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Cysteine-rich secretory protein-1: structure and function
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·*Review*·

Structure and function of epididymal protein cysteine-rich secretory protein-1

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Abstract

Cysteine-rich secretory protein-1 (CRISP-1) is a glycoprotein secreted by the epididymal epithelium. It is a member of a large family of proteins characterized by two conserved domains and a set of 16 conserved cysteine residues. In mammals, CRISP-1 inhibits sperm-egg fusion and can suppress sperm capacitation. The molecular mechanism of action of the mammalian CRISP proteins remains unknown, but certain non-mammalian CRISP proteins can block ion channels. In the rat, CRISP-1 comprises two forms referred to as Proteins D and E. Recent work in our laboratory demonstrates that the D form of CRISP-1 associates transiently with the sperm surface, whereas the E form binds tightly. When the spermatozoa are washed, the E form of CRISP-1 persists on the sperm surface after all D form has dissociated. Cross-linking studies demonstrate different protein-protein interaction patterns for D and E, although no binding partners for either protein have yet been identified. Mass spectrometric analyses revealed a potential post-translational modification on the E form that is not present on the D form. This is the only discernable difference between Proteins D and E, and presumably is responsible for the difference in behavior of these two forms of rat CRISP-1. These studies demonstrate that the more abundant D form interacts with spermatozoa transiently, possibly with a specific receptor on the sperm surface, consistent with a capacitation-suppressing function during sperm transit and storage in the epididymis, and also confirm a tightly bound population of the E form that could act in the female reproductive tract. *(Asian J Androl 2007 July; 9: 508–514)*

Keywords: cysteine-rich secretory protein-1; epididymis; sperm; capacitation

1 The cysteine-rich secretory protein (CRISP) family

The cysteine-rich secretory proteins (CRISP) derive their name from the relatively high abundance of cysteine residues (16) that are found in, and conserved among, the members of the family [1, 2]. They are members of the CRISP/Antigen 5/PR-1 (CAP) superfamily of proteins that are expressed in mammals, reptiles, insects, plants and fungi and which all share in common the highly conserved CAP domain [3–9]. With a few exceptions, the CRISP proteins are expressed primarily in the reproductive tracts of mammals and in the venoms of various snakes and lizards [1, 9, 10]. The structural details of the CRISP proteins have been determined

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by crystal structure analysis of snake venom CRISP proteins and the NMR solution structure of the cysteine-rich domain of mammalian CRISP-2 [3, 11–13]. These studies demonstrate that the CRISP proteins comprise two distinct domains. The amino terminal half of the protein contains the CAP domain and is highly similar to the structure determined for the pathogenesis-related (PR) protein PR14a [11, 14, 15]. The carboxyl terminal half of the protein is cysteine-rich, containing 10 of the 16 conserved cysteines, and has been referred to as the cysteine-rich domain (CRD) [11]. The distal part of this domain contains six conserved cysteines and is structurally related to a family of cysteine-rich toxin proteins found in sea urchins (i.e. Bgk and Shk) [3, 11]. The proximal region contains four cysteines and appears to act as a linker region between the CAP domain and the CRD. The high sequence similarity between the CRISP family members allows relatively safe extrapolation of these established structures to other family members.

Most of what are known about the molecular mechanism of the CRISP proteins have been derived from studies of the toxin members of the CRISP family found in various snakes and lizards, and most recently from a study of the CRD of mammalian CRISP-2 (also called Tpx-1) [10, 13, 16–20]. These studies demonstrate that the CRISP proteins have ion channel blocking activity. Helothermine, one of the most extensively studied CRISP toxins found in the venom of the Mexican beaded lizard, blocks both voltage-gated Ca^{2+} and K^+ channels, as well as ryanodine receptor Ca^{2+} channels [10, 17, 18]. Several snake venom CRISPs (i.e. natrin, PsTx, pseudecin) have been shown to block Ca^{2+} and K^+ channels and others have been implicated in such activity by their ability to block smooth muscle contraction [5, 16, 19–21]. The region of the CRISP protein required for channel blocking activity has not been determined for the venom CRISP to date. However, it has been recently shown that the CRD from mammalian CRISP-2 alters Ca^{2+} movement through the ryanodine receptor channel [3]. This study is important because it demonstrates a molecular activity of a mammalian CRISP protein that is similar to that demonstrated for toxin CRISP proteins and it also demonstrates that this activity lies in the CRISP domain. This result is consistent with the structural similarity of the CRISP domain to the ion channel-blocking proteins Bgk and Shk produced by sea urchins [22, 23]. On the basis of the finding that the CRD of mammalian CRISP-2 has channel-blocking activity, it has been proposed that this region of the protein be referred to as the ion channel regulator (ICR) domain [3]. The various domain features of the CRISP proteins are illustrated in Figure 1.

