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Impaired sperm fertilizing ability in mice lacking Cysteine-RIch Secretory Protein 1 (CRISP1)

Vanina G. Da Ros [a], Julieta A. Maldera [a], William D. Willis [b], Débora J. Cohen [a], Eugenia H. Goulding [b], Diego M. Gelman [c], Marcelo Rubinstein [c,d], Edward M. Eddy [b], Patricia S. Cuasnicu [a,*]

[a] Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, Buenos Aires, 1428, Argentina
[b] Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA
[c] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Buenos Aires, 1428, Argentina
[d] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, 1428, Argentina

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ABSTRACT

Mammalian fertilization is a complex multi-step process mediated by different molecules present on both gametes. Epididymal protein CRISP1, a member of the Cysteine-RIch Secretory Protein (CRISP) family, was identified by our laboratory and postulated to participate in both sperm–zona pellucida (ZP) interaction and gamete fusion by binding to egg-complementary sites. To elucidate the functional role of CRISP1 *in vivo*, we disrupted the *Crisp1* gene and evaluated the effect on animal fertility and several sperm parameters. Male and female $Crisp1^{-/-}$ animals exhibited no differences in fertility compared to controls. Sperm motility and the ability to undergo a spontaneous or progesterone-induced acrosome reaction were neither affected in $Crisp1^{-/-}$ mice. However, the level of protein tyrosine phosphorylation during capacitation was clearly lower in mutant sperm than in controls. *In vitro* fertilization assays showed that $Crisp1^{-/-}$ sperm also exhibited a significantly reduced ability to penetrate both ZP-intact and ZP-free eggs. Moreover, when ZP-free eggs were simultaneously inseminated with $Crisp1^{+/+}$ and $Crisp1^{-/-}$ sperm in a competition assay, the mutant sperm exhibited a greater disadvantage in their fusion ability. Finally, the finding that the fusion ability of $Crisp1^{-/-}$ sperm was further inhibited by the presence of CRISP1 or CRISP2 during gamete co-incubation, supports that another CRISP cooperates with CRISP1 during fertilization and might compensate for its lack in the mutant mice. Together, these results indicate that CRISP proteins are players in the mammalian fertilization process. To our knowledge this is the first knockout mice generated for a CRISP protein. The information obtained might have important functional implications for other members of the widely distributed and evolutionarily conserved CRISP family.

Introduction

In most invertebrates and non-mammalian vertebrates the spermatozoa that leave the testes already have the ability to fertilize the egg. In mammals, however, testicular sperm become competent to fertilize only after undergoing a series of changes during their transit through the male and female reproductive tracts known as maturation and capacitation, respectively (Yanagimachi, 1994). After passing through the cumulus oophorus that surrounds the egg, capacitated sperm bind to the zona pellucida (ZP), undergo the acrosome reaction (AR), penetrate the ZP and, finally, fuse with the egg plasma membrane. Evidence indicates that most of these events are mediated by specific molecules present on both gametes. One such protein is rat epididymal protein DE, described by our laboratory (Cameo and Blaquier, 1976) and also known as CRISP1 for being the first identified member of the highly conserved Cysteine-RIch Secretory Protein (CRISP) family. Other members of the family subsequently identified include CRISP2, expressed in the testes (see Busso et al., 2007b), CRISP3, with a wider tissue distribution, including non-reproductive organs (Kjeldsen et al., 1996; Udby et al., 2005), and the more recently identified CRISP4, exclusively expressed in the epididymis (Jalkanen et al., 2005; Nolan et al., 2006). In addition, non-mammalian members have also been described in venoms from lizards, snakes and snails, and in eggs and embryos from frogs (see Yamazaki and Morita, 2004). All CRISP family members are characterized by the presence of 16 conserved cysteine residues, ten of which are located in the C-terminal region of the molecule. Crystal structure studies revealed that CRISP proteins contain an extended and loose N-terminal domain, referred to as PR-1 (plant Pathogenesis-Related domain), and a compact C-terminal domain, known as CRD (Cysteine-Rich Domain) (Guo et al., 2005). Evidence suggests that CRISP members have evolved to perform a variety of functions that rely on these different domains (Guo et al., 2005).

* Corresponding author. Fax: +54 11 4786 2564.
E-mail address: cuasnicu@dna.uba.ar (P.S. Cuasnicu).

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The first functional role for a CRISP member was described for rat CRISP1 (rCRISP1) (Rochwerger et al., 1992) which associates with the dorsal region of the sperm head during epididymal maturation (Kohane et al., 1980). Although a substantial amount of rCRISP1 is released during capacitation (Cohen et al., 2000b), part of the protein remains on the sperm surface and migrates to the equatorial segment (Rochwerger and Cuasnicu, 1992), the region through which the sperm fuses with the egg (Yanagimachi, 1994). *In vitro* studies demonstrated that the presence of either a specific antibody against rCRISP1 or purified rCRISP during gamete co-incubation significantly inhibited penetration of ZP-free eggs, supporting the participation of rCRISP1 in gamete fusion through its binding to egg-complementary sites (Rochwerger et al., 1992). Interestingly, recent studies showed that the egg-binding ability of rCRISP1 resides in a specific region of 12 amino acids within the PR-1 domain known as Signature 2 which corresponds to a highly conserved sequence present in all CRISP family members (Ellerman et al., 2006). As described for rCRISP1, evidence supports the involvement of both the mouse and the human CRISP1 homologues in gamete fusion through their binding to complementary sites on their eggs (Cohen et al., 2000a, 2001).

