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Molecular mechanisms involved in gamete interaction[1]

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Abstract

Epididymal protein DE and testicular protein Tpx-1 are two cysteine-rich secretory proteins also known as CRISP1 and CRISP2, respectively. DE/CRISP1 is localized on the equatorial segment of acrosome-reacted sperm and participates in rat gamete fusion through its binding to egg-complementary sites. Recent results using bacterially-expressed recombinant fragments of DE as well as synthetic peptides revealed that the ability of DE to bind to the egg surface and inhibit gamete fusion resides in a region of 12 amino acids corresponding to an evolutionary conserved motif of the CRISP family named Signature 2. Interestingly, protein Tpx-1 exhibits only two substitutions in Signature 2 when compared to DE, and was also capable of binding to the rat egg, opening the possibility for a role of Tpx-1 in gamete fusion. Results showed the ability of recombinant Tpx-1 to significantly inhibit zona-free egg penetration, supporting the participation of this protein in gamete fusion through its interaction with egg-binding sites. Subsequent in vitro competition studies showed that incubation of zona-free eggs with a fixed concentration of recombinant Tpx-1 and increasing amounts of DE, gradually reduced the binding of recTpx-1 to the egg, indicating that both CRISPs would be sharing the egg complementary sites. The possible participation of both epididymal DE/CRISP1 and testicular Tpx-1/CRISP2 in gamete fusion provides important information on the molecular mechanisms involved in this process and supports the idea of a functional cooperation between homologue molecules as a mechanism to ensure the success of fertilization.

Keywords: sperm, egg, gamete fusion, fertilization, CRISP.

Introduction

Epididymal protein DE and testicular protein Tpx-1 are members of the CRISP (Cysteine-RIch Secretory Protein) family, a large group of secreted proteins with molecular weights of about 20-30 kDa, characterized by the presence of sixteen conserved cysteine residues, ten of which are clustered in the C-terminal domain of the molecule. Being the two first members described, DE was given the name of CRISP1 while Tpx-1 was denominated CRISP2. Since then, other members of this family have been identified in different mammalian tissues: CRISP3, presenting a wider tissue distribution than the other CRISPs including reproductive (prostate and ovary) and non-reproductive (salivary gland, pancreas, thymus and colon) organs (Haendler *et al*., 1993; Kjeldsen *et al*., 1996; Udby *et al*., 2005), and the recently described CRISP4 which is exclusively expressed in the epididymis (Jalkanen *et al*., 2005). 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 CRISPs are present in plants, insect and fungi (Fernandez *et al*., 1997; Henriksen *et al*., 2001; Serrano *et al*., 2004, Yamazaki and Morita, 2004). Although CRISPs 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.

DE/CRISP1 and Tpx-1/CRISP2 in gamete fusion

DE was originally identified in our laboratory in the rat epididymis (Cameo and Blaquier, 1976). After its secretion into the epididymal lumen, DE associates with the sperm surface during epididymal maturation, and migrates from the dorsal to the equatorial segment of the acrosome concomitantly with the occurrence of the acrosome reaction (Rochwerger and Cuasnicu, 1992). The relocation of DE to the equatorial segment, the fusogenic region of the sperm head, opened the possibility for a role of this protein in gamete fusion. The finding that the 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 complementary sites localized to the fusogenic area of the egg surface (Rochwerger *et al*., 1992). Subsequent studies revealed that homologous proteins for DE in mouse and human would also participate in sperm-egg fusion through their interaction with binding sites on the surface of the corresponding eggs (Cohen *et al*., 2000; 2001).

