Rapid sperm acrosome reaction in the absence of acrosomal CD46 expression in promiscuous field mice (*Apodemus*)

Peter M Johnson, Leanne E Clift, Petra Andrlikova¹, Michaela Jursova², Brian F Flanagan, Joanne A Cummerson, Pavel Stopka¹ and Katerina Dvorakova-Hortova²

Division of Immunology, School of Infection and Host Defence, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, UK, Departments of ¹Zoology and ²Developmental Biology, Faculty of Science, Charles University, Vinicna 7, Prague, Czech Republic

Correspondence should be addressed to P M Johnson; Email: mq22@liv.ac.uk

Abstract

There is pronounced promiscuity and sperm competition in long-tailed field mice (*Apodemus sylvaticus*). These mice have evolved unusual sperm behaviour favouring rapid fertilisation, including dynamic formation of sperm trains and their subsequent dissociation. The cell surface complement regulatory (CReg) protein CD46 is broadly expressed in eutherian mammals other than rodents, in which it is expressed solely on the spermatozoal acrosomal membrane. Ablation of the CD46 gene has been associated with a faster acrosome reaction (AR) rate in inbred laboratory mice. Here, we demonstrate that wild-caught field mice of three species, *A. sylvaticus*, *A. flavicollis* and *A. microps*, exhibit a more rapid AR than wild-caught house mice *Mus musculus* or inbred laboratory BALB/c mice. We also demonstrate that wild-caught field mice of these three species, unlike house mice, produce alternatively spliced transcripts of testicular CD46 mRNA lacking exons 5–7 or 6–7, together with an extended 3'- and often truncated 5'-utr, leading to failure to express any sperm CD46 protein in both the testis and epididymis. Male field mice may therefore have traded expression of this CReg protein for acrosomal instability, providing a novel genus-specific strategy to favour rapid fertilisation and competitive advantage in the promiscuous reproductive behaviour of wild field mice.

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Introduction

Field mice are highly territorial and there is a pronounced sperm competition for individual mating success in their natural environment, reflected in males by their disproportionately large testicles (Breed & Taylor 2000, Bryja & Stopka 2005). In *Apodemus sylvaticus* (*A. sylvaticus*, long-tailed field mouse; wood mouse), mechanisms have evolved to facilitate rapid fertilisation, such as dynamic formation of sperm 'trains' with significantly increased progressive motility (Moore *et al.* 2002). Up to 50% of these spermatozoa may undergo a spontaneous acrosome reaction (AR) within an hour, which is also believed to be associated with the essential dissociation of sperm trains (Moore *et al.* 2002). Rapid fertilisation would temporally assist individual males within this highly promiscuous species and represent a mechanism selected by *A. sylvaticus* males to compete between each other at the cellular level.

CD46 (membrane cofactor protein) is a widely expressed cell surface protein in humans and most other mammals and is a key complement regulatory (CReg) protein protecting host cells from complement-mediated damage. Human CD46 is a type I transmembrane glycoprotein composed primarily of four extracellular domains, termed

short consensus repeats (SCRs), and occurs in many isoforms generated by alternative splicing of three short exons encoding serine/threonine/proline (STP)-rich regions and two exons encoding different cytoplasmic tail (CYT) regions (Liszewski & Atkinson 1992). CD46 cross-linking initiates intracellular signalling events in various cell types, including T lymphocytes (Wong *et al.* 1997, Wang *et al.* 2000, Marie *et al.* 2002, Kemper *et al.* 2003, Liszewski *et al.* 2005). Furthermore, CD46 functions as a cell surface receptor for a diverse range of microbial pathogens (Cattaneo 2004, Liszewski *et al.* 2005).

However, in sperm, CD46 is expressed solely as an unusual lower molecular weight hypoglycosylated isoform localised to the acrosomal membrane and becomes surface exposed only after sperm have acrosome reacted (Anderson *et al.* 1989, Cervoni *et al.* 1992, Riley *et al.* 2002a). The AR is a critical Ca²⁺-dependent event that follows capacitation, enabling sperm to be fusion competent after exocytosis of the single giant enzyme-rich secretory vesicle, the acrosome. This results in morphological change and remodelling of the anterior sperm head. Monoclonal antibodies to the first SCR (SCR1) ectodomain of CD46 block the complement-independent interaction of human sperm with zona-free oocytes *in vitro* (Anderson *et al.* 1989, 1993, Okabe *et al.*

1990, Taylor *et al.* 1994, D'Cruz *et al.* 1997). New World monkeys selectively express an alternatively spliced CD46 isoform lacking SCR1 on all cells except sperm (Riley *et al.* 2002b), further supporting a key role for the SCR1 ectodomain of CD46 in fertilisation.

