Cloning and analysis of monkey fertilin reveals novel α subunit isoforms

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The fertilin complex, a heterodimeric surface membrane glycoprotein found on the head region of mammalian spermatozoa, has been reported to mediate membrane fusion with the egg plasma membrane during fertilization. We have employed PCR to generate products corresponding to monkey (*Macaca fascicularis*) fertilins from testis cDNA. These PCR products have been used to isolate full-length fertilin cDNA clones corresponding to both α and β subunits. Both monkey fertilin α and β cDNAs encode proteins which belong to an expanding family of metalloproteinase-like, disintegrin-like, cysteine-rich, mam-

malian proteins. Surprisingly, cDNAs for two fertilin α isoforms, α I and α II, were isolated, encoding proteins of 905 and 825 residues respectively. The predicted fertilin α isoforms share extensive identity, but differ significantly towards their N- and C-termini, suggesting the possibility of more than one type of fertilin complex in primates. Alignment of monkey α and β fertilins with their respective guinea-pig counterparts suggests a high level of overall structural conservation throughout, whilst providing new insights into the domain function of each.

INTRODUCTION

Fertilization in mammals involves several distinct sperm surface components which mediate sperm–egg recognition and fusion [1–3]. In addition to those components responsible for preliminary interactions between spermatozoa and the zona pellucida (an external oocytic glycoprotein matrix), at least one surface antigen, the fertilin complex (formerly known as PH30), is reported to be involved in recognition of the oolema plasma membrane and possibly also in sperm–oolema membrane fusion [4]. The fertilin complex is a heterodimeric glycoprotein, comprising α and β subunits that are synthesized in the testis as larger precursors and one of which (the β subunit) undergoes final epididymal processing coincidental with the acquisition by spermatozoa of full fertilizing ability [5].

Recently, full-length cDNA sequences corresponding to the precursors of both guinea-pig fertilin subunits have been determined and their respective deduced protein products shown to be similar not only to each other, but also to a series of snake venom polypeptides incorporating metalloproteinase (type IV collagenase) and/or disintegrin activities [6,7]. It is not yet known whether the metalloproteinase-like domains of fertilin precursors possess proteinase activity, and they are processed away in the mature fertilin complex (although this does not preclude a role in the immature complex). However, the disintegrin domain is present on the β subunit of fertilin on spermatozoa that have undergone the full extent of their epididymal processing [6]. By analogy to the venom disintegrins to which it is similar, this β fertilin domain may interact with one or more $\alpha_{\text{IIb}}\beta_3$ -like integrin receptor moieties on the plasma membrane of a recipient egg [6,8]. Indeed, not only does the egg possess at least one type of integrin [9] but, like integrin function, fertilization is Ca^{2+} -dependent. Moreover, short peptides mapping to the disintegrin region of the fertilin β subunit also inhibit sperm–egg fusion, possibly by competing with the fertilin complex for its integrin receptor on the egg surface [10].

It is now apparent that fertilins α and β are members of a

metalloproteinase-like, disintegrin-like, cysteine-rich (MDC), family [11–13]. Of these, at least two (tMDC I and tMDC II) are expressed in the mammalian testis and are predicted to be type I integral membrane proteins with primary structures akin to those of the fertilin subunits. An additional MDC family member, epididymal apical protein I (EAP I) is synthesized exclusively in the caput epididymis and is located along the apical membrane of epididymal epithelial cells where, unlike either fertilin subunit, it exists in at least one form that does not appear to be substantially processed [14].

Whereas fertilins have hitherto been studied using a guinea-pig model, EAP I, tMDC I and tMDC II have been characterized from the monkey *Macaca fascicularis* [12–14]. Elucidating the relationship, both functional and evolutionary, between the fertilin subunits and other male reproductive tract MDC family members is therefore complicated by species differences. We have addressed this problem by cloning *M. fascicularis* fertilins α and β and analysing their corresponding transcripts in the male reproductive tract. These data confirm that both fertilin subunits are distinct from EAP I and provide strong evidence for the existence of at least one additional fertilin α species in primates. We compare monkey and guinea-pig homologues and discuss the functional implications of their alignments.

