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Epididymal proteins and male contraception
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·*Review*·

Participation of epididymal cysteine-rich secretory proteins in sperm-egg fusion and their potential use for male fertility regulation

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Abstract

Rat protein DE is an androgen-dependent cysteine-rich secretory protein (CRISP) synthesized by proximal epididymal regions. DE, also known as CRISP-1, is localized on the equatorial segment of acrosome-reacted spermatozoa and participates in gamete fusion through binding to egg complementary sites. Immunization of rats with DE inhibits fertility and sperm fusion ability, suggesting that DE represents a good epididymal contraceptive target. Recombinant DE fragments and synthetic peptides revealed that DE binds to the egg via a 12-amino acid region of an evolutionarily conserved motif, Signature 2 (S2). The ability of other CRISP to bind to the rat egg was correlated with their S2 amino acid sequences. Although testicular protein Tpx-1 (CRISP-2) was capable of binding to rodent eggs, human epididymal AEG-related protein (ARP) and helothermine (from lizard saliva) were not. The S2 region presented only two substitutions in Tpx-1 and four in ARP and helothermine, compared with the DE S2, suggesting that this amino acid sequence was relevant for egg interaction. Studies with Tpx-1 and anti-Tpx-1 revealed the participation of this protein in gamete fusion through binding to complementary sites in the egg. In competition studies, DE reduced binding of Tpx-1 dose-dependently, indicating that both CRISP share the egg complementary sites. That anti-DE and anti-Tpx-1 inhibit sperm-egg fusion while recognizing only the corresponding proteins, suggests functional cooperation between these homologous CRISP to ensure fertilization success. These results increase our understanding of the molecular mechanisms of gamete fusion and contribute to the development of new and safer fertility regulating methods. *(Asian J Androl 2007 July; 9: 528–532)*

Keywords: contraception; cysteine-rich secretory protein; epididymis; gamete fusion; sperm

1 Introduction

There is a need to develop new family planning methods that meet the different needs and preferences of people at different times in their reproductive lives. This is particularly critical for men given that choices available are limited to condoms and vasectomy. In this regard, the epididymis is a good target for contraception because it is not itself involved in sperm production or hormone synthesis, and contraceptive strategies directed towards this organ are unlikely to produce adverse effects. Therefore, specific interference with the acquisition of the sperm fertilizing ability that occurs during epididymal maturation represents an attractive approach to the development of new and safer contraceptive methods. The present article focuses on the results obtained in our laboratory aimed at studying both the involvement of epididymal proteins in sperm–egg interaction and their potential use for fertility regulation.

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2 Epididymal protein DE

Epididymal protein DE was first described by our laboratory [1]. This protein of 32 kDa contains 10% carbohydrates [2, 3], is synthesized in an androgen-dependent manner by the proximal segments of the epididymis, and associates with the sperm surface during epididymal maturation [4, 5]. DE is a member of the cysteine-rich secretory protein (CRISP) family, a large group of secreted proteins with molecular weights of approximately 20–30 kDa, characterized by the presence of 16 conserved cysteine residues, 10 of which are clustered in the C-terminal domain of the molecule. Because it was the first described member of the CRISP family, DE is also known as CRISP-1. Since then, other members of the family have been identified in different mammalian tissues: CRISP-2, also known as Tpx-1, which is expressed in the testis and is synthesized exclusively in the developing spermatids [6, 7], CRISP-3, with a wider tissue distribution than the other CRISP, including reproductive (prostate and ovary) and non-reproductive (salivary gland, pancreas, thymus and colon) organs [8–10], and the recently described CRISP-4, which is exclusively expressed in the epididymis [11, 12]. Other members of the family are present in salivary secretions of certain snakes and lizards, and several proteins with significant homology to the N-terminal domain of CRISP are present in plants, insect and fungi. A recent crystallographic analysis of several CRISP family members revealed that CRISP are modular proteins formed by two domains: a plant pathogenesis-related domain (PR-1) and a cysteine-rich domain (CRD), connected by a short hinge [13]. Although CRISP are found across a broad variety of living forms and exhibit diverse biological functions, the molecular mechanisms underlying these functions remain unknown for most of the CRISP family members.