In line with this concept, but in contrast to other eutherian mammals, there has been loss of systemic expression of CD46 in rodents and its CReg role has instead been subsumed by Crry, a rodent-specific membrane CReg protein (Hosokawa *et al.* 1996, Miwa *et al.* 1998, Tsujimura *et al.* 1998, Mead *et al.* 1999). Thus, CD46 protein is expressed in rodents only by mature sperm and spermatozoal precursors in testes with active spermatogenesis (Mizuno *et al.* 2004). Additionally, anti-CD46 antibody may not block *in vivo* fertilisation in the rat (Mizuno *et al.* 2007). The CD46 gene in rodents contains 11 functional exons, in comparison with 14 in humans, and includes a single STP and a single CYT exon; alternative splicing of rodent membrane-bound CD46 has rarely been described.

Since disruption of the CD46 gene in inbred laboratory mice may cause an accelerated AR compared with wild-type mice (Inoue *et al.* 2003), we hypothesised that there could also be differences in CD46 gene expression in sperm of wild field mice. Here, we have demonstrated that wild-caught *A. sylvaticus* exhibit a more rapid AR than house mice *Mus musculus*. Furthermore, we show that *A. sylvaticus* fail to express any CD46 protein in both the testis and the epididymis. We extend these observations also to show that two further species within the *Apodemus* genus (*A. flavicollis* and *A. microps*) behave similarly to *A. sylvaticus*.

Results

Accelerated AR in sperm of wild field mice

In order to assess the rate of AR under *in vitro* fertilising conditions for sperm of wild-caught field mice

(*Apodemus*), and to compare with that for wild-caught house mice (*M. musculus*), we used Alexa peanut agglutinin (PNA) lectin selective for an intact acrosome. The AR rate for spermatozoa collected from the very distal region of the cauda epididymis was measured at selected times during sperm incubation in M2 fertilising medium. Two subspecies of house mice (*M. musculus musculus* and *M. musculus domesticus*), as well as BALB/c mice, were used as reference controls for three field mouse species (*A. sylvaticus*, *A. microps* (pygmy field mouse) and *A. flavicollis* (yellow-necked field mouse)). There was a significantly faster mean rate of AR by sperm of all three species of wild-caught field mice compared with the two subspecies of wild-caught house mice and also BALB/c mice (Fig. 1). There was no obvious difference in viability or motility of sperm between field and house mice in these experiments. The increased rate of AR in field mice is genus specific (ANOVA, $F=30.1$, d.f.=5, $P<0.000001$). *Post hoc* Tukey test revealed that wild house mice had significantly lower rates of AR compared with any of the three species within the field mouse genus (*A. microps* $P<0.01$; *A. flavicollis* $P<0.0003$; *A. sylvaticus* $P<0.0001$). Furthermore, there was no difference in the rate of AR between *M. m. musculus*, *M. m. domesticus* and inbred BALB/c ($P>0.6$). There was also no difference between *A. microps* and *A. flavicollis* ($P=0.68$).

Alternatively spliced *A. sylvaticus* testicular CD46 mRNA

Since wild field mice are comparable with inbred CD46^{-/-} gene knockout mice regarding a more rapid AR rate (Inoue *et al.* 2003), we investigated whether they were also phenotypically homologous with respect to expression of mature CD46 protein. Conserved primer pairs were designed using published mouse (*M. musculus*) and rat (*Rattus norvegicus*) CD46 consensus gene sequences from within exons, flanking individual exons or spanning

Figure 1 The acrosome reaction is significantly faster in three different species of wild-caught field mice (*Apodemus*) compared with house mice (*Mus musculus*). Each datum point represents eight separate experimental observations.

individual intron/exon divides. These primers were used in PCR on genomic DNA from wild-caught *A. sylvaticus*, followed by sequencing of amplicons, to derive full or partial sequences of *A. sylvaticus* CD46 exons 1, 2, 3, 5 and 11. Further sets of exon-specific primers were then designed to span separate exonic sequences and used in RT-PCR to interrogate testicular mRNA from *A. sylvaticus* compared with BALB/c mice. It became clear that there is a significant sequence deletion of 432 nucleotides in *A. sylvaticus* compared with BALB/c testicular CD46 cDNA (Fig. 2; lanes 2 and 3). Sequencing demonstrated that exons 5–7 were absent. Further PCR analysis using a primer for exon 5 revealed that some transcripts instead lacked only exons 6–7 (Fig. 2; lanes 4 and 5). After obtaining partial *A. sylvaticus* mRNA sequence information, this approach was repeated with additional sets of primers, supplemented by both 5'- and 3'-RACE, in order to derive the full-length *A. sylvaticus* CD46 testicular cDNA sequence. This complete sequence, lacking exons 6 and 7, is shown in Fig. 3, together with the predicted *A. sylvaticus* amino acid sequence. Compared with published sequences (Miwa *et al.* 1998, Tsujimura *et al.* 1998, Mead *et al.* 1999), *A. sylvaticus* CD46 shows 91.2% nucleotide homology with *Mus*, and 86.2% with *Rattus*; 4.0% of nucleotides were specific for *A. sylvaticus*. This sequence is compatible with the phylogenetic position of *Apodemus* between the *Rattus* and *Mus* genera (Michaux *et al.* 2002). The *A. sylvaticus* CD46 testicular cDNA sequence is in-frame, has no premature stop codon and retains characteristic conserved sequences for CD46. Primers used for PCR, RT-PCR and RACE are listed in Table 1.