MATERIALS AND METHODS

Materials

All chemicals and reagents were of AnalaR grade or higher. *Taq* DNA polymerase was supplied by Boehringer Mannheim, Lewes, E. Sussex, U.K. Other DNA-modifying enzymes, oligo(dT)-primed cDNA synthesis kit, oligo(dT)_{12–18} and dephosphorylated, *Sma*I-cut pUC18 were from Pharmacia, Milton Keynes, U.K. Bacteriological media were from Difco, Detroit, MI, U.S.A. Nitrocellulose membranes for library screening and Hybond-N for Northern blot analysis were respectively from Schleicher and Schüll and Amersham International (Amersham, Bucks., U.K.). Microdialysis membranes (0.025 μm pore diameter) were from

Abbreviations used: MDC, metalloproteinase-like, disintegrin-like, cysteine-rich; EAP I, epididymal apical protein I.

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The nucleotide sequence data reported in this paper appear in the EMBL Nucleotide Sequence Database under the accession numbers: X79808 (fertilin α I), X79809 (fertilin α II) and X77653 (fertilin β).

Millipore. Radiochemicals ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$; each at 3000 Ci.mmol⁻¹) and random 14-mer DNA labelling kit were provided by Du Pont-NEN, Stevenage, U.K. Oligo(dT)-cellulose (Collaborative Biomedical Products) was used for the enrichment of polyadenylated RNA.

A sexually mature monkey (*Macaca fascicularis*) or guinea-pig (*Cavia cobaya*) was killed and fresh tissue was collected immediately, flash frozen in liquid N₂ and stored at -70 °C until required for RNA extraction.

Isolation of RNA

Total RNA was extracted from fresh samples (approx. 2 g) using an SDS/proteinase K-based method. Polyadenylated RNA was affinity purified from approx. 10 mg of *M. fascicularis* total testis RNA on an oligo(dT)-cellulose column, essentially as described elsewhere [15]. Ribosomal-RNA-enriched flow-through was retained for use as size markers in Northern blotting. Initial estimates of RNA concentration were obtained spectrophotometrically at 260 nm.

Reverse transcriptase PCR amplification of fertilin and EAP I sequences

The sequences of rat and monkey EAP I are similar [14], permitting the design of two redundant oligonucleotide primers for amplification of part of the guinea-pig EAP I homologue. These primers were 5'GAAGCCAGGATTYACTTGTGY-AGAAG3' and 5'CATYTGASATCTTCTCYTCACAGGG3'. The design of primers for the amplification of monkey fertilin sequences was more critical, since guinea-pig α and β subunits exhibit only limited sequence identity and no fertilin species homologues had been described [6,14]. However, we reasoned that fertilins α and β are distantly related (since each possesses a disintegrin domain, albeit processed away in the mature α subunit [6]) and that regions conserved between them might also be conserved in homologues from other species. Nucleotide sequences coding for mature guinea-pig fertilins α and β were therefore aligned optimally and two stretches of maximal sequence conservation chosen for the design of PCR primers, with particular reference to the sequence of fertilin α . The primers were 5'GGGKRATATWTTTGAAACTGTGG3' (corresponding to nt 183–206 of Figure 1 in ref. [6]) and 5'TGGCAGTGCCTTWGTTGTTGC3' (corresponding to nt 542–521 of Figure 1 in ref. [6]). Oligo(dT)-primed cDNA, synthesized using either *M. fascicularis* testis polyadenylated RNA (1 μg) or guinea-pig total RNA (2 μg), was then used to programme a PCR reaction containing the appropriate primers and employing the following parameters: 94 °C, 90 s; cool to 58 °C over 90 s; keep at 58 °C, 2 min; 72 °C, 2 min; 30 cycles. Products were cloned into pUC18 and their sequences determined using a Du Pont Genesis 2000 automated sequencer.