To elucidate the molecular mechanisms underlying DE function, structure-function studies using both native and bacterially-expressed recombinant DE were performed in our laboratory. These studies showed that the activity of this protein does not involve carbohydrates, and resides in the polypeptidic region of the molecule (Ellerman *et al*., 2002). However, the

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analysis of the amino acid sequence of the protein indicated the lack of functional domains that could explain its involvement in gamete fusion. In order to identify the binding domain of DE, recombinant fragments of the protein were expressed in a prokaryotic system, and evaluated for their ability to both bind to the egg surface and interfere with gamete fusion. Indirect immunofluorescence (IIF) 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 led us to circumscribe this activity to a region of 45-amino acids (114-158). 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 corresponding to each of these motifs were produced: Peptide 1 (P1): GHYTQVVWNST and Peptide 2 (P2): FYVCHYCPGGNY. The use of these peptides in IIF and sperm-egg fusion assays indicated that P2 but not P1 was capable of binding to the egg and interfering with gamete fusion. The lack of both egg labeling and fusion inhibition observed with a peptide containing the same amino acids than P2 but in a different order, confirmed the relevance of the Signature 2 region for the binding of DE to the egg.

These results strongly suggested that the ability of DE to bind to the egg surface resides in the 12-amino acid region corresponding to Signature 2 of the CRISP family. To study the specificity of this interaction, we analyzed the ability of several CRISP proteins to interact with rat eggs as well as the amino acid sequence of their corresponding Signature 2 regions. While mouse Tpx-1 (CRISP2) was capable of binding to the rat egg, ARP, a human epididymal CRISP (Hayashi *et al*., 1996; Kratzschmar *et al*., 1996), as well as helothermine, a CRISP from lizard saliva (Morrissette *et al*., 1995), were unable to recognize the rodent gamete. In correlation with this, the Signature 2 region presented only two substitutions in mouse Tpx-1, and four in both human ARP and helothermine, when compared to Signature 2 in rat DE. These results indicate that the egg-binding site of DE resides in an evolutionarily conserved region of the molecule (Ellerman *et al*., 2006).

The observation that Tpx-1 is able to bind to the rat egg opened the possibility for the participation of this protein in gamete fusion. In this regard, although there is consensus on the specific expression of Tpx-1 in rodent male germ cells and its presence in mature sperm, the possible function of this sperm protein has not yet been clearly established. Previous results from our laboratory indicate that human Tpx-1 (TPX1) would be an intra-acrosomal protein potentially relevant for gamete interaction (Busso *et al*., 2005). These observations together with the binding of Tpx-1 to the egg surface, led us to explore the participation of Tpx-1 in gamete fusion. We observed that the incubation of zona-free rat 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 to controls.

The similar localization of DE- and Tpx-1-binding sites on the egg, and the high homology of the corresponding Signature 2 regions, suggest that both proteins might interact with the same egg binding sites. To evaluate this possibility, in vitro competition studies were performed on zona-free eggs incubated in medium containing a fixed concentration of recTpx-1 and increasing concentrations of DE. The binding of each of the proteins was evaluated by IIF using the corresponding primary antibodies. Results showed that exposure of eggs to increasing concentrations of DE resulted in a gradual decrease in the level of fluorescent staining for recTpx-1, indicating that both proteins share binding sites on the egg surface.

Although the results of the competition studies might be due to the use of two proteins highly homologous in their egg-binding domains, it is also possible that both DE and Tpx-1 are required to mediate gamete fusion through their interaction with the egg. This possibility is further supported by recent evidence indicating the functional cooperation of homologous molecules during fertilization. In this regard, fertilin β and cyritestin, two members of the ADAM family, have both been involved in sperm-ZP interaction (Nishimura *et al*., 2001). Similarly, CD81, an egg protein belonging to the tetraspanin family, has been shown to replace the function of its homologous protein CD9 during sperm-egg fusion (Kaji *et al*., 2002).

Conclusions

Together, our results support the participation of both epididymal DE/CRISP1 and testicular Tpx-1/CRISP2 in gamete fusion through their binding to the egg surface. These results provide important information on the molecular mechanisms underlying gamete fusion and support the idea of a functional cooperation between homologue molecules as a mechanism to ensure the success of fertilization.

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