Thus, two separate mRNA transcripts have been identified for *A. sylvaticus* testicular CD46, one lacking exons 5–7 and the other lacking exons 6 and 7. These would be predicted to encode CD46 proteins that exclude the SCR3 (exon 5), SCR4 (exon 6) and STP (exon 7) regions. Although alternative splicing of CD46 is common in primates, it has not been described for transmembrane CD46 in rodents other than some guinea pig CD46 cDNA

Figure 2 CD46 transcripts are shorter in *A. sylvaticus* than in BALB/c mice. PCR of cDNA prepared from testicular extracts. 1, Ladder. 2 and 3, CD46 exon 2–11 primer set giving products of 822nt (BALB/c) and 390nt (*A. sylvaticus*) respectively. 4 and 5, CD46 exon 5–11 primer set giving products of 585nt (BALB/c) and 326nt (*A. sylvaticus*) respectively. 6 and 7, primer set corresponding to nt 972–1412 of *M. m. musculus* ADAM2 (A disintegrin and metalloprotease 2) cDNA, used as a positive control for testicular cDNA, giving a 440nt product for both BALB/c and *A. sylvaticus*. B, BALB/c; As, *A. sylvaticus*.

clones that lacked the exon encoding either the SCR4 or the STP domain, which were identified in addition to full-length cDNA clones (Hosokawa *et al.* 1996).

In *A. sylvaticus* CD46 mRNA, there is a 54nt sequence extension to the 3'-utr that is AU-rich (74.1%; Fig. 3). AU-rich elements in the 3'-utr are associated with destabilisation of RNA and can provide mechanisms for suppressing gene expression initiated by deadenylation and subsequent degradation through exonuclease complexes. There has been recent recognition of the relative importance of the 5'–3' decay pathway (Stoecklin *et al.* 2006). In line with this concept, although RT-PCR using a 5'-terminal exon 1 primer amplified *A. sylvaticus* testicular CD46 cDNA, use of 5'-RACE often identified amplicons lacking between 165 and 199nt from exon 1 and the 5'-terminal sequence of exon 2, and hence without the ribosomal binding site and translation start codon. In contrast, amplicons produced by 5'-RACE reactions in *Mus* consistently included the complete exon 1 and 2 sequences. This raised the possibility that CD46 is not translated in the *A. sylvaticus* testis.

CD46 protein is not expressed in *A. sylvaticus* testis or sperm

Accordingly, we raised a rabbit antiserum against a 14-mer peptide (PFEAMELKGTPLKLY) that was homologous with amino acids 50–63 of the predicted *A. sylvaticus* CD46 SCR1 amino acid sequence, but with a single amino acid difference from the published *M. musculus* sequence (PFEAMELKGTPLKLF; Miwa *et al.* 1998, Tsujimura *et al.* 1998). The peptide was conjugated to keyhole limpet haemocyanin (KLH) for immunisation and antiserum bleeds screened by ELISA against peptide conjugated to BSA. High-titre antibody was affinity purified using non-conjugated peptide immobilised to an agarose gel support. The immune antiserum gave an OD_{405 nm} 1.40±0.01 at 1:3200 dilution in ELISA against BSA-peptide, whereas it was 0.11±0.01 at the same dilution against BSA alone; pre-immune serum gave OD_{405 nm} values of 0.13±0.01 and 0.12±0.01 at 1:3200 dilution against BSA-peptide and BSA alone respectively. We also included a polyclonal rat anti-rat CD46 antiserum (Mizuno *et al.* 2007).