In addition to its role in gamete fusion, recent experiments in which rat and mouse ZP-intact eggs were inseminated with capacitated sperm in the presence of either anti-rCRISP1 or purified rCRISP1 revealed a novel role for the protein in the previous step of sperm–ZP interaction (Busso et al., 2007a). Although this activity resides in the peptidic region of the molecule, the domain within which it is located is still unknown.

Evidence for another functional role for rCRISP1 comes from experiments showing an inhibitory activity of the protein on both sperm protein tyrosine phosphorylation and progesterone-induced AR during capacitation (Roberts et al., 2003). Based on the finding that CRISP proteins from snake venoms possess an ion-channel regulatory activity located in the CRD, it was suggested that rCRISP1 might interfere with the uptake of ions required for this process (Roberts et al., 2006). Considering that part of the protein is released during capacitation (Cohen et al., 2000b), rCRISP1 might act as a decapacitation factor, regulating ion-channels through its CRD.

In an effort to elucidate the functional role of CRISP1 in the fertilization process, we generated mice containing a targeted disruption of the Crisp1 gene. While their fertility was normal, the mutant animals produced sperm with impaired fertilizing ability.

Materials and methods

Generation of CRISP1-deficient mice

Exon and intron nomenclature are those from Schwidetzky et al. (1997) and nucleotide numbers in the *Crisp1* genomic sequence are those from Mouse Genomics Informatics accession IDs: MGI: 102553. Different genomic clones encoding the *Crisp1* gene were isolated from a Lambda mouse 129/SvEv genomic library (Stratagene, La Jolla, CA) and used to construct the homologous recombination arms of the targeting vector. The 5′ arm consisted of a 1.4 kb *Sca*I/*Nco*I fragment containing part of intron A (nucleotides 4212:5626), and the 3′arm was a 4.7 kb *Eco*RV/*Xba*I fragment spanning from intron B to intron D (nucleotides 7687:12417). Both arms were subcloned in a pUC vector containing neomycin resistance (neo^r) and thymidine kinase (tk) cassettes (see Fig. 1A). The *Sal*I-linearized *Crisp1* targeting vector was electroporated into TC-1 embryonic stem (ES) cells, and neomycin (G418)- and ganciclovir-resistant colonies were selected. Homologous recombination was assessed by PCR using the following primers: forward 5′-CAAACCAGTAGCCAACATCAAAGTAA-3′ and reverse 5′-CTGCACGA-GACTAGTGAGACGT-3′ that correspond to sequences in intron A (outside the targeting sequence) and neo^r, respectively. Genotypes were confirmed by Southern blot after digestion with *Pvu*II, using as a probe a PCR product (primers 5′-GTTTCTCCATCTGG-CAGTGACTT-3′ and 5′-GAGAGGGAGAGGCAATAACACAGT-3′) comprising *Crisp1* exon 3 and part of intron C.

Four different ES cell clones were injected into C57BL/6N mouse blastocysts that were then transferred into pseudopregnant foster mothers. Chimeric males produced from two independent ES cell clones were backcrossed with C57BL/6N females to obtain F1 heterozygotes, and intercrossing between heterozygous mice produced F2 offspring. Genotypes were determined by PCR using 3 primers for amplification of both a Crisp1 gene-specific 894 bp fragment (forward 5′-AGACAAAGAGACCACCAACAGATT-3′ and reverse 5′-AGTACAGCAGCCAAGAAGAACAG-3′) and a neo^r gene-specific 611 bp fragment (reverse 5′-CTACCCGCTTCCATTGCTC-3′). No differences in phenotypes were detected among CRISP1-deficient mice derived from the two different ES cell clones.

The animals were housed on a 12h-12h light-dark cycle with *ad libitum* access to food and water. All protocols were in accordance with the *Guide for Care and Use of Laboratory Animals* published by the National Institute of Health and studies performed at NIEHS were approved by the NIEHS Animal Care and Use Committee.

RT-PCR analysis

Total RNA from epididymis was isolated with Trizol® (Gibco BRL, Rockville, MD), according to the manufacturer's recommendations, reverse transcribed and subjected to PCR using primers complementary to exons 2 and 8 of the *Crisp1* gene (forward 5′-AAGCCATCAGAATTCCAAGATAGCTCTCAG-3′ and reverse 5′-CTGCTGCAGGTCTGGAAT-TATTTCAATGTC-3′).

Immunoblot analysis

Mouse epididymides and testes were homogenized in 1.5 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF). The homogenates were then centrifuged twice at 10,000×*g* for 20 min at 4 °C and the supernatants were dialyzed against 50 mM Tris–HCl buffer (pH 6.8). Protein samples (50 μg/lane) were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Towbin et al., 1979). After blocking with 2% skim milk in PBS, the membranes were probed with either anti-rCRISP1 (Kohane et al., 1983) (1:200) or anti-CRISP2 polyclonal antibody (kindly provided by Dr. D. Hardy, Texas Tech University) (1:200), or anti-β tubulin monoclonal antibody (1:40,000; clone D66, Sigma, St. Louis, MO, USA) followed by the corresponding peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected by an ECL Western Blotting kit (GE Healthcare UK Ltd, Buckinghamshire, England).