3 Participation of DE in sperm–egg fusion

Originally localized in the dorsal region of the acrosome, DE migrates to the equatorial segment as the acrosome reaction occurs [14]. The relocation of DE to the equatorial segment, the region through which the sperm fuses with the egg [15, 16] opened the possibility of a role for DE in sperm-egg fusion. The finding that exposure of zona-free rat eggs to purified DE produced a significant reduction in the percentage of egg penetration without affecting the first step of sperm–egg binding indicated that this protein participates in an event subsequent to binding and leading to fusion, through its interaction with complementary sites localized on the egg surface [17]. Indirect immunofluorescence studies show that these DE-binding sites are localized over the entire egg surface with the exception of the area overlying the meiotic spindle [17], a region through which fusion rarely occurs. Therefore, while DE is localized on the fusogenic region of the sperm head, the DE-binding components are localized on the fusogenic area of the egg surface.

Sequential extraction of proteins from epididymal sperm revealed the existence of two populations of DE bound to sperm: a major (70%) population loosely associated with sperm by ionic interactions, which is released from the cells during capacitation and, therefore, is proposed to act as a decapacitation factor [5, 18]; and a minor (30%), tightly bound population, which behaves as an integral protein, remains on sperm after capacitation and corresponds to the protein that migrates to the equatorial segment and participates in gamete fusion (Figure 1) [18].

4 Relevance of DE for fertility

Having established the participation of DE in fertilization, the question arose as to whether this epididymal protein was relevant for fertility. Male and female rats were then immunized with purified DE and analyzed for their subsequent fertility. In this regard, it is important to note that this immunological approach not only provides information on the relevance of a protein for fertility but also represents an excellent tool to neutralize a protein and to identify a potential target for contraception. Results indicated that immunization of rats with DE produced specific antibodies against the

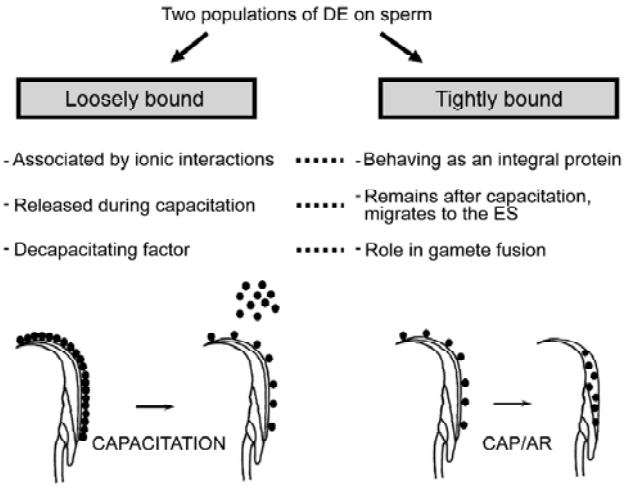

Figure 1. Schematic representation of the relationship between the association of protein DE with spermatozoa, and its behavior and function. Protein DE is represented by a black dot (•). ES, equatorial segment. CAP: capacitation; AR: acrosome reaction.

protein in over 90% of the animals and a significant and reversible inhibition of fertility in both sexes [19].

Subsequent studies confirmed the absence of pathological effects on the reproductive organs and revealed that fertility inhibition would involve the entry of the antibodies into the reproductive tract, their interaction with sperm and their specific interference with the sperm fertilizing ability [20, 21]. Together, these results support both the relevance of DE for fertility and its potential use for contraceptive development.

5 Participation of other epididymal CRISP in gamete fusion

An analysis of the complete sequence of DE showed that it exhibits significant homology (84%) with murine epididymal protein AEG-1/CRISP-1 [8, 22], suggesting its possible involvement in gamete fusion. Results from our laboratory showed that this homologous protein also participates in sperm-egg fusion through its interaction with complementary sites on the surface of the murine egg [23].