In immunocytochemistry on permeabilised epididymal sperm from wild-caught *M. musculus* as well as inbred BALB/c mice, the rabbit anti-CD46 peptide antibody clearly stained only the acrosomal region, whereas there was no corresponding staining of wild-caught *A. sylvaticus* sperm (Figs 4A and 5). This acrosomal staining of *M. musculus* sperm was completely inhibited by pre-incubation of the rabbit anti-CD46 peptide antibody with a 50 molar excess of either the free peptide or the BSA-peptide conjugate. Similarly, the rat anti-rat CD46 antiserum stained the acrosomal region of epididymal sperm from wild-caught *M. musculus*, inbred BALB/c mice and Wistar rats but gave no corresponding staining of

↗Signal peptide	
M T A S P L V P V S T H P	13
agtttccctttcagtcctggaacATGACAGCGTCGCCTCTTGTGCCAGTCTCAACGCATC	60
R R K R K S Y T F F W C S L G I Y T E A	33
CCCGTCGTAGAAGGAAGAGCTACACTTTCTTCTGGTGCCTCCCTGGGCATCTATACAGAGG	120
↗SCR1	
L L F L L S G L S D A C E L P P P F E A	53
CCCTTCTGTTTCTGCTGTCTGGTTTATCTGATGCCTGTGAACACCACCACATTGAAG	180
M E L K G T P K L Y Y A V G E R I E Y K	73
CTATGGAACCAAGGGTACACCTAACTCTATTATGCAGTTGGAGAAAGAATAGAATATA	240
C K K G Y L H L S P Y L M I A T C E P N	93
AGTGTAAGAAAGGATACCTTCATCTGTCTCCATACCTGATGATTGCTACCTGTGAGCCAA	300
↗SCR2	
H T W V P I S D V G C I K V Q C I M L Q	113
ATCATACATGGGTCCCTATTTTCTGATGTTGGTTGTATCAAAGTTCAGTGATTATGTTAC	360
N P S F G K V H Y I D G R F S W G A R V	133
AGAACCCTTCATTTGGCAAGTACACTACATAGATGGCAGATTTTCATGGGGTGCCTCGAG	420
K F T C M E G Y Y L A G M S V L H C V L	153
TAAATTTTACTTGTATGGAAGGTTATTACTTAGCTGGTATGTCAGTTCTACACTGTGTGC	480
↗SCR3	
K S D G A Y W N G H P P S C E K V Y C L	173
TAAAGTGATGGTGCATCTGGAATGGCCATCCTCCAAGTTGTGAAAAGGTTTATTGTT	540
P P P K I K N G T H T F T D I N V F K Y	193
TACCACCTCCAAAATAAAAAATGGAACACACCTTTACAGATATAAATGTATTCAAAT	600
H E A V I Y S C D P N P G P D K F S L V	213
ACCATGAAGCAGTAATTTACAGTTGTGATCCTAACCCAGGGCCAGATAAGTTTCCCTTG	660
G T S M L F C A D H N T Y S N N P P E C	233
TTGGAACAAGCATGCTATTCTGTGCTGACCATAACACCTATAGTAACAACCTCCAGAGT	720
↗UK	
K G Y P N L R E G I F G Q E L D A W I V	253
GTAAAGGATATCCTAATCTCCGTGAAGGAATATTGGACAAGAATTAGATGCGTGGATTG	780
A L I V I I S I V G V L E I C L I M L R	273
TTGCTTTGATCGTTATTATTTCATTGTTGGAGTTTGGTAATTTGTCTCATCATGCTCA	840
↗CYT	
Y F E H R K K R N V S A A R *	287
GGTATTTTGTAGCAGGAAGAAAAGAAATGTATCTGCAGCAAGATGAatatcccatgtgt	900
gcaagtcattactgttccatTTTTTgaaaactggatcttcatgtctgtggcaaaagcaaaatt	960
gtatatTTTgcaggagctTTTgtttgtgtaaatgtgtatgtgttttctaattgaaatgaaatc	1020
tgtgtttataaac	1033

Figure 3 Complete nucleotide sequence of *A. sylvaticus* CD46 cDNA lacking exons 6 and 7, together with the predicted amino acid sequence. Exon 5 (corresponding to SCR3) immediately precedes where exons 6 and 7 are deleted. SCR, short consensus repeat region; UK, region of unknown function; TM, transmembrane region; CYT, cytoplasmic tail region.

A. sylvaticus sperm (Fig. 4B). In addition, both the rabbit anti-CD46 peptide antibody and the rat anti-rat CD46 antiserum gave clear immunohistochemical staining of spermatids and spermatozoa in *M. musculus* and BALB/c mouse testis but no staining in *A. sylvaticus* testis (Fig. 4C). Furthermore, seven separate mouse monoclonal antibodies (MM1, 2, 3, 4, 5, 8 and 9) against rat CD46 (Mizuno *et al.* 2004, 2005, Mizuno *et al.* unpublished observations) clearly stained Wistar rat but not *A. sylvaticus* epididymal sperm (data not shown).