Assessment of fertility

Individual *Crisp1*$^{-/-}$ males (2 to 6 months old) or their *Crisp1*$^{+/+}$ and *Crisp1*$^{+/-}$ littermates were housed with 2 *Crisp1*$^{+/+}$ females of proven fertility for 2 weeks. Each male was bred with at least 6 females. Mating was confirmed by detection of a copulatory plug. The fertility of each male was analyzed for average litter size, average pup weight and the time from the beginning of mating to the delivery of the pups. For evaluation of fertility between *Crisp1*$^{-/-}$ partners, each *Crisp1*$^{-/-}$ male was housed with a *Crisp1*$^{-/-}$ and a *Crisp1*$^{+/+}$ female for 2 weeks.

In vitro sperm capacitation

Mouse sperm were recovered by incising the cauda epididymides in 300 μl of capacitation medium (Fraser and Drury, 1975) supplemented with 0.3% of bovine serum albumin (BSA) under paraffin oil. Aliquots of the suspension were added to 300 μl of fresh medium previously placed in tissue culture dishes to give a final concentration of 0.1–1×10^7 cells/ml. Sperm suspensions were then incubated for 90–120 min under paraffin oil at 37 °C in an atmosphere of 5% CO_2 in air.

In vitro sperm determinations

a) *Morphology and motility assessment:* 10 μl of sperm suspensions was placed on pre-warmed slides and analyzed subjectively under a light microscope (400×).
b) *Spontaneous and progesterone-induced AR*: for induction of the AR, progesterone (15 μM final concentration; Sigma) in dimethylsulfoxide (DMSO) was added to sperm for the last 15 min of incubation. The acrosomal status was evaluated by Coomassie brilliant blue staining as previously described (Busso et al., 2007b).
c) *Protein tyrosine phosphorylation:* sperm collected at different capacitation times were washed with PBS and resuspended in Laemmli sample buffer (Laemmli, 1970). After a 5 min incubation, samples were boiled and centrifuged at 5000×*g* for 5 min. The supernatants were recovered and boiled in the presence of 70 mM 2-β-mercaptoethanol (Sigma). Solubilized proteins (corresponding to 0.5–1×10^6 spermatozoa/lane) were separated by SDS–PAGE, transferred onto nitrocellulose, and immunoblotted with the anti-phosphotyrosine monoclonal antibody (1:10,000; clone 4G10; Upstate, Lake Placid, NY) as previously described (Da Ros et al., 2004).

In vitro fertilization assays

a) *Recovery and treatment of oocytes*: immature *Crisp1*$^{+/+}$ female mice were super-ovulated by an injection (i.p.) of equine chorionic gonadotropin (eCG; 5 UI; Syntex, Argentina), followed by the administration (i.p.) of human chorionic gonadotropin (hCG; 5UI; Sigma) 48 h later. Oocytes were collected from the oviducts of superovulated animals 12–13 h after hCG administration. Cumulus cells were removed by incubating the oocyte–cumulus complexes for 3 min in 0.3 mg/ml hyaluronidase (type IV; Sigma). The ZP were dissolved by treating the oocytes with acid Tyrode solution (pH 2.5) for 10–20 s (Nicolson et al., 1975). For experiments involving the effect of different CRISP proteins on gamete fusion, ZP-free oocytes were incubated for 30 min before insemination with medium alone or medium containing either native rCRISP1 (6 μM) (Garberi et al., 1979, 1982), bacterially

