The Netherlands). Both primers were used in PCR reactions with human testis cDNA as template and the resulting PCR products were resolved on low melting point agarose gels, excised, gel purified and ligated into the pBAD/TOPO Thiofusion vector using buffer and conditions supplied by the manufacturer, prior to transformation into E. coli TOP10 cells (Invitrogen). Plasmids were purified from the resulting recombinant clones using a Qiagen plasmid mini-preparation kit (Qiagen Ltd, West Sussex, UK) and inserts were completely sequenced on both DNA strands.

Following induction of positive clones in the presence of 0.02% arabinose for 4 h at 37°C, cells were lysed and the total soluble protein fraction was retained following centrifugation. Affinity purification of recombinant protein was achieved by passage over a nickel-charged agarose gel (ProBond; Invitrogen) and elution with 350 mmol/l imidazole.

Specific polyclonal antisera were raised to the purified recombinant protein in two rabbits by Charles River UK Ltd, Margate, Kent, UK.

### Western blot analysis of human tMDC III

Plasma membrane-enriched fractions of human ejaculated sperm were prepared by detergent extraction with 1% (w/v) Triton X-100 (Sigma Chemical Co. Ltd, Poole, UK) and vortex mixing (Jones, 1986). A total of 1 mmol/l 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF; Sigma) was included in the preparation. Proteins were separated by electrophoresis under reducing conditions on 12% (w/v) polyacrylamide gels containing sodium dodecyl sulphate, then electrobotted onto polyvinylidene difluoride membranes (PolyScreen; NEN Life Science Products, Brussels, Belgium). Blots were probed as previously described (Frayne et al., 1998) with preimmune sera and antisera raised against the recombinant human tMDC III protein.

### Results

#### Sequence analysis of human tMDC III cDNA

The entire coding region of human tMDC III was obtained as a series of three overlapping PCR fragments (Figure 1). Initially we tested a range of primers that had previously been designed for sequencing the entire coding region of macaque tMDC III. Using human testis cDNA as the template, two of these primers (P1 and P2: 5′-ACCTCAGATATGTGCTTCTG-3′ and 5′-TACCAAGTCAAC-TTTCCA-3′; see Figure 1) gave a PCR product of 904 bp. DNA sequence analysis of this PCR product and comparison with the macaque tMDC III sequence (Frayne et al., 1998) showed that this fragment comprised the middle region of the tMDC III transcript.

To obtain the 3′ end of the human tMDC III transcript, a forward primer (P3: 5′-GACTAAATGCGCTCAGAAGCT-3′) was designed to the 3′ end of the above middle fragment and a reverse primer (P4) was based on the 3′ non-coding region of the macaque tMDC III sequence (5′-AAAGCACATTGTGCTTTCAGTTA-3′). These primers generated a PCR product of 1152 bp.
Finally, the 5' end of the human tMDC III transcript was obtained using a reverse primer (P5) designed to the 5' end of the middle fragment (5'-CTTGCTGAAGATTACTTG-3') and a forward primer (P6) designed to the 5' non-coding region of the macaque sequence (5'-CTTGTTGAGCCATG-3'). These primers generated a PCR product of 417 bp.

Compilation of the sequence data from these three overlapping PCR products provided the entire 739 aa residue coding region of human tMDC III (Figure 2). When this contig was used to search a human expressed sequence tag (EST) database and the human genome database [National Centre for Biotechnology Information (NCBI), National Library of Medicine, National Institute of Health, USA], additional sequence information corresponding to the 5' non-coding region (ESTs BI464271 and BG722982; 35 nucleotides) and 3' non-coding region (EST AI206708 and human genome contig NT_008045.2; 90 nucleotides) was obtained. This additional sequence information is included in the final sequence (Figure 2).

In common with most other MDC proteins, the complete human tMDC III cDNA sequence encodes a signal peptide, pro-domain, metalloproteinase-like domain, disintegrin-like domain, cysteine-rich domain, transmembrane domain and cytoplasmic tail (see Figure 1). The coding region shares 94.2% sequence identity with macaque tMDC III, confirming its identity as the human tMDC III orthologue. Human, macaque, rat and mouse tMDC III all contain an ECD tripeptide motif within their putative integrin-binding disintegrin-like domain (Figure 3). Interestingly, ECD is also conserved in all species orthologues of fertilin β and is the most common XCD tripeptide (where X is one of a limited, but as yet unde

**Tissue distribution of human tMDC III transcripts**

In the rat, transcripts for tMDC III are abundantly and exclusively expressed by spermatogenic cells, as shown by analysis of a wide variety of tissues, and testes from a range of prepubertal animals (Frayne et al., 1997). Transcripts were not detectable in somatic cells, even using a highly sensitive RT–PCR approach. In the macaque, tMDC III transcripts are similarly abundantly expressed in the testis (Frayne et al., 1998), although a very low level of transcript was detected in a small number of additional tissues (skeletal muscle, uterus and ovary), the physiological significance of which is question-

### Figure 1. Cloning and sequencing strategy for human tMDC III cDNA. The coding region of human tMDC III cDNA was amplified as a series of overlapping PCR fragments, using primers P1–P6, as described in the text. 5' and 3' non-coding regions were obtained from EST and genomic clones. The domain organization of the tMDC III coding region is also indicated.