CD46 protein is not expressed in other *Apodemus* testis or sperm

We then investigated spermatozoal and testicular expression of CD46 protein, as well as CD46 transcripts, in a variety of other wild-caught *Apodemus* and *Mus* taxa

within the muroid clade. As with *A. sylvaticus*, expression of CD46 protein could not be detected immunocytochemically in either testicular or epididymal sperm of the other field mice, *A. flavicollis* and *A. microps*, using both the rabbit anti-CD46 peptide antibody and rat anti-rat CD46 polyclonal antiserum, whereas *M. m. musculus* and *M. m. domesticus* house mice clearly expressed this protein – i.e. the same immunostaining patterns as depicted in Figs 4 and 5 (data not shown). Similarly, both house mice subspecies expressed identical testicular CD46 mRNA transcripts to sequences derived from inbred BALB/c mice (Fig. 6A). In contrast, both *A. flavicollis* and *A. microps* field mice expressed comparable alternatively spliced testicular CD46 mRNA sequences to *A. sylvaticus* in respect of the exon 5–7 deletion (Fig. 6B) and the AU-rich (74.1% and 75.9% respectively) 3'-utr sequence extension (Fig. 6C).

Table 1 Primers used for PCR, RT-PCR, 5'-RACE and 3'-RACE reactions where amplicons were subsequently sequenced.

Primer sequence	Primer description
<i>PCR primer sequences</i>	
CCGATTGGCCTAGCTAGC	CD46 5'-flanking to exon 1, sense
CGACACAGCCTCTCTCCT	CD46 intron 1, anti-sense
GTCTCTAGATGCCTGTGAAC	CD46 intron 1-exon 2, sense
GCCCAATTAACCTGGTCAT	CD46 intron 2, anti-sense
TTGATTCAAGTGCAACTGG	CD46 intron 2, sense
TATTTAACTCGAGCACC	CD46 exon 3, anti-sense
TGGAACACACACCTTTAC	CD46 exon 5, sense
CTTTACACTCCGGAGGGT	CD46 exon 5, anti-sense
AAATGTATCTGCAGCAAG	CD46 exon 11, sense
GAAGCTCCTGCAAATAT	CD46 exon 11, anti-sense
<i>RT-PCR primer sequences</i>	
CCTTCTGTTTCTGCTGTCT	CD46 exon 1, sense
AATCATACATGGGTCCCTA	CD46 exon 2, sense
TAGGGACCCATGTATGATT	CD46 exon 2, anti-sense
ACTACATAGATGGCAG	CD46 exon 3, sense
CTGCCATCTATGTAGT	CD46 exon 3, anti-sense
ATTGTTTACCACCTCCA	CD46 exon 5, sense
TGGAACACACACCTTTAC	CD46 exon 5, sense
CGATCAAAGCAACAATC	CD46 exon 9, anti-sense
TTCATCTTGCTGCAGATAC	CD46 exon 11, anti-sense
AGCTGCTGAGCCTCAGCATG	ADAM2, sense
ATGTGCCGTTGCAGTACTCT	ADAM2, anti-sense
<i>3'-RACE primer sequences</i>	
TGGAACACACACCTTTAC	Gene-specific primer (GSP) 1, CD46 exon 5
AAATGTATCTGCAGCAAG	GSP2, CD46 exon 11
GACTCGAGTCGACATCGA	Adaptor primer (AP)
GACTCGAGTCGACATCGA(T) ₁₇ G	AP-polyT-G
<i>5'-RACE primer sequences</i>	
AATTTAGCTCGAGCACC	GSP3, CD46 exon 3
CTGCCATCTATGTAGT	GSP4, CD46 exon 3
TAGGGACCCATGTATGATT	GSP5, CD46 exon 2
GACTCGAGTCGACATCGA	AP
GACTCGAGTCGACATCGA(G) ₁₇	AP-polyG

Like *A. sylvaticus*, but unlike both house mice subspecies, 5'-RACE reactions in the two other *Apodemus* species often terminated prematurely in exon 2.

Polymorphism in *Apodemus testicular CD46* mRNA

There was minor nucleotide sequence polymorphism between the three *Apodemus* taxa (see Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/, for full *Apodemus* taxa CD46 sequence information compared with *M. musculus*). In addition, complete sequencing of testicular CD46 mRNA transcripts from five wild-caught *A. sylvaticus* mice identified further polymorphisms between individuals involving seven nucleotide positions in the coding region and four nucleotide positions in non-coding flanking regions (also see Supplementary Figure 1). Comparable polymorphisms were also noted for *A. flavicollis* and *A. microps*. This was unexpected for *Apodemus* since genetic polymorphism has not previously been described for the coding region of rodent CD46. Analysis of the