Recombinant methods and construction and storage of *M. fascicularis* testis cDNA library

Unless indicated, recombinant methods were essentially as described previously [16]. Construction of a monkey cDNA library was as follows. *M. fascicularis* testis polyadenylated RNA (5 μg)

was used as template in a Pharmacia cDNA synthesis reaction according to the instructions of the vendor, with oligo(dT)_{12–18}-primed first-strand cDNA synthesis. Linker-adapted cDNA was then fractionated on a 3 ml Sephacryl S-1000 column and an aliquot of one early fraction ligated into *Eco*RI-digested, dephosphorylated pAT153 vector DNA [17]. The resultant ligation was then subjected to microdialysis, and half of it used to electroporate *Escherichia coli* XL1-Blue cells [18] at a transformation equivalent of 4.3×10^9 transformants per μg of supercoiled pUC19. After plating out and incubation for 12 h at 37 °C, approx. 2×10^8 independent recombinant transformants were harvested in 6 ml of 15% (v/v) glycerol broth, mixed gently and rapidly frozen in liquid N₂ in 100 μl aliquots. Library aliquots were stored at -70 °C and, when required, diluted to yield a predetermined optimum plating density (approx. 3×10^4 colonies per 22 cm $\times$ 22 cm plate).

Identification of monkey fertilin cDNA clones

For each screen, approx. 1.4×10^5 independent *M. fascicularis* cDNA clones were transferred to four nitrocellulose filters (22 cm $\times$ 22 cm) and screened for those whose cDNA inserts hybridized to monkey fertilin fragments that had been gel-purified and labelled to a high specific radioactivity with ³²P. Hybridization was at 60 °C for 40 h in hybridization solution containing 1 ng/ml heat-denatured probe. Hybridization solution contained 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7) and 0.5% (w/v) SDS. Filters were then washed at 60 °C for 20 min in 2 $\times$ SSC/0.5% SDS, followed by 20 min in 1 $\times$ SSC/0.5% SDS and 10 min in 0.2 $\times$ SSC/0.5% SDS, and then subjected to autoradiography with an intensifying screen at -70 °C. Hybridization was verified by secondary screening and clones were purified following tertiary screening under identical hybridization conditions. Plasmid DNAs from positive clones identified in this way were then isolated and the cDNA inserts of the largest were subjected to sequence analysis using a Du Pont Genesis 2000 automated sequencer. Sequences were determined on both strands using a custom primer walking strategy. Sequence (≥ 300 nt) of the ends of all clones were determined and shown to align with that of their respective full-length consensus. Sequences were compiled and aligned using the LASERGENE suite of programs (DNASTAR, West Ealing, London, U.K.) and subjected to database searches using the FASTA algorithm [19].

High stringency Northern blot analysis of RNA

Analysis of RNA was achieved by capillary transfer of RNA samples that had been fractionated on a 1.1% (w/v) agarose gel (containing formaldehyde as denaturant) on to Hybond-N filters [16]. Filters were prehybridized at 42 °C in bags for approx. 4 h in hybridization solution containing 50% (v/v) formamide. The bags were then drained and replenished with fresh hybridization solution containing 50% (v/v) formamide and ~2 ng/ml $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ -labelled probe, and hybridization was allowed to proceed for 16–20 h at 42 °C. Filters were then washed at 65 °C for 20 min in 2 $\times$ SSC/0.5% SDS, followed by 20 min in 1 $\times$ SSC/0.5% SDS and 10 min in 0.2 $\times$ SSC/0.5% SDS. Hybridization was detected following autoradiography at -70 °C with an intensifying screen.

All DNA probes were prepared by labelling recombinant

Figure 1 Nucleotide and deduced amino acid sequences of monkey fertilin α (top) and α II

Vertical bars mark positions identical in both sequences. The extended metalloproteinase consensus Zn²⁺ binding motif (residues 373–386 in α), putative integrin ligand, ECD (residues 505–507 in α) and potential membrane-spacing domains (residues 744–766 in α) are indicated with horizontal bars. The nucleotide sequence of fertilin α contains a putative 3' consensus polyadenylation/cleavage signal (nt 3664–3669).

Figure 1 For legend see opposite.

The putative integrin ligand, ECD (residues 451–453), and a hydrophobic potential transmembrane region (residues 689–709) are underlined.

fascicularis testis cDNA library was re-screened with pmT-Fertilin α I-S4 cDNA and a probe containing sequences unique to the 3' end of α I. This re-screen yielded five clones not previously isolated, four of whose sequences were perfectly superimposable on that of α I, and one on that of α II (results not shown), indicating that two, non-sibling, full-length representatives of α I and α II had been isolated. No non- α I/non- α II clones were isolated.