Given that many members of the CAP superfamily do not encode an ICR/CRISP domain, including the wasp venoms, the plant pathogenesis resistance proteins, and the mammalian GliPR proteins, it is likely that the CAP domain performs an independent function. This is best exemplified by comparing the *Xenopus laevis* proteins XCRISP and Allurin. XCRISP, a true CRISP protein, is involved in the degradation of the vitelline envelope and the ICR/CRD is required for this activity [7]. Allurin, a CAP protein without the ICR/CRD, is a chemo-attractant for spermatozoa and this activity must reside in the CAP domain [24]. Mammalian CRISP-1 inhibits sperm-egg fusion when incubated with zona-free eggs and capacitated spermatozoa [25, 26]. Although the molecular mechanism responsible for this inhibition is not known, the region of CRISP-1 responsible for blocking sperm-egg fusion has been localized to the CAP domain [26]. This is the first activity reported for a CRISP protein that is confined to the highly conserved region of the PR domain.

Figure 1. The domain organization of the cysteine-rich secretory protein (CRISP) is shown with the primary amino acid sequence of Stecrisp, a representative toxin CRISP protein that has been crystallized and its structure determined [11]. The CRISP/Antigen 5/PR-1 (CAP) domain comprises the majority of the sequence beginning at the amino terminus. The cysteine rich domain (CRD) is shown with its hinge and ion channel regulatory (ICR) regions. The 16 conserved cysteines are highlighted in gray and the pattern of disulfide bonds are shown by gray lines. The cysteine-cysteine disulfide bonds are contained within each domain of the protein. The dotted lines connect adjacent amino acids at the boundaries of the domains.

2 The mammalian *Crisp* gene family

The first CRISP protein described, which later became known as CRISP-1, was found in the rat epididymis by Cameo and Blaquier [27]. Later, a second CRISP protein was discovered (TPX-1/CRISP-2) in the testis, followed by a third (CRISP-3) expressed in several different tissues, including the salivary glands, prostate and B cells [2, 28]. As the CRISP proteins were characterized from other species, their number designations (1, 2 or 3) were based, at least in part, on their tissue distribution patterns. Therefore, the CRISP protein expressed in the human epididymis was designated CRISP-1, as were those from other species, and these have been assumed to be orthologous to the murine *Crisp-1* genes [29–32]. The recent discovery of a fourth CRISP protein (designated CRISP-4) in the mouse and, subsequently, rat, has illuminated a flaw in the assumption that murine (rat and mouse) *Crisp-1* is orthologous to human and other mammalian *Crisp-1* genes [33, 34]. Murine CRISP-4 is also produced in the epididymis, but in the more proximal caput epididymidis, and has much higher amino acid sequence similarity to human CRISP-1 than does murine CRISP-1 [33, 34]. The relationship of these mammalian *Crisp* genes and the syntenic regions of their respective genomes is illustrated in Figure 2. The discovery of murine *Crisp-4* and the careful analysis of the human, murine and rat genome, suggests that *Crisp-1* in the murine species is an additional epididymal form of *Crisp* that might not be present in humans and other mammals. Additionally, mouse CRISP-1 and CRISP-3 might be the products of a gene duplication event in that they are over 75% identical and are adjacent to each other in the mouse genome. There is no such duplication apparent in the rat genome and, therefore, there might be no equivalent *Crisp-3* gene in the rat. The presence of four *Crisp* genes might be unique to the murine genome.

The discovery of the CRISP-4 proteins in the murine epididymis introduces two caveats into the study of CRISP proteins in the rat or murine epididymis. First, as the true ortholog to human *Crisp-1*, murine *Crisp-4* genes and their products are the most relevant model system with which to study the role of human CRISP-1 in reproductive biology. Second, the presence of both CRISP-1 and CRISP-4 proteins must be considered and ultimately accounted for in the pursuit of CRISP functionalities in the murine epididymis. In contrast, the testicular CRISP-2 protein is well conserved from species to species and its testicular expression is very consistent [28, 31, 35–37]. On the basis of sequence analysis in non-murine species, human *Crisp-3* does not appear to be a true ortholog of *Crisp-1* in the rat or of either *Crisp-1* or *Crisp-3* in the mouse, as might be expected. However, much work remains to be done on the categorization of these members of the *Crisp* family.