**Sequence and expression of human tMDC III**

**Identification of tMDC III on human sperm using specific antisera**

Western blot analyses were performed on ejaculated human sperm protein extracts using two human tMDC III-specific polyclonal antisera raised to the disintegrin plus cysteine-rich domains of recombinant human tMDC. Both antisera detected a protein on mature sperm (Figure 5) of a size compatible with a processed mature product which had lost its pro- and metalloproteinase-like domains as a result of epididymal processing. Such processing has previously been demonstrated for a number of other MDC proteins (e.g. fertilin α, fertilin β, tMDC I and tMDC II) in a range of different species, thereby focusing attention on the function of the retained disintegrin-like domain in each of these mature proteins. No protein bands were detected on parallel blots probed with preimmune sera from the same rabbits.

Having demonstrated an association of tMDC III with human sperm, attempts were made to localize it by indirect immunofluorescence of live or fixed sperm. Despite using a variety of experimental conditions, both anti-tMDC III antisera failed to specifically label either intact or permeabilized sperm cells, even though the specificity and efficacy of these antisera had been demonstrated in Western blotting applications. This is not an unusual problem; there are many documented examples of antisera which are perfectly usable for Western blotting, but fail to work in immunolocalization studies. However, we have found that this is particularly prevalent with antisera raised against recombinant MDC proteins (Frayne et al., 1998). We believe that this may be attributed to their extremely cysteine-rich nature, the specific disulphide bond configuration of which is undoubtedly important in the protein’s native conformation. Since bacterially-expressed MDC proteins will have a large proportion of either free cysteine residues or inappropriate disulphide bonds (depending on isolation buffer conditions), and further incorrect disulphide bond formation could occur following immunisation of rabbits, the resulting
Figure 2. Nucleotide sequence of human tMDC III cDNA. The complete nucleotide sequence (accession no.: AJ133004), derived from a series of overlapping PCR fragments, is shown together with the deduced amino acid sequence. The metalloproteinase-like, disintegrin-like and transmembrane domains are indicated by horizontal bars and the putative integrin-binding ECD tripeptide motif is boxed.

Discussion

Of those MDC proteins shown to be expressed on mammalian sperm (see Table I), only the genes for fertilin β, and now tMDC III, have been found to be functional in the human.

Interestingly, interrogation of the human genome database indicates that these two functional, testis-specific, MDC proteins are located immediately adjacent to each other on human chromosome 8. It is therefore highly likely that one has arisen by gene duplication of the other; consistent with a closely related role in sperm–oocyte binding.

In further support of a related function, fertilin β and tMDC III each contain an ECD tripeptide motif in the putative integrin-binding disintegrin loop; a feature which is totally conserved between all
**Sequence and expression of human tMDC III**

**Figure 3.** Sequence alignment of the putative integrin-binding loops of an RGD- and an ECD-containing snake venom disintegrin (A); and the disintegrin-like domains of four tMDC III orthologues (B), testis-abundant MDC proteins (C), epididymis-abundant MDC proteins (D) and non-reproductive MDC proteins (E). All sequences were obtained from protein sequence databases. Highly conserved residues, including the totally-conserved cysteine residues are boxed in grey, and the XCD tripeptide motif is indicated in inverse text.

**Figure 4.** Tissue distribution of human tMDC III transcripts. Human tMDC III transcripts were detected by RT–PCR as described in Materials and methods using cDNAs for a range of human tissues as templates, with primers flanking a 1152 bp region of the transcript (A). Parallel PCR reactions (B) using GAPDH primers were carried out to ensure cDNA integrity and equivalence of concentration in each sample.

**Figure 5.** Western blot analysis of human tMDC III. Proteins extracted from plasma membrane-enriched fractions of human ejaculated sperm were separated by polyacrylamide gel electrophoresis under reducing conditions, blotted and probed with two different polyclonal antisera (1 and 2) raised against recombinant human tMDC III disintegrin plus cysteine-rich domain (see Materials and methods), as well as the corresponding preimmune sera.

Species orthologues. Significantly, the same tripeptide is also found in a number of snake venom disintegrin domains which inhibit platelet aggregation by binding to an integrin (Jia et al., 1997). This has led a number of investigators to examine the ability of ECD-containing peptide mimics to inhibit sperm–oocyte interactions in vitro; an approach which has provided the greatest body of evidence in support of a role for fertilin β in oolemma binding (Almeida et al., 1995; Evans et al., 1995; Linder and Heinlein, 1997; Pyluck et al., 1997; Yuan et al., 1997). It is therefore of significant interest that tMDC III also contains an ECD tripeptide motif, conserved throughout all of its orthologues, suggesting that it may have similar integrin-mediated, oolemma-binding properties to fertilin β.

Although fertilin β and tMDC III are the only MDC proteins which have been shown to be expressed on human sperm, transcripts for...
four additional, testis-specific, human MDC proteins have recently been reported (ADAM 20, 21, 29 and 30) (Van Huijsduijnen, 1998; Cerretti et al., 1999). Whilst not yet localized to sperm, each of these deduced proteins contains an ECD tripeptide motif like fertilin β and tMDC III. However ADAM 20 and 30 have potentially active metalloproteinase domains, suggesting that they may play an alternative role in spermatogenesis or fertilization.

In conclusion, fertilin β and tMDC III share much in common. In the human, their genes are immediately adjacent to each other on chromosome 8, suggesting duplication from a common gene. Both proteins have the same tissue specificity, show developmentally-regulated expression, are present on sperm and contain an identical putative integrin-binding motif (ECD). It is therefore tempting to suggest that tMDC III is an attractive oocyte-binding candidate, particularly in the human where three of the alternative MDC genes (tMDC I/cyritestin, fertilin α, tMDC II) are non-functional. Whilst definitive evidence for such a role must clearly await further experimentation, this will be difficult to establish in animal model systems where the presence of tMDC I, fertilin α and tMDC II may compromise the ability of tMDC III to display its full effect.

References

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