DE also exhibits homology (40%) with a human epididymal protein described by two independent laboratories and named ARP (AEG-related protein) [24] or hCRISP-1 [25]. However, recent results showed that this protein presents a higher homology (53%) with epididymal CRISP-4 [12]. The absence of a protein more related to DE in humans, together with the strict epididymal origin of ARP/hCRISP, its molecular weight, and its localization on the sperm head [24], suggest that it could correspond to the molecule performing, in humans, a function equivalent to that of DE in rodents. Although the weak association of ARP/hCRISP to the sperm surface [25] raised the question of whether this protein would have a role in fertilization, sequential protein extraction experiments carried out in our laboratory also indicated the existence of another population of ARP, tightly associated with human sperm [26]. The involvement of ARP in gamete fusion was then evaluated by investigating the effect of an antibody against the recombinant human protein [24] on the ability of capacitated human sperm to penetrate zona-free hamster eggs. Results showed that the antibody significantly decreased the sperm's ability to penetrate the eggs without affecting sperm viability/motility, the occurrence of the acrosome reaction or the binding of sperm to the hamster oolemma [26]. Subsequent immunofluorescence experiments revealed the existence of binding sites for ARP on the surface of zona-free human eggs supporting the idea that ARP could be the functional homologue of DE in humans.

6 Structure-function analysis of DE

The results obtained in our studies indicate that DE and its functional homologues in mouse and human participate in gamete fusion through their binding to complementary sites on the egg surface. However, the molecular mechanisms involved in these interactions remained unknown. The successful expression of recombinant DE in a prokaryotic system [27] led us to perform structure-function studies aimed to elucidate the molecular mechanisms underlying DE function. These studies showed that the activity of the protein does not involve carbohydrates, and resides in the polypeptidic region of the molecule [27]. However, the analysis of the amino acid sequence of DE indicated a lack of known functional domains that could explain its involvement in gamete fusion. To identify the binding domain of DE, recombinant fragments of the protein were expressed in a prokaryotic system based on the structure of recombinant mouse CRISP-1, and evaluated for their ability to bind to the egg surface and interfere with gamete fusion. Indirect immunofluorescence and sperm-egg fusion experiments using a first series of fragments revealed that the egg binding ability of DE is contained within the N-terminal domain of the molecule. Subsequent experiments using a new series of recombinant fragments circumscribed this activity to a region of 45-amino acids (114–158) [28]. Interestingly, the analysis of this region revealed that it contains the two feature motifs of the CRISP family named Signature 1 and Signature 2. To investigate whether these motifs were involved in the egg binding ability of DE, two synthetic peptides with the amino acid sequence of these motifs were produced: Peptide 1 (P1): GHYTQVVWNST and Peptide 2 (P2): FYVCHYCPGGNY. The use of these peptides in biological assays indicated that P2 but not P1 was capable of binding to the egg and interfering with gamete fusion [28]. The lack of egg labeling and fusion inhibition observed with a peptide containing the same amino acids as P2 but in a different order, confirmed the relevance of the S2 region for the binding of DE to the egg. To our knowledge, these results constitute the first evidence describing a functional role for the motif of the CRISP family and succeeding in delimiting the activity of a CRISP protein to such a small region. Moreover, the finding that the activity of DE resides in only 12 amino acids represents an important contribution for the future design of new and safer fertility regulating methods.

Considering the modular structure of CRISP proteins, our results indicate that the egg-binding ability of DE resides within the PR-1 domain of the molecule. The involvement of the CRD domain in other potential func-

tions of DE, however, cannot be excluded. Recent evidence indicates that CRISP proteins from snake venom [29] as well as murine Tpx-1/CRISP-2 [30], possess an ion-channel regulatory activity located in the CRD. In this regard, it is interesting to mention that DE has been shown to have an inhibitory activity on sperm protein tyrosine phosphorylation [31], a capacitation-associated event that depends on the regulation of several ion channels [32]. In view of this, it is likely that DE acts as a decapacitation factor regulating ion channels through the CRD. According to all these observations, the biological roles of DE would not only be exerted by two different populations of the protein (i.e. loosely/tightly bound to sperm), but would also reside in different domains of the protein (Figure 2).