3 Protein D and E forms of rat CRISP-1

As first described by Cameo and Blaquier [27], CRISP-1 was initially thought to be two proteins, which were given the designation as bands D and E, corresponding to their electrophoretic mobility on non-denaturing polyacrylamide gels. Shortly after this report, Lea and French [38] reported the isolation of an acidic epi-

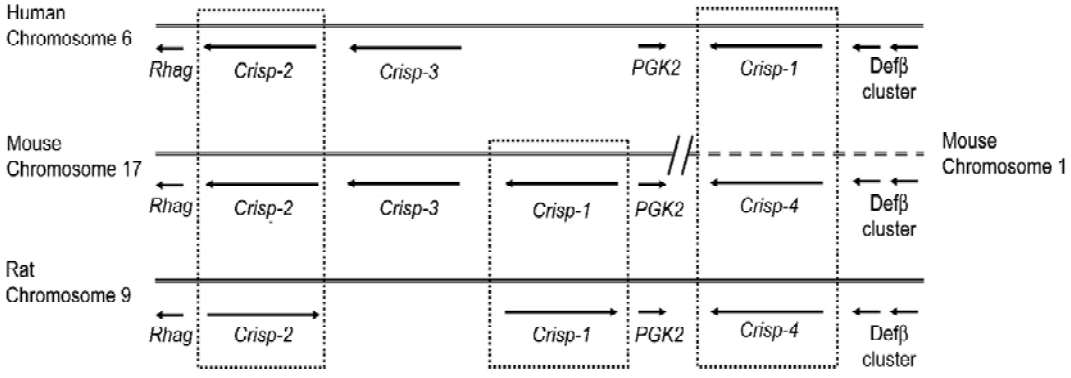

Figure 2. The syntenic regions encoding the *Crisp* gene cluster in the human, murine and rat genomes are highly conserved. Each is flanked by the *Rhag* gene and a cluster of beta defensin genes, with the *PGK-2* gene contained within. This region in the murine genome is split between chromosome 17 and 1. Sequence comparison illustrates that the *Crisp-2* genes in all three species are true orthologs, whereas the human *Crisp-1* gene is the ortholog of the mouse and rat *Crisp-4* genes (boxes with dotted lines). The murine and rat *Crisp-1* genes are highly similar and appear to be orthologs, but the murine and human *Crisp-3* genes are not. Only the murine genome appears to harbor four *Crisp* genes and the high degree of similarity between the murine *Crisp-1* and *Crisp-3* genes suggests that they might represent a gene duplication event in the mouse.

didymal glycoprotein, referred to as AEG, which turned out to be proteins D and E. The D and E proteins were carefully characterized by Brooks [39] and no apparent biochemical or immunological differences could be demonstrated between them. Consequently, proteins D and E were assumed to be isoforms of the same protein and the name "Protein DE" became affixed to the protein.

Although initially believed to be immunogenically identical, there is at least one epitope that distinguishes the Protein E form of CRISP-1 from the Protein D form. This epitope is recognized by monoclonal antibody 4E9 [40]. The 4E9 antibody was not developed by immunization with CRISP-1 polypeptides but by screening hybridomas created after immunizing mice with a fraction of rat epididymal proteins that bound to a ricin affinity column. The protein recognized by mAb 4E9 turned out to be the E form of rat CRISP-1 [41, 42]. The epitope for mAb 4E9 was shown to be located at the amino terminus of the protein that contributed approximately 2 kDa to the molecular weight based on relative mobility in SDS-PAGE [42]. Recent comparative mass spectroscopy (MS) analysis of tryptic fragments of purified CRISP-1 D and E revealed that the first tryptic cleavage site in the N-terminus of the D form is uncleaved in the E form (Figure 3A). The N-terminal peptide fragment in the E form, covering the combined first and second tryptic peptides from the D form, was positively identified yet contained an additional 203 dalton substituent (Figure 3B). This added mass found in a peptide containing one serine and four threonine residues is consistent with the hypothesis that the E form of rat CRISP-1 contains an O-linked glycosylation containing at least one N-acetylglucosamine or N-acetylgalactosamine. These two differences near the amino terminus revealed by mass spectrometry (MS/MS) analyses suggests that they might be contributing, at least in part, to the unique 4E9 epitope on Protein E (Figure 3C). A detailed analysis of the glycosylation of both Proteins D and E is currently underway to test the hypotheses generated by this initial observation.