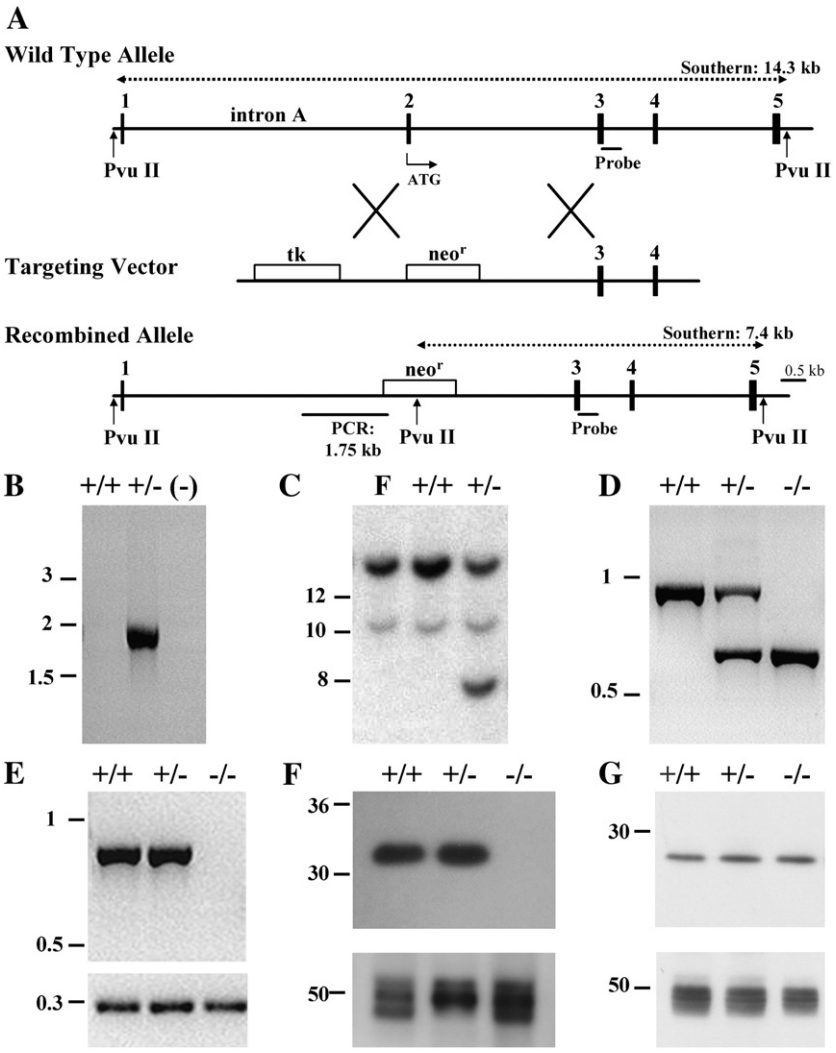

Fig. 1. Disruption of the mouse *Crisp1* locus. (A) Schematic representation of the wild type allele, targeting vector and recombined allele. The black rectangles and the numbers above them denote the exons in the *Crisp1* gene. The open boxes represent the negative (tk) and positive (neo^r) selection cassettes. The locations of the PCR product, the *Pvu* II restriction sites and the probe used for the Southern blot are denoted. (B) Genomic DNA from wild type and drug-resistant ES cells was amplified by PCR using primers within intron A and the neo^r gene, resulting in a 1.75 kb-fragment in the mutated allele (+/−) but not in the wild type allele (+/+) or in the no DNA control (−). An ethidium bromide-stained 1% agarose gel of PCR products is shown. (C) Genomic DNA samples from ES cells that were positive in the 5′ PCR were analyzed by Southern blot. As indicated in panel A, the probe hybridized with a 14.3 kb fragment in the *Pvu* II-digested DNA of wild type ES cells (+/+), targeted ES cells (+/−), and feeder layer cells (F), and with a 7.4 kb fragment in targeted ES cells (+/−). (D) DNA samples of *Crisp1*$^{+/+}$, *Crisp1*$^{+/-}$ and *Crisp1*$^{-/-}$ mice were amplified using a combination of 3 primers. Amplicons of 894 bp or 611 bp were produced from the wild type or mutated allele, respectively as shown in an ethidium bromide-stained 2% agarose gel. (E) Total epididymal RNA was subjected to RT-PCR using primers complementary to exons 2 and 8. Products were separated on a 1.5% agarose gel and stained with ethidium bromide. PCR primers for actin were used as template controls (bottom panel). (F) Epididymal proteins were separated on a 12% SDS gel, transferred to nitrocellulose and immunoblotted using anti-rCRISP1. (G) Testicular protein extracts were separated on a 15% SDS gel, transferred to nitrocellulose and immunoblotted using anti-CRISP2. β-tubulin was used as loading control in F and G (bottom panels). Size (kbp) or molecular weight (kDa) standards are shown on the left of each panel.

expressed mouse CRISP2 (mCRISP2, 30 μM) (Busso et al., 2007b) or bacterially expressed human CRISP1 (hCRISP1, 30 μM) (Hayashi et al., 1996). In parallel, protein-incubated eggs were subjected to indirect immunofluorescence as previously described (Ellerman et al., 2006) using anti-rCRISP1 (1:50) or anti-MBP (1:100) for native or recombinant proteins, respectively.

b) *Gamete co-incubation*: cumulus-intact and ZP-intact eggs were inseminated with capacitated sperm (final concentration 0.5–2×10^5 sperm/ml) and gametes were co-incubated for 3 h at 37 °C in an atmosphere of 5% CO_2 in air. ZP-free eggs were inseminated with capacitated sperm (final concentration: 0.5–1×10^4 cells/ml) and gametes co-incubated for 1 h. In all cases, eggs were then washed, fixed with 2% paraformaldehyde, and stained with 1 μg/μl Hoechst 33342 (Sigma). Finally, eggs were mounted on slides and analyzed under a UV microscope (250×). Eggs were considered fertilized if at least one decondensing sperm nucleus could be observed in the ooplasm. For competition assays, sperm from *Crisp1*$^{+/+}$ and *Crisp1*$^{-/-}$ mice were capacitated and, during the last 15 min of incubation, one of the two populations was exposed to 100 nM MitoTracker® Green FM (Invitrogen Corporation, Carlsband, CA). Subsequently, the two populations were used individually or mixed in equal concentration to inseminate ZP-free eggs. Gamete co-incubation and determination of the percentage of penetrated eggs were conducted as described above, with the exception that in this case eggs were not fixed. The same results were obtained independently of the sperm population that was stained.

Calculations and statistical analysis

Fertility parameters levels were analyzed by the *t*-student test. The percentages of motile sperm and fertilized eggs were analyzed by the chi-square (χ^2) test. AR rates were analyzed using two-way ANOVA. Results were considered significantly different at $P<0.05$.

Results

Generation of CRISP1-deficient mice

To investigate the biological role of CRISP1 *in vivo*, homologous recombination in ES cells was used to generate a mouse line carrying a null mutation in the *Crisp1* gene. The targeting vector was designed to replace a 2 kb region of the gene containing the ATG initiation site,

with neo^r cassette after homologous recombination (Fig. 1A). The correct occurrence of recombination in the ES cells was assessed by PCR for the 5′ arm (Fig. 1B), and by Southern blot using a 3′ probe (Fig. 1C). ES cells lines were injected into blastocysts, and the chimeric animals carrying the mutated *Crisp1* allele were mated to produce the heterozygous mice which were intercrossed to produce the *Crisp1*$^{-/-}$ homozygotes. Genotyping of the progeny was carried out by PCR yielding fragments of 894 bp and 611 bp in wild type and targeted alleles, respectively (Fig. 1D). The obtained genotypes approximated the expected Mendelian distribution (*Crisp1*$^{+/+}$: 21%; *Crisp1*$^{+/-}$: 51%; and *Crisp1*$^{-/-}$: 28%; $n=365$), indicating that the mutation had no embryonic-lethal effect.