The fact that the egg-binding ability of DE resides in an evolutionarily conserved region of the protein raised the question of how this common region might possess the necessary specificity for interacting with the different eggs. To answer this question, we analyzed the ability of several CRISP proteins to interact with rat eggs in relation to the amino acid sequences of their corresponding S2 regions. Although testicular murine Tpx-1 (CRISP-2) was capable of binding to the rat egg, human ARP and helothermine, a CRISP from lizard saliva [33], were unable to recognize the rodent gamete. In correlation with this, the S2 region presented only two substitutions in murine Tpx-1, and four in both human ARP and helothermine, when compared with S2 in rat DE. These results suggest that differences in the amino acid sequence of this region might be responsible for the specificity of the binding of each CRISP to its target egg [28].

The observation that murine Tpx-1 was able to bind to the rat egg surface, opened the possibility for a role of this protein in gamete fusion. The incubation of zona-free eggs with different concentrations of bacterially-expressed recombinant Tpx-1 (recTpx-1) prior to insemination produced a significant and dose-dependent decrease in the percentage of penetrated eggs compared with controls, suggesting that Tpx-1 would participate in gamete fusion through its interaction with complementary sites on the egg surface. Considering that the S2 region of these two proteins differed in only two amino acids, the possibility existed that both proteins would be interacting with the same binding sites on the egg. *In vitro* competition studies in which zona-free murine eggs were incubated with a fixed concentration of recTpx-1 and increasing amounts of DE showed a gradual decrease in the binding of recTpx-1 to the egg, suggesting that both proteins interact with common egg complementary sites [34]. Therefore, to examine the specific participation of Tpx-1 in gamete fusion we evaluated the effect of an antibody against this protein (anti-Tpx-1) on murine *in vitro* fertilization, knowing that this antibody does not cross-react with DE. Results showed that anti-Tpx-1 significantly decreases the percentage of penetrated eggs with a coincident accumulation of perivitelline sperm, supporting the specific participation of Tpx-1 at the sperm-egg fusion level [34].

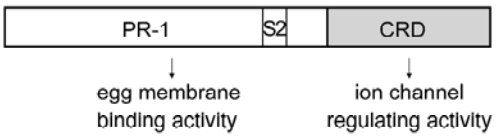

Figure 2. Schematic diagram illustrating the structural domains of cysteine-rich secretory proteins (CRISP) and their relationship with proposed functional activities of DE. CRISP members contain a plant pathogenesis-related (PR-1) domain and a cysteine-rich domain (CRD), connected by a short hinge region. The egg-binding site of DE resides in the Signature 2 (S2) region located in the PR-1 domain, whereas the proposed ion-channel regulating activity (decapacitation activity) resides in the CRD.

Together, the results obtained suggest the involvement of both epididymal DE/CRISP-1 and testicular Tpx-1/CRISP-2 in gamete fusion, supporting the idea of a functional cooperation between homologue molecules as a mechanism to ensure the success of fertilization. Nevertheless, the lack of cross-reaction of anti-DE with Tpx-1 confirmed that the inhibition of fertility in DE-immunized animals would be a result of a specific interference with the epididymal protein.

7 Conclusion

In summary, the results obtained indicate that epididymal protein DE/CRISP-1 fulfills many of the requisites for an epididymal contraceptive target: (i) it is an epididymal specific protein; (ii) it is localized on the sperm surface being accessible for its blockage in the male tract; (iii) it is relevant for fertility, as demonstrated by the immunization studies; (iv) it plays a specific role in fertilization (sperm–egg fusion) and capacitation; (v) its active site has been identified and resides in a discrete region of the molecule (12 amino acids); and (vi) it is a member of a highly evolutionarily conserved family (CRISP) with functional homologues in other species, such as mouse and human.

The relevance of ARP/hCRISP-1 for human fertility is currently being investigated in our laboratory by immunization studies carried out on a non-human primate model. We believe these results will contribute to a better understanding of the molecular mechanisms involved in fertilization as well as to the development of

new and safer methods of fertility regulation.

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