Although the molecular differences between the Protein D and E forms of CRISP-1 appear relatively subtle, the sperm binding characteristics of Proteins D and E, and likely their activities, are very different [43, 44]. We have shown that Protein D is readily removed by gentle washing and, therefore**,** appears to interact transiently with the sperm surface [44]. We have also shown that exogenous CRISP-1 will inhibit capacitation when rat spermatozoa are incubated under capacitating conditions [44]. We have recently refined these observations and shown that Protein D exists in a binding equilibrium with the sperm surface that is concentration-dependent (Roberts *et al.*, unpublished data). In these same experiments we have shown that the degree of Protein D binding correlates well with the degree of capacitation inhibition that is achieved by Protein D. In contrast, Protein E binds essentially irreversibly to the sperm surface [43, 44]. In addition, the binding of Protein E to the spermatozoa appears to require other proteins produced by the epididymal epithelium and it is only the smallest

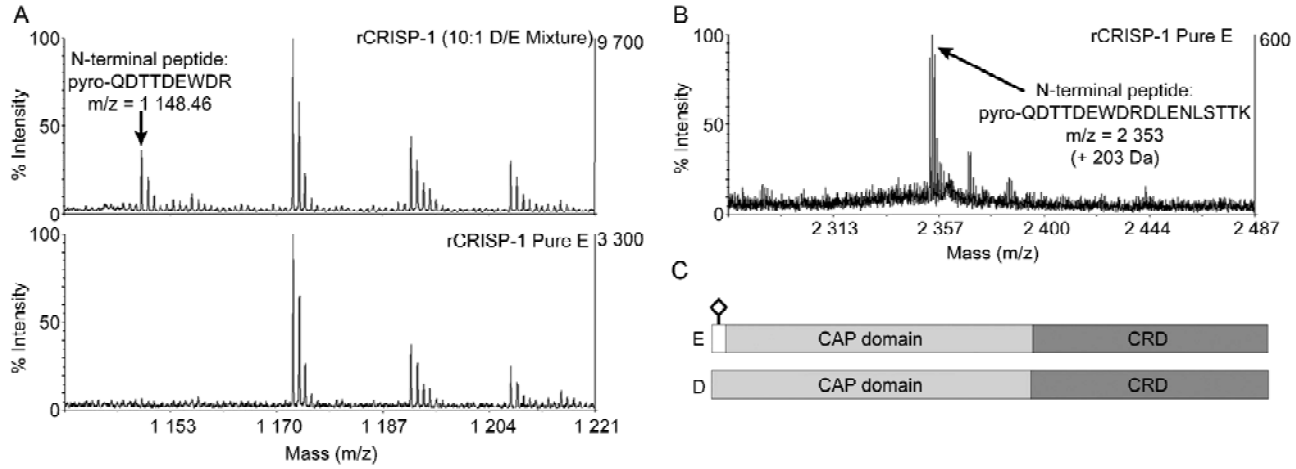

Figure 3. Mass spectrometry (MS) analysis of rat cysteine-rich secretory protein-1 (CRISP-1), a mixture of proteins D and E forms containing the D form in at least 10-fold excess, and of purified rat Protein E. The absence of the N-terminal tryptic peptide in the E form is apparent (A). The first tryptic peptide of pure Protein E was found in the spectrum but was found to carry an adduct of 203 daltons (B). The weight of this adduct is consistent with an N-acetylglucosamine or N-acetylgalactosamine residue that may modify one of the four threonines or the serine present in this peptide. The location of this adduct is illustrated in (C). CAP, CRISP/Antigen 5/PR-1; CRD, cysteine-rich domain; rCRISP-1, rat CRISP-1.