Male and female *Crisp1*$^{-/-}$ mice developed normally with no significant differences in behaviour, body size and health condition compared to controls. RT-PCR analysis revealed the absence of *Crisp1* transcripts in epididymal tissue from *Crisp1*$^{-/-}$ mice (Fig. 1E). In agreement with this, Western blot analysis confirmed the lack of CRISP1 protein in *Crisp1*$^{-/-}$ epididymis (Fig. 1F), indicating that the deletion produced a null allele. In all cases, *Crisp1* mRNA and CRISP1 protein were detected in *Crisp1*$^{+/+}$ and *Crisp1*$^{+/-}$ littermates (Figs. 1E and F).

The observations that some targeted gene deletions resulted in a modified expression of other family members (Drabent et al., 2000; Stein et al., 2005) led us to analyze the expression of testicular CRISP2, an intra-acrosomal protein also reported to be involved in mouse gamete fusion through its binding to the same egg-complementary sites than CRISP1 (Busso et al., 2007b). Western blot analysis revealed the presence of CRISP2 in testes from *Crisp1*$^{-/-}$ animals (Fig. 1G) with levels (by densitometry) similar to those corresponding to *Crisp1*$^{+/+}$ and Crisp1$^{+/-}$ mice (data not shown).

Fertility of CRISP1-deficient mice

To study the effect of the absence of CRISP1 on fertility, adult males of the three genotypes were bred with control females for 2 weeks. All male animals were sexually active as assessed by the presence of copulatory plugs in their female partners. As shown in Table 1, *Crisp1*$^{-/-}$ animals presented no significant differences in the average litter size compared to *Crisp1*$^{+/-}$ or *Crisp1*$^{+/+}$ mice. In addition, the average pup weight and the number of days from the beginning of the mating to the delivery were similar among the three genotypes (Table 1). No differences were either observed in the average litter size when *Crisp1*$^{-/-}$ males were bred with *Crisp1*$^{-/-}$ females (*Crisp1*$^{-/-}$×*Crisp1*$^{+/+}$: 6.75±0.25, *Crisp1*$^{-/-}$×*Crisp1*$^{-/-}$: 6.0±1.8, $n=4$, NS).

Sperm analysis in CRISP1-deficient mice

Sperm from *Crisp1*$^{-/-}$ mice were subjected to *in vitro* analysis for potential differences that could have been overcome under *in vivo* conditions. Histological examination of testicular and epididymal tissue from *Crisp1*$^{-/-}$ mice showed seminiferous and epididymal tubules with normal diameters and abundant spermatogenic cells in the epithelium and sperm in the lumen (data not shown). Moreover, analysis of epididymal sperm from *Crisp1*$^{-/-}$ mice revealed no differences in their number, shape or size compared to those from *Crisp1*$^{+/+}$ or Crisp1$^{+/-}$ mice (data not shown).

Table 1
Fertility of CRISP1-deficient mice[a]

Genotype	Litter size (# of pups)	Pup weight (g)	Time to delivery (days)
Crisp1$^{+/+}$	7.3±0.3 (6)	1.41±0.10	27.4±3
Crisp1$^{+/-}$	6.3±0.5 (7)	1.55±0.04	31.7±3
Crisp1$^{-/-}$	6.5±0.4 (12)	1.48±0.04	28.5±2

[a] Data represent the mean value±SEM of at least 5 independent experiments. The number of mated males is shown in brackets. No statistically significant differences were found among genotypes for any of the variables.

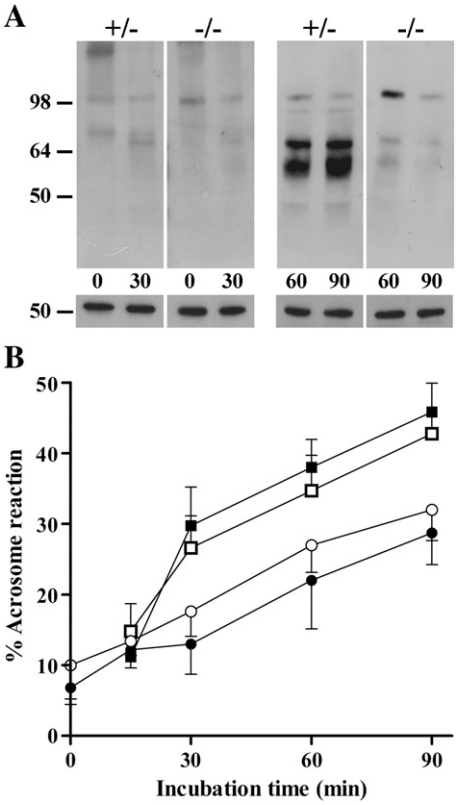

Fig. 2. Assessment of sperm capacitation. (A) Epididymal sperm from *Crisp1*$^{+/-}$ and *Crisp1*$^{-/-}$ mice were subjected to capacitation, aliquots were removed every 30 min and sperm proteins were analyzed by Western blotting using an anti-phosphotyrosine monoclonal antibody. β-tubulin was used as loading control (bottom panel). The blot shown is representative of 7 independent experiments. (B) Epididymal sperm from *Crisp1*$^{+/-}$ (black) and *Crisp1*$^{-/-}$ (white) mice were capacitated in the absence (circles) or presence (squares) of 15 μM progesterone during the last 15 min of incubation. Aliquots were removed at different time points and cells subjected to Coomassie Blue staining to analyze the acrosomal status. Results represent the mean±SEM of 7 independent experiments.