Figure 4 CD46 protein is expressed by *M. m. musculus* but not *A. sylvaticus* sperm. Immunostaining for CD46 (green) in the acrosomal region of sperm from *M. m. musculus* (left panels) and *A. sylvaticus* (right panels). (A) Affinity-purified rabbit polyclonal antibody against *A. sylvaticus* CD46 SCR1 peptide on epididymal sperm; (B) rat polyclonal antiserum against rat CD46 on epididymal sperm; (C) rat polyclonal antiserum against rat CD46 on testicular spermatozoa. Nuclei counterstained with DAPI (blue) or propidium iodide (red). 5 µm cryosection. Scale bars represent 10 µm.

origin of individual *A. sylvaticus* mice revealed a correlation with having been caught in either western (Bohemia) or eastern (Moravia) Czech Republic. Since the majority (71%) of the polymorphisms within the coding region would result in an amino acid change in any translated protein (i.e. non-synonymous changes), they may represent positive selection driving evolution of gamete recognition proteins (Levitan & Ferrell 2006) in competitive breeding within the *Apodemus* taxa prior to the advent of failure to express CD46 protein, as may also be the case for the unique CD46 splicing. These events

Figure 5 Affinity-purified rabbit polyclonal antibody against *A. sylvaticus* CD46 SCR1 peptide on epididymal sperm (left panels) and corresponding phase-contrast micrographs showing the morphological status of the acrosomal region (right panels); (A) BALB/c sperm; (B) *A. sylvaticus* sperm. Scale bars represent 8 μ m.

could have occurred independently during the rapid species radiation into ecologically specialised taxa (Serizawa *et al.* 2000).

Discussion

In the present study, we have demonstrated a rapid AR and the absence of spermatozoal CD46 in three species of wild-caught field mice (*Apodemus*). In contrast, wild-caught house mice (*M. musculus*) express CD46 and take longer to undergo the AR, behaving exactly the same as inbred BALB/c mice. Thus, field mice, in comparison with house mice, are naturally occurring null phenotypic analogues to CD46^{-/-} gene knockout mice (Inoue *et al.* 2003) with respect to a more rapid mean AR rate and higher fertilising ability. Similarly, these field mice do not exhibit any abnormalities in spermatogenesis or fertilisation, further supporting the concept that the primary role of CD46 on acrosome-reacted sperm is not protection from attack by the abundant complement in the oviducts. Instead, spermatozoal CD46 in rodents is more likely associated with acrosomal integrity within the sperm head, and the absence of molecular expression of CD46 protein in field mice may favour rapid fertilisation and individual competitive advantage in their promiscuous reproductive behaviour. The mechanism by which the presence of acrosomal membrane CD46 stabilises this organelle is unknown. This could involve actin reorganisation following protein kinase signalling directly upon CD46 stimulation (Wong *et al.* 1997, Zaffan *et al.* 2001) or specific CD46 binding to β 1-integrins and subsequent indirect associations through the integrin tail anchor

Figure 6 Different species of field mice (*Apodemus*) all have shorter CD46 transcripts with a 3'-utr extension compared with house mice (*M. musculus*). PCR of cDNA prepared from testicular extracts. (A) 1, Ladder. 2–5, CD46 exon 2–11 primer set giving a product of 822nt for *M. m. domesticus* and *M. m. musculus*, as well as BALB/c mice, compared with a product of 390nt for *A. sylvaticus*. (B) 1, Ladder. 2–5, CD46 exon 2–11 primer set giving a product of 390nt for *A. flavicollis*, *A. microps* and *A. sylvaticus*, but a product of 822nt for BALB/c mice. (C) 1, Ladder. 2–5, 3'-RACE giving a product of 204nt for *A. flavicollis*, *A. microps* and *A. sylvaticus*, compared with a product of 150nt for BALB/c mice. Af, *A. flavicollis*; Am, *A. microps*; As, *A. sylvaticus*; B, BALB/c.

to the surrounding cortical cytoskeleton during its restructuring throughout capacitation (Lozahic *et al.* 2000, Kurita-Taniguchi *et al.* 2002, Rezcallah *et al.* 2005). The induction of a calcium flux on CD46 signalling (Källström *et al.* 1998) may be particularly relevant to the acrosomal membrane. These issues relating to acrosomal stability could be restricted to the rodent superfamily *Muroidea* with the apical hook-shaped falciform sperm head morphology that is unique within eutherian mammals.