The sequences of fertilins α I and α II cDNAs encode hypothetical translation products of 905 and 825 amino acid residues (with predicted M_r s of 99308 and 91609) respectively. Although they are identical for the majority of their respective potential coding regions (nt 702–2492 in α I; Figure 1), the 5' and 3' ends of α I and α II cDNAs are distinct, yet related (Figure 1). At the 5' end, this disparity is most pronounced in the predicted non-coding region but extends, albeit decreasingly, into the coding portion [the N-terminal parts of the α I and α II proteins predicted to precede the common core are 60% identical (Figure 1 and 3)]. Although the 5' ends of both α I and α II conserve a candidate initiating Met codon (nt 350–352 in α I; Figure 1), neither isoform possesses a strong candidate N-terminal signal peptide [21] when this Met is assumed to correspond to the start of fertilin α translation; this feature has also been observed for guinea-pig fertilin α [7]. Alignment of the α I and α II 3' ends reveals maximum disparity between putative C-terminal coding regions, due largely to an extensive repetitive insertion in α I relative to α II (Figure 1). This glutamate-rich, proline-rich, α I domain ([P/LPPGEEA]₇[AAPP{E}]₁₁; amino acid residue 706 to end in Figure 1) is reminiscent of the C-terminus of the guinea-pig fertilin α subunit (Figure 3; [6]). Preliminary hydropathy analysis of α I and α II predicted proteins (results not shown) suggests that each possesses a single membrane-spanning domain, typical of type I integral membrane proteins (Figures 1 and 3).

We believe that the class of relatively weakly hybridizing cDNA clones represents the *M. fascicularis* fertilin β homologue, based on sequence similarities to the guinea-pig β subunit (Figures 2 and 3). The full-length sequence of monkey fertilin β cDNA includes an open reading frame of 2204 nt encoding a protein of 735 amino acid residues (Figure 2). The N-terminal Met of this putative protein is part of a plausible 16-residue signal peptide [21] and hydropathy analysis (results not shown) reveals a second hydrophobic stretch consistent with a transmembrane domain (residues 687–707; Figure 2). Although the sequences of fertilin β clones are largely congruous, they differ at six single nucleotide positions throughout which do not affect the primary structure of their predicted protein products (Figure 2). These differences presumably represent a series of polymorphic variants. A similar pattern of differences has been described for *Crotalus atrox* venom haemorrhagic proteins a–d [22].

Northern blot analysis of fertilin transcripts

Polyadenylated RNA from *M. fascicularis* testis or epididymis was analysed for fertilin transcripts using a probe derived from α I (pmT-Fertilin α I-S8) or β (pmT-Fertilin β I-W5) subunit cDNAs (Figure 4). In each case hybridizing transcript was detected in the testis, but not the epididymis, and was of a size consistent with that determined for the corresponding near-full-length cDNA clones when allowance is made for a poly(A) tail (Figures 1, 2 and 4). To determine the relative steady-state levels of detectable α I and α II transcripts, we probed Northern blots of *M. fascicularis* polyadenylated testis RNA with fragments corresponding to the unique 5' ends of each of the two fertilin α cDNAs (Figure 5). Northern blot analyses revealed species of 3.8 and 3.4 kb,

Figure 4 Expression of fertilin α and β mRNAs in monkey testis and epididymis

Polyadenylated RNA (1 μ g per lane) from monkey testis and epididymis was analysed by Northern blotting using probes derived from pmT-Fertilin α I-S8 and pmT-Fertilin β -W5 cDNAs for fertilins α and β respectively. The relative positions of 18 S (1.87 kb) and 28 S (5.03 kb) ribosomal RNAs are indicated.

Figure 5 Expression of testicular fertilin mRNAs containing either α I or α II 5' sequences

Fragments derived from pmT-Fertilin α I-S8 and pmT-Fertilin α II-S5' (corresponding to the unique 5' sequences of fertilin α I and α II respectively; see the Materials and methods section) were used in equal amounts and specific activities to probe Northern blots of monkey testis polyadenylated RNA (1 μ g each). Control slot-blot analysis revealed that probes hybridized to complementary templates with equivalent efficiency and cross-hybridized at $\sim 1/20$ of this efficiency (results not shown). The relative positions of 18 S and 28 S ribosomal RNAs are indicated.

corresponding to transcripts containing α I and α II 5' sequences respectively, and present at similar steady-state levels (Figure 5).