Sperm capacitation and AR

Having established that sperm lacking CRISP1 did not present apparent structural and/or morphological differences compared to controls, several functional parameters were analyzed. Motility of fresh (*Crisp1*$^{+/+}$ 66.7±4.5%; *Crisp1*$^{+/-}$ 58.0±6.6%; *Crisp1*$^{-/-}$ 66.7±4.3%; NS) or capacitated (*Crisp1*$^{+/+}$ 61.3±5.1%; *Crisp1*$^{-/-}$ 55.3±2.9%; *Crisp1*$^{-/-}$ 63.8±4.2%; NS) epididymal sperm was not significantly different among the three groups. However, evaluation of protein tyrosine phosphorylation during capacitation revealed that *Crisp1*$^{-/-}$ sperm exhibited levels clearly lower than those of the controls at the end of the incubation (Fig. 2A). In spite of this, sperm from *Crisp1*$^{-/-}$ and *Crisp1*$^{+/-}$ mice showed a similar time-dependent increase in their ability to undergo the spontaneous or progesterone-induced AR during capacitation (Fig. 2B).

Sperm fertilizing ability

To evaluate whether the absence of CRISP1 affected the sperm fertilizing ability, sperm from *Crisp1*$^{-/-}$ and *Crisp1*$^{+/-}$ mice were capacitated and used in *in vitro* fertilization assays. *Crisp1*$^{-/-}$ sperm were as capable as control sperm of fertilizing cumulus-intact eggs (Fig. 3A). However, when sperm were co-incubated with ZP-intact eggs devoid of cumulus cells, the percentage of penetrated eggs was significantly lower for *Crisp1*$^{-/-}$ than for *Crisp1*$^{+/-}$ mice (Fig. 3B) with no accumulation of sperm in the perivitelline space. To examine whether

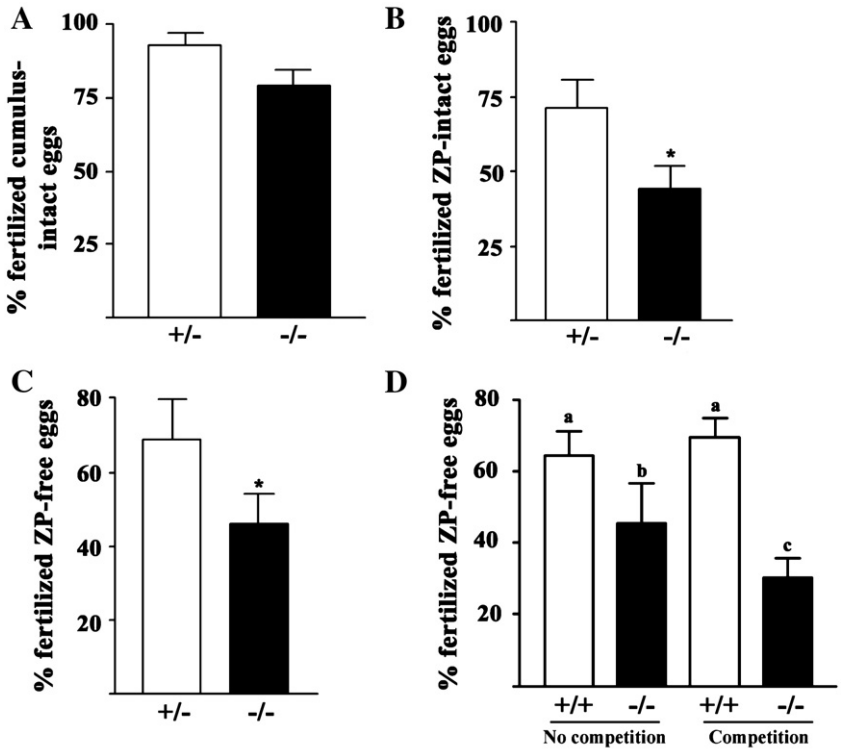

Fig. 3. Evaluation of sperm fertilizing ability. *Crisp1*$^{-/-}$ capacitated sperm were used to inseminate either cumulus–oocytes complexes (A), ZP-intact eggs (B) or ZP-free eggs (C), obtained from *Crisp1*$^{+/+}$ females. (D) *Crisp1*$^{+/+}$ or *Crisp1*$^{-/-}$ capacitated sperm were stained with MitoTracker® Green FM and then used individually (no competition) or in combination (competition) to inseminate ZP-free eggs. Results represent the mean ± SEM of at least 5 independent experiments. A and B (*, $p=0.0002$), C (*, $p=0.0001$) and D (a vs. b, $p<0.05$; a vs. c, $p<0.0001$; b vs. c, $p<0.05$).

sperm lacking CRISP1 showed any defects in their fusogenic ability, oocytes devoid of both cumulus and ZP were used. In this case, *Crisp1*$^{-/-}$ sperm also exhibited a significant reduction in the fertilizing ability compared to controls (Fig. 3C). This effect was further investigated by simultaneously inseminating ZP-free eggs with *Crisp1*$^{+/+}$ and *Crisp1*$^{-/-}$ sperm in a competition assay. Under these conditions, sperm from *Crisp1*$^{-/-}$ mice showed a greater reduction in their fusion ability compared to that observed in a non-competitive situation (Fig. 3D).