It appears that the genus *Apodemus* has developed during phylogeny to exceptionally select two CD46 transcripts with exons 5, 6 and 7 spliced out, a 54nt 3'-utr sequence extension and often a 5'-terminal sequence deletion, which together prevent functional CD46 protein translation and expression on sperm. An additional non-exclusive hypothesis is that field mouse sperm CD46 might become rapidly ubiquitinated and undergo proteosomal degradation within the acrosome; it has been reported that inhibition of mouse sperm proteosomal activity may block acrosomal exocytosis (Pasten *et al.* 2005). Nevertheless, the consequence is that expression of CD46 is traded in field mouse sperm cells for an accelerated AR, fast-tracking zona pellucida (ZP) penetration through a high concentration of lytic enzymes at the fertilisation site. This is in line with a concept that males may trade aspects of immune

competence for competitive sperm advantage in certain species (Simmons & Roberts 2005). Sperm would still be protected against locally activated complement by expression of other CReg proteins and, indeed, CD46-independent deposition of activated C3 on the acrosome-reacted sperm head may be beneficial for fertilisation at least in humans (Riley-Vargas *et al.* 2005).

CD46 has been utilised as a cell surface receptor by numerous pathogenic viruses and bacteria (Cattaneo 2004, Liszewski *et al.* 2005), and differential tissue expression of CD46 could be a result of selective pressure from these pathogens. In promiscuous field mouse species, with high exposure to genital pathogens, it may be advantageous not to express certain pathogen receptors such as CD46. Thus, there could be pathogen-driven rather than reproduction-driven selective loss of spermatozoal CD46 in field mice. However, the short period of time between surface exposure of spermatozoal CD46 and potential fertilisation would argue against the former mechanism.

As spermatozoa reside in the female reproductive tract, progressively more undergo capacitation followed by the AR, such that over 40% have acrosome reacted by the time sperm are in the oviducts of inbred mice (Klemm & Engel 1991). Sperm competition influences sperm function by increasing the proportion that capacitate, selecting for sperm that are more sensitive to signals released by the ovum to undergo the AR at the site of fertilisation (Gomendio *et al.* 2006). This would be expected to be exaggerated in wild field mice, and the consequence of premature loss of the acrosomal content would be compatible with other reproductive biological traits of *Apodemus*. There is pronounced sperm competition for individual mating success, reflected by the more promiscuous behaviour and large testicles of field mice as well as sperm morphological adaptations in this species-rich rodent subfamily (Breed & Taylor 2000, Bryja & Stopka 2005, Immler *et al.* 2007). These factors are reflected in mechanisms that have evolved in field mice to facilitate rapid fertilisation, such as dynamic sperm 'trains' and an AR completed within a 60-min period for greater than 50% of sperm, which may assist both dissociation of these trains (Moore *et al.* 2002) and bypass the primary binding of acrosome-unreacted sperm to ZP (Myles *et al.* 1987, Yamagata *et al.* 2002). The release of acrosomal contents within a short time period can facilitate ZP structural weakening and sperm passage, as well as binding of acrosome-reacted sperm to ZP independently of zona protein 3 (Talbot *et al.* 2003). It has also been demonstrated that only acrosome-reacted sperm can bind to and penetrate the ZP in the guinea pig (Huang *et al.* 1981), and that acrosome-reacted mouse sperm can fertilise ZP-free eggs and produce normal offspring (Naito *et al.* 1992).

In conclusion, we have demonstrated unique transcriptional forms of CD46 in wild field mice, not found in other rodents, resulting in loss of CD46 translation in sperm. This

may be associated with acrosomal instability, and indeed we have shown that the AR is completed faster in field mice compared with house mice. This contributes a novel molecular basis underlying a genus-specific strategy for more rapid fertilisation and increased competitive advantage in promiscuous field mice.

Materials and Methods

Mice

Male mice were caught from regions of Central Bohemia and South Moravia, Czech Republic (*A. sylvaticus*, *A. flavicollis* and *A. microps*), from around Buskovice, Czech Republic (*M. m. musculus*) and around Straas, Germany (*M. m. domesticus*). At the time of experiments, all mice were at reproductive age. These mice were maintained in a pathogen-free facility for wild mice at the Department of Zoology, Charles University, Prague, Czech Republic. Laboratory BALB/c mice and Wistar rats were maintained in animal facilities in the Department of Developmental Biology, Charles University, Prague, Czech Republic. All animal procedures were carried out in accordance with the Animal (Scientific Procedure) Regulations and subjected to review by the local ethical committee.

AR rate measurement

Spermatozoa were recovered from the cauda epididymis by placing its very distal region into PBS for 10 min at 37 °C in 5% CO₂ in air. Epididymal sperm were capacitated at a concentration of 5×10^6 in M2 fertilisation medium (Sigma-Aldrich) under paraffin oil. At times 5, 10, 20, 40, 60, 80 and 120 min, spermatozoa were collected for evaluation of acrosomal status by staining with 5 µM Alexa PNA lectin (Molecular Probes, Prague, Czech Republic) and immediately assessed under the epifluorescent microscope. Sperm motility and viability (Sperm Viability Kit; Molecular Probes) was examined throughout all experiments using an inverted microscope with a stage thermostatically controlled at 37 °C; sperm viability was always > 86%.