Fertilin sequence alignments

Optimal alignment of *M. fascicularis* and guinea-pig fertilin amino acid sequences and the polypeptide sequences of venom proteinase [23] and disintegrin [24] from *Trimeresurus* sp. revealed a framework of conserved cysteine residues throughout the *M. fascicularis* fertilin preproteins (Figure 3). These alignments

exhibited greatest dissonance at either end, with increased similarity (> 30%) in the interior proteinase- and disintegrin-like domains (Figure 3). Indeed, the fertilin α sequence includes an MXHEXGHXLGXHD extended consensus (residues 373–386; Figures 1 and 3), corresponding to the active-site helix of the venom proteinase adamalysin II, HR2A (to which it is identical) and thermolysin [25]. The apparent absence of a sequence resembling this motif in the analogous region of pre-fertilin β suggests that it does not bind zinc and cannot function as a metalloproteinase.

Predicted sequence conservation is also high in the disintegrin-like domains (Figure 3), although neither monkey fertilin subunit possesses the most common potential integrin ligand, RGD, in this region. By analogy to smaller venom disintegrins, Cys residues 499 and 519 of fertilin α I (and corresponding residues in other fertilin subunits; Figure 3) would form a loop presenting the integrin ligand. While it is not clear how sequences between these residues align, we favour an alignment anchored at the aspartate residue in *T. gramineus* disintegrin RGD that produces an ECD fertilin counterpart (Figure 3). Further support for the role of ECD as a potential integrin ligand is provided by the *T. flavoviridis* disintegrin-containing haemorrhagic venom peptide, HR1B [26], which also possesses this tripeptide in place of RGD. However, since the ECD tripeptide is conserved amongst several members of the testicular MDC family (including fertilin species homologues), it cannot be sufficient for the specificity of interactions with integrin receptors.

DISCUSSION

We have reported the cloning and sequence analysis of the monkey (*M. fascicularis*) homologues of the α and β subunits of a guinea-pig complex, the fertilin complex, thought to be active in sperm–oolema recognition and fusion. The global domain organization of both monkey counterparts conforms to that of other members of the male reproductive tract MDC family, including EAP I [14], tMDC I ([12]; also known as cyritestin [27]), tMDC II [13] and guinea-pig pro-fertilins α and β [7]. Each precursor protein possesses a zinc-proteinase-like, disintegrin-like, cysteine-rich and transmembrane domain leading to a heterologous C-terminus. However, the cloning of fertilin subunits from a different species has allowed not only the first detailed comparison of regions of purported importance in their guinea-pig homologues, but also the identification of a novel fertilin isoform.

The regions of monkey fertilin subunits that map to the N-termini of their mature, fully processed guinea-pig counterparts either share a high level of sequence identity or exhibit conservative amino acid substitutions, suggesting that they are ultimately processed in a similar fashion. The processing of guinea-pig fertilin α has apparently gone to completion soon after its synthesis in the testis [5], and conservation of the extended metalloproteinase-like catalytic consensus in monkey fertilin α therefore suggests a possible functional role for this putative proteinase domain. This role might be to participate in a cascade of proteolytic activation involving other endoproteases, conceivably other MDC family members, expressed at high levels in the testis [28,29]. Alternatively, it might involve an autocatalytic activity analogous to the self-processing of pro-Kex2 endoprotease at a dibasic site [30]. The biological consequence of fertilin α proteolysis might be to liberate spermatis from cognate integrin(s) at their point of anchorage on Sertoli cells in the testis [31].

In contrast to the fertilin α subunit, monkey fertilin β is highly unlikely to possess proteinase activity. Final epididymal pro-

cessing of fertilin β could, instead, be mediated by a specific epididymal proteinase that does not require a dibasic substrate [since pro-fertilin β lacks a dibasic pair near the region likely to correspond to the N-terminus of the mature monkey β subunit (Figure 3)]. Such processing permits an additional level of regulation, possibly important to spermatozoa which are terminally differentiated cells with severely restricted transcriptional and translational ability.