Considering that sperm–egg fusion is inhibited by the presence of rCRISP1 during gamete co-incubation, we investigated whether this effect was still observed when the eggs were inseminated with sperm lacking CRISP1. An inhibitory effect under these conditions would be consistent with the participation of other CRISP protein/s in gamete fusion, through their binding to common egg-complementary sites. To investigate this possibility, ZP-free eggs and sperm from *Crisp1*$^{-/-}$ mice were co-incubated in the presence of rCRISP1 or other CRISP homologue proteins available to our laboratory, including mCRISP2, and hCRISP1. Results revealed that rCRISP1 and mCRISP2 which are capable of binding to the mouse egg significantly inhibited egg penetration (Fig. 4). By contrast, hCRISP1 failed to both bind to the egg and affect gamete interaction even when used at the same concentration as mCRISP2 (Fig. 4).

Discussion

A mouse line bearing a targeted deletion of the *Crisp1* gene was generated to elucidate the functional role of epididymal protein CRISP1 in the fertilization process. When *Crisp1*$^{-/-}$ males were bred with females of proven fertility, they were found to be fertile. Mating of *Crisp1*$^{-/-}$ males with *Crisp1*$^{-/-}$ females also showed normal fertility rates indicating that the genetic modification did not affect male or female fertility. This result adds to a growing number of reports of fertile mice lacking proteins proposed to be involved in the fertilization process (Okabe and Cummins, 2007). Based on the observation that these mice often exhibited other reproductive deficiencies (Baba et al., 2002; Lu and Shur, 1997), *Crisp1*$^{-/-}$ males were evaluated for evidence of changes in various parameters related to reproductive function. No differences compared to controls were observed in the testes or epididymides at histological level and no

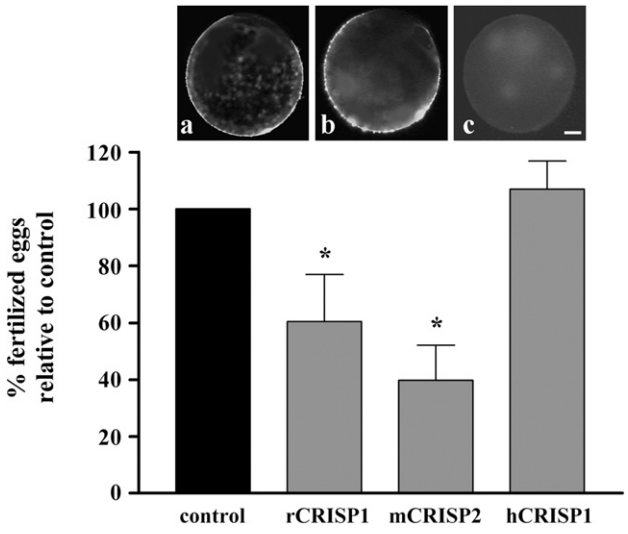

Fig. 4. Evaluation of the involvement of other CRISP proteins in gamete fusion. Capacitated *Crisp1*$^{-/-}$ sperm were co-incubated with *Crisp1*$^{+/+}$ ZP-free eggs in medium alone (control) or in the presence of 6 μM rCRISP1, 30 μM mCRISP2, or 30 μM hCRISP1. Results are expressed as relative to control (assigned a value of 100%) and represent the mean ± SEM of at least 4 independent experiments (*, $p<0.02$). As a control, eggs incubated with rCRISP1 (a), mCRISP2 (b), or hCRISP1 (c), were subjected to indirect immunofluorescence. Scale bar = 10 μm.

apparent changes were detected in the morphology or motility of epididymal sperm either before or after capacitation. Because it has been reported that the presence of CRISP1 during rat sperm capacitation inhibits protein tyrosine phosphorylation (Roberts et al., 2003), we investigated whether the absence of the protein in the mutant mice produced an increase in the levels of this capacitation-dependent event. Capacitated sperm from *Crisp1*$^{-/-}$ mice exhibited, however, clearly lower levels of tyrosine phosphorylation than controls, suggesting that CRISP1 might play a regulatory role during mouse sperm capacitation different from that proposed for the rat (Roberts et al., 2006). An alternative possibility is that the decrease in tyrosine phosphorylation is due to an indirect effect produced by the absence of CRISP1 in the sperm plasma membrane. The molecular mechanism underlying this inhibition is at present under investigation. In spite of the lower levels of tyrosine phosphorylation in *Crisp1*$^{-/-}$ mice, no differences were observed in either the spontaneous or progesterone-induced AR when compared to controls. These observations are in agreement with previous reports showing normal levels of AR even in mutant sperm in which tyrosine phosphorylation was completely abolished (Xie et al., 2006). As far as we know, this is the first report showing a significant decrease in tyrosine phosphorylation accompanied by normal levels of fertility. These results suggest that protein tyrosine phosphorylation is either not required or, required in low levels such as those observed in our study, to achieve spontaneous or progesterone-induced AR or normal fertility.