form (approximately 22 kDa) of Protein E that binds to the spermatozoon (Roberts *et al*., unpublished data). The different characteristics of binding imply that Protein D and Protein E are binding to different acceptor molecules on the surface of the spermatozoon. In fact, when rat spermatozoa are exposed to a potent, non-specific protein cross-linking agent and then Proteins D and E are analyzed by western blotting, it is apparent that the two forms of CRISP-1 bind to, or are in close proximity to, different proteins on the sperm surface (Figure 4). The Protein D form of CRISP-1, predominately detected by anti-peptide antibody CAP-A, detects a cross-linked complex at greater than 250 kDa (Figure 4A, arrow head). MS analysis shows that this complex contains CRISP-1. The identity of potential binding partners or near neighbors is now being determined. When this same western blot is stripped and re-probed with monoclonal antibody 4E9, specific for the Protein E form of CRISP-1, the pattern of cross-linked proteins is entirely different (Figure 4B), indicating that the protein D and E forms of CRISP-1 interact with, or are situated near, distinct sperm proteins. The molecular determinant(s) of the different mechanisms by which Protein E binds to spermatozoa, compared with Protein D, remain to be established. Given the distinct difference in binding behavior between Proteins D and E, it appears unlikely that Protein E has the same sperm function as does Protein D.

4 Summary

The CRISP family of CAP proteins are expressed in the reproductive tissues of mammals, as well as in a few additional tissues, and in the venoms of various reptiles. Many of the venom toxins, and recently the mammalian CRISP-2 protein, have been shown to have ion channel-blocking activity. Rat CRISP-1 is produced in two isoforms: Proteins D and E. The transient association of Protein D with spermatozoa can be correlated with its inhibitory effect on capacitation, while the binding of Protein E to spermatozoa, which is essentially irreversible, does not appear to be consistent with the observed effect of CRISP-1 on capacitation. Cross-linking of Protein D and E to spermatozoa also produces very dissimilar patterns, indicating independent mechanisms whereby these two forms of CRISP-1 associate with the sperm surface. We have recently identified two differences between the Protein D and E forms of rat CRISP-1: a loss of trypsin cleavage at the position 9 arginine and a 203 dalton substituent in the first 18 amino acids of the E form. It remains to be determined if these distinctions contribute to the differences in behavior of Proteins D and E.

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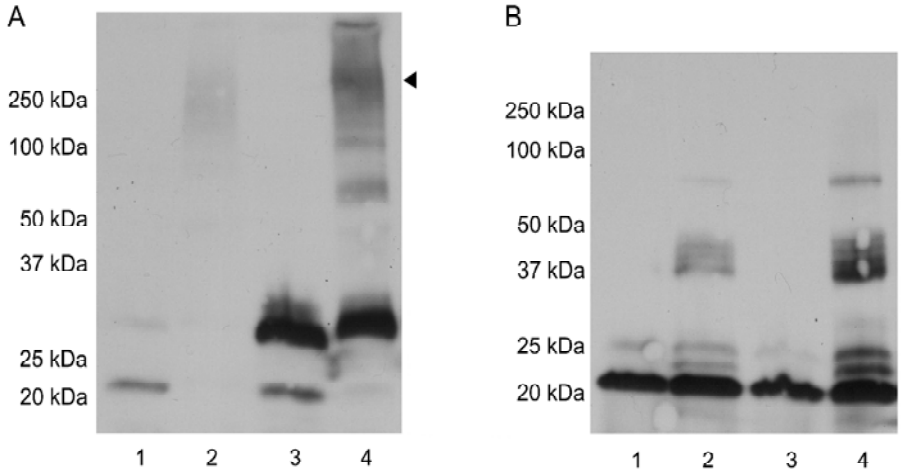

Figure 4. Cross-linking of the Protein D and E forms of rat cysteine-rich secretory protein-1 (CRISP-1) to rat spermatozoa. Rat spermatozoa were washed (lanes 1 and 2) or incubated with exogenous CRISP-1 followed by a brief cold wash (lanes 3 and 4), and then incubated with (lanes 2 and 4) or without (lanes 1 and 3) a homo-bifunctional chemical cross-linker. The spermatozoa were subsequently extracted with detergent to remove all surface and membrane proteins, and the extracts were analyzed by Western blotting with the CAP-A antibody for detection of the D form (A) and mAb 4E9 for detection of the E form of CRISP-1 (B). Successful cross-linking of Protein D requires incubation of spermatozoa with exogenous CRISP-1. The most abundant cross-linked band for Protein D is above 250 kDa. This band was shown by mass spectrometry (MS) analysis to contain CRISP-1. The pattern of cross-linking of Protein E is different from Protein D and, as expected, Protein E can be cross-linked without incubation of spermatozoa with exogenous CRISP-1. These results suggest that the D and E forms associate with different proteins on the surface of the spermatozoon.

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