We have produced an alignment of fertilin disintegrins which shows the tripeptide ECD corresponding to the integrin-binding RGD motif found in many venom proteins (residues 451–453 in monkey fertilin β ; Figure 3). It has been argued by Wolfsberg et al. [7] that the RGD analogue within guinea-pig fertilin β lies two residues N-terminal to this (corresponding to FDE in monkey fertilin β ; residues 449–451; Figure 3). This motif is less well conserved than ECD, and in guinea-pig pro-fertilin α is VGP (Figure 3; [7]). Although such variability could account for the apparent specificity of integrin binding exhibited by fertilins, it is also possible that integrin binding itself is mediated by ECD and that nearby residues account for the observed specificity. Recent *in vitro* studies confirm that short peptides corresponding to this region in guinea-pig fertilin β bind eggs and block sperm–egg fusion [32]; unfortunately, most test peptides contained the ECD motif in addition to N-terminal residues, making functional distinction between the two difficult. However, the ability of sperm to adhere to and penetrate zona pellucida-free hamster eggs is inhibited by the peptide RGDV, but not GRGES [10]. Recent sequence analysis of a wide range of related disintegrin-containing monkey proteins has indicated that ECD is the only highly conserved tripeptide motif in this region, further supporting its choice as the most likely integrin-binding ligand ([13]; A. C. F. Perry, R. Jones and L. Hall, unpublished work).

Following interaction with the egg, it has been proposed that sperm–egg membrane fusion is mediated by a short fusogenic peptide present in mature fertilin α (corresponding to residues 600–622 in fertilin α I; Figure 1) that might describe a 'sided' helix (Figure 3). However, at the core of this region lies a six-residue stretch (CGKLIC) that is identical in both monkey and guinea-pig fertilins α and β (Figure 3), implying an important role that is not specific to the α subunit. Also, the analogous region in the monkey counterpart is not as likely to present a 'sided' helix, due to the presence of highly polar residues in the putative helix turn described by AQHT (residues 614–617 in fertilin α I; Figure 1). Moreover, the internalization of recombinant filamentous phages bearing an RGD motif [33] suggests that integrin–disintegrin interaction can result in the internalization of large structures in the absence of additional membrane-fusing components.

The demonstration that there are at least two monkey fertilin α transcripts (α I and α II) raises the possibility that spermatozoa may differ qualitatively in their fertilin composition, and consequently in the properties endowed by the different fertilin α N- and C-terminal domains. Although predicted C-terminal differences between monkey fertilin isoforms α I and α II could account for 8000-*M*, (Figure 3), there is no strong evidence for fertilin α subunits exhibiting such a difference in the guinea-pig [5]. This argues that fertilin α isoforms are not present in the guinea-pig, that previous immunological techniques detect only one isoform (either because it is immunodominant or simply more abundant) or that glycosylation masks isoform size differences (mature guinea-pig-fertilin α is a glycoprotein; [5]). Assuming that differences between α I and α II are manifested in their C-terminal sequences, they might correlate with characteristic signal transduction and/or internalization properties; the C-terminal domains of both α I and α II contain Tyr and Ser residues. Indeed,

regulation of other receptors is mediated via C-terminal variants that arise from differential splicing [34]. If fertilins αI and αII were the product of a single gene, then such differential splicing would presumably have faithfully to match exon(s) for the unique 5' region with the correct 3' exon(s) to generate αI and αII mRNAs. Such 'two-step' differential splicing would require that the first splicing decision determined the fate of the second, since we have no direct evidence for the existence of transcripts containing sequences corresponding to the 5' end of αI and the 3' end of αII (or vice versa). Alternatively, αI and αII could arise from separate genes that each share a significant proportion of identical exons. This lack of evidence for evolutionary drift between the identical exons shared by αI and αII might imply that their respective genes are the products of a relatively recent duplication.

This work was supported in part by the Medical Research Council (U.K.) and the Agricultural and Food Research Council (U.K.). We thank Helen Barker for assistance with automated DNA sequencing.

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