

Crisp proteins and sperm chemotaxis: discovery in amphibians and explorations in mammals

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ABSTRACT Crisp proteins appear to play multiple roles in the life history of sperm. One of these roles is to act as a sperm chemoattractant. Allurin, a 21 kDa Crisp protein rapidly released from the egg jelly of at least two frogs, *X. laevis* and *X. tropicalis*, elicits directed motility in both homospecific and heterospecific sperm. In *X. tropicalis*, allurin is coded for by the newly documented *Crisp A* gene. Recently, the observation that allurin can also elicit chemotaxis in mouse sperm raises the question of whether allurin-like proteins might act as sperm chemoattractants in mammals. Although an allurin gene has yet to be documented in mammals, Crisp proteins truncated post-translationally appear to exist in both the male and female reproductive tract of mammals.

KEY WORDS: *fertilization, sperm motility, allurin, egg jelly, extracellular matrix*

Introduction

Fertilization consists of a carefully coordinated series of events requiring sperm-egg communication at every step (Wassarman *et al.*, 2001; Primakoff and Myles, 2002; Evans and Florman, 2002; Hoodbry and Dean, 2004). The earliest step of sperm-egg communication is chemotaxis of the sperm toward the egg in response to an egg or ovum associated factor (Eisenbach, 2007; Eisenbach and Giojalas, 2006; Kaupp *et al.*, 2006). Since the source of the signal is typically the egg extracellular matrix, the accessory cells surrounding the egg or the egg itself, sperm need only to detect an ascending chemical gradient of the signal and follow it to its origin. To follow the gradient, the sperm must be equipped with a receptor for the chemotactic factor and an intracellular control system that modulates the flagellar movement such that the sperm is either turned toward the egg periodically (in mammals) or is changed in its pattern of swimming such that the sperm sweeps out a trajectory that brings it ever closer to the egg (as in invertebrates).

The chemotactic factors used for such communication are extremely diverse (see Table 1). Some of the earliest and best studied sperm chemoattractants are the peptides resact and speract found in the egg jelly of sea urchins (Ward *et al.*, 1985; Shimomura *et al.*, 1986; Dangott *et al.*, 1989; Garbers, 1989; Garbers *et al.*, 1994; Suzuki, 1995; Shiba *et al.*, 2005). Sperm detection of such peptides begins with their binding to a guanylate

cyclase receptor, production of an intracellular cGMP signal that in turn leads to a cascade of ionic events including opening of potassium channels, hyperpolarization of the plasma membrane, initiation of calcium waves, and activation of adenylate cyclase and cyclic AMP dependent kinase (Kaupp *et al.*, 2003; Bohmer *et al.*, 2005; Strunker *et al.*, 2006; Darszon *et al.*, 2006, 2007; Wood *et al.*, 2005, 2007; Darszon *et al.*, this issue). These events result in an increase in dynein heavy chain ATPase activation, a subsequent increase in flagellar beat frequency and an alteration in the pattern of flagellar dynein activity resulting in asymmetric bends in the flagellum and turning of the sperm (Cook *et al.*, 1994; Brokaw, 1979, 1999, 2002; Shiba *et al.*, 2005).

Sperm chemotaxis has been studied extensively in a number of marine species with external fertilization including sea urchins, starfish, ascidians, abalone, and coral (Miller, 1985; Neill and Vacquier, 2004; Nishigaki *et al.*, 1996; Yoshida *et al.*, 1993, 2002; Ishikawa *et al.*, 2004; Riffell *et al.*, 2002, 2004; Matsumoto *et al.*, 2003; Morita *et al.*, 2006; Shiba *et al.*, 2006). The chemical identity of the attractants used ranges from peptides to proteins to lipids to sulfated steroids (see Table 1). Species specificity of marine sperm chemoattractants has been documented in a number of these organisms and has been found to be very high in some brittle stars and mussels while being familial in organisms such as starfish and sea cucumbers. Some level of chemotactic specificity is thought to be required in these organisms because fertilization is external; gametes from multiple species are spawned simulta-

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neously and gametes are broadly distributed by currents, and consequently, chemotaxis may play an important role in gamete recognition in these species (Miller, 1985; Babcock, 1995).

Among vertebrates, sperm chemotaxis has been studied principally in mammals. In all mammalian species studied, follicular fluid has been demonstrated to elicit sperm chemotaxis although the full spectrum of factors involved remains unclear (Ralt et al., 1991; Cohen-Dayag et al., 1995; Oliveira et al., 1999). The evidence for progesterone to be an important chemoattractant is strongest (Teves et al., 2006; Villanueva-Diaz et al., 1995). Follicular fluid contains progesterone, the egg and surrounding cumulus cells have been shown to produce progesterone, and sperm from a number of mammalian species including humans chemotax toward relatively low concentrations of progesterone *in vitro* (Sun et al., 2005; Teves et al., 2006). Since progesterone also initiates hyperactivated sperm motility and triggers the acrosome reaction at higher concentrations *in vitro*, this steroid clearly has multiple important roles in mammalian sperm physiology (Eisenbach and Giojalas, 2006; Eisenbach et al., this issue). In contrast to marine invertebrates, heterospecific chemotaxis of mammalian sperm to follicular fluid has been reported for a relatively diverse group of mammals, implying that mammals likely do not rely on chemotaxis as a mechanism to prevent interspecies fertilization or to mediate species-specific gamete recognition (Sun et al., 2003).

In vitro studies have indicated that odorants such as bourgeonal and lylal also have sperm chemoattractant activity but the biological role of these agents *in vivo* remains unclear as these compounds have yet to be isolated from follicular fluid or egg extracellular matrix components. Bourgeonal and lylal bind to G-protein coupled odorant receptors designated as hOR17-4 and mOR23 respectively. In humans, it has been demonstrated that hOR17-

4 activation is coupled to a cAMP-mediated-signaling cascade; ultimately calcium influx occurs and flagellar beating is modified resulting in directed motility (Spehr et al., 2003, 2006; Fukuda et al., 2004; Spehr et al., this issue). Calcium influx also occurs in mouse sperm in response to mOR23 activation by the small aromatic aldehyde lylal and this too results in modification of flagellar beating and directed movement.

A relatively large void exists in our knowledge of sperm chemotaxis mechanisms between the exquisite biochemical and cellular studies in marine invertebrates and the critical physiological studies of chemotaxis in mammals. More recently knowledge of sperm chemotactic mechanisms has been augmented by studies in fish and amphibians. Motility