Extraction of nucleic acid

Splenic DNA was extracted using the DNeasy Tissue Kit (Qiagen), and RNA extracted from mouse testes using ice-cold Trizol reagent (Invitrogen).

Reverse transcription

Testicular RNA was reverse transcribed using the Superscript II reverse transcriptase protocol (Invitrogen).

PCR

PCRs were conducted according to the 2× ReddyMix PCR master mix protocol (ABgene, Epsom, UK). A murine ADAM2 (fertilin-β) primer set was used in positive control reactions.

Using 2% agarose gel electrophoresis, 10 µl CD46 and 2 µl ADAM2 PCR products were visualised and purified using the QIAquick PCR purification kit gel extraction protocol (Qiagen). In order to confirm sequence identity, or to define new *Apodemus* sequences, PCR and RACE amplicons were sequenced by primer extension at Lark Technologies (Takeley, UK) using an ABI PRISM Sequence Detection System.

3'-RACE

First-strand cDNA synthesis and amplification of target cDNA steps were identical to the Invitrogen 3'-RACE kit protocol, except that a different adaptor primer (AP) and AP-polyT-G primer were used (see Table 1). An additional nested PCR step was added in which 0.1% of the product from amplification of target cDNA was re-amplified using a second gene-specific primer (GSP).

5'-RACE

First-strand cDNA synthesis was conducted according to the Invitrogen 5'-RACE kit protocol, except that cDNA was not RNase treated and was ethanol precipitated. Tailing of cDNA was also conducted following this protocol, except that 16.5 µl purified cDNA was used instead of 6.5 µl and samples incubated with terminal deoxynucleotide transferase for 30 min instead of 10 min. PCR of tailed cDNA was also conducted according to the Invitrogen 5'-RACE kit protocol, except that a different AP-polyG primer was employed (see Table 1) and the tailed cDNA was used at a 1:40 dilution. An additional nested PCR step was added in which 0.1% of the product from amplification of tailed cDNA was re-amplified using a second GSP and AP instead of AP-polyG.

Antibodies

An antiserum was raised in a New Zealand white rabbit against a 14-mer peptide specific to an *A. sylvaticus* CD46 SCR1 amino acid sequence (PFEAMELKGTPLY) predicted from the derived cDNA sequence. This peptide was selected based on the prediction that it would be both antigenic and exposed on the surface of CD46 using the Lasergene analytical package (DNASTAR Inc., Madison, WI, USA). The peptide was synthesised using the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester method and conjugated via an additional N-terminal cysteine to KLH by Sigma-Aldrich. Antiserum bleeds were screened by ELISA against peptide conjugated to BSA. High-titre antibody was affinity purified on non-conjugated peptide immobilised to an agarose gel support using a Sulfolink kit (Perbio Science, Cramlington, UK) and eluted with 100 mM glycine buffer (pH 2.8).

A polyclonal antiserum raised against a recombinant rat CD46 SCR123 fusion protein by immunisation of female rats was a kind gift of B P Morgan, C L Harris and M Mizuno, Cardiff University School of Medicine (Mizuno *et al.* 2007). Seven separate mouse monoclonal antibodies (MM1, 2, 3, 4, 5, 8 and 9) raised against the rat CD46 SCR123 fusion protein (Mizuno *et al.* 2004, 2005, Mizuno *et al.* unpublished observations) were also a kind gift of B P Morgan, C L Harris and M Mizuno. Secondary antibodies

conjugated with Alexa Fluor 488 (Molecular Probes) were used at 1:1000 dilution.

Immunohistochemical analysis

In parallel with the preparation of epididymal sperm smears, testes were also collected for 5 µm cryosections. Samples were permeabilised for 10 min in acetone pre-cooled at -20 °C, air-dried, blocked for 1 h with 3% goat serum and then incubated with primary followed by secondary antibodies at optimised dilutions in phosphate-buffered isotonic saline plus 1% BSA. Pre-immune sera, irrelevant isotype-matched monoclonal antibodies and secondary antibodies (used without primary antibodies) served as negative controls. The monoclonal antibody 18.6, which recognises an acrosome-associated antigen (Moore *et al.* 1987), was also used as a positive control and was a kind gift of H D M Moore, University of Sheffield. DNA was stained through the Vectashield mounting medium including either 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (Vector Laboratories, Peterborough, UK). Slides were examined using an epifluorescence microscope (Olympus, Prague, Czech Republic) and images were taken using a TCS SP2 RS high-speed confocal/two-photon system (Leica, Prague, Czech Republic) for live cell imaging and dynamics.

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