In vitro fertilization experiments showed that *Crisp1*$^{-/-}$ sperm were as capable as control sperm of fertilizing cumulus-intact eggs. The normal fertilization levels seen under these conditions might be due to the known beneficial effects of the cumulus matrix for fertilization (Yanagimachi, 1994). After removal of the cumulus cells, however, *Crisp1*$^{-/-}$ sperm exhibited a fertilizing ability significantly lower than controls. Although we cannot exclude the possibility that this inhibition is due to an effect on sperm capacitation (Nolan et al., 2004; Xie et al., 2006), the observation that sperm from *Crisp1*$^{-/-}$ mice exhibited normal rates of both spontaneous and induced AR, supports an inhibitory effect at the sperm–ZP level. These results are consistent with our recent report proposing a novel role for CRISP1 in sperm–ZP interaction (Busso et al., 2007a).

In agreement with the postulated role of rCRISP1 in gamete fusion (Cohen et al., 2000a, 2001; Rochwerger et al., 1992), *in vitro* assays using ZP-free eggs showed a significant reduction in the fusion ability of *Crisp1*$^{-/-}$ sperm which became even more evident when mutant sperm were subjected to a competitive fertilization assay. This effect is probably not due to the decrease in protein tyrosine phosphorylation because normal levels of gamete fusion have been reported for sperm that have not undergone this event during capacitation (Nolan et al., 2004; Xie et al., 2006). Thus, the overall results of the *in vitro* fertilization studies indicate that sperm lacking CRISP1 present a disadvantage in their ability to both interact with the ZP and fuse with the egg.

Despite the compromised sperm fertilizing ability, *Crisp1*$^{-/-}$ males were fertile. In this regard, it has been reported that different phenotypes can arise from the same mutation depending on the genetic background. For example, female mice bearing a mutation in the CD81 tetraspanin were fertile when originally derived but showed a reduced fertility after backcrossing onto homogenous background (Rubinstein et al., 2006). Thus, the possibility that CRISP1 is essential for male fertility in a different genetic background, cannot be ruled out.

Our observations in *Crisp1*$^{-/-}$ mice might also be due to other CRISP proteins compensating for the lack of CRISP1. This possibility was explored by examining the inhibitory effect of different CRISP proteins on the fusion ability of *Crisp1*$^{-/-}$ sperm. The finding that rCRISP1 and mCRISP2 but not hCRISP1 significantly reduced ZP-free egg penetration by *Crisp1*$^{-/-}$ sperm indicates that a CRISP protein with an egg-binding site homologous to CRISP1 might also be involved in gamete fusion. In this regard, recent experiments using an antibody against CRISP2 (which does not cross react with CRISP1) as well as CRISP1 and CRISP2 proteins in competitive studies, revealed the involvement of CRISP2 in mouse gamete fusion through its interaction with the same egg-binding sites than CRISP1 (Busso et al., 2007b). Together, these results support CRISP2 as a candidate molecule to cooperate with CRISP1 during fertilization. Furthermore, the egg-binding site of mCRISP2 (Signature 2) is only 2 amino acids different from that corresponding to rCRISP1 (Ellerman et al., 2006), suggesting that CRISP1 and CRISP2 might act through a similar molecular mechanism being capable of compensating for each other. A similar situation has been reported for the tetraspanin CD151 knockout mice in which the lack of the protein could be compensated by BAB22942 (Wright et al., 2004), a homologous molecule exhibiting differences in only 2 out of the 11 amino acids of the active site (Berditchevski et al., 2001).

Another member of the CRISP family, CRISP4, has recently been identified in mouse (Jalkanen et al., 2005) and rat (Nolan et al., 2006) epididymides. Although the function of this protein remains unknown, its high homology with hCRISP1 (now considered the human ortholog of rodent CRISP4), opens the possibility that mouse CRISP4 may also be involved in sperm–egg fusion as proposed for hCRISP1 (Cohen et al., 2001). However, the observation that hCRISP1 (with only 1 amino acid different in its Signature 2 compared to rodent CRISP4), failed to both bind to mouse eggs and inhibit their penetration by *Crisp1*$^{-/-}$ sperm does not support CRISP4 as the candidate molecule to compensate for the lack of CRISP1. Nevertheless, it is important to note that the protein compensating for CRISP1 in mutant sperm (i.e. CRISP2, CRISP4 and/or another CRISP) would do so only partially as judged by the significantly impaired fertilizing ability of *Crisp1*$^{-/-}$ sperm. The generation of knockout animals for CRISP2 and CRISP4 will provide important information in this regard.

Previous observations from our laboratory showing that immunization of male and female rats with rCRISP1 significantly inhibited fertility in both sexes supported the relevance of CRISP1 for animal fertility and its potential use for fertility regulation (Cohen et al., 2007). Our present findings do not rule out the possibility of developing a contraceptive approach to block or inhibit CRISP1 protein function in normal adult individuals, a situation clearly different from that corresponding to a knockout animal in which compensatory mechanisms might arise during prenatal or postnatal life.

Together, the results of the present work indicate that CRISP1 is a player in the fertilization system. To our knowledge, this is the first knockout mice generated for a CRISP protein. Continued effort to understand CRISP family function will not only provide a step towards understanding the molecular mechanisms underlying mammalian fertilization but will also provide more information about the versatile capabilities of this growing and evolutionarily conserved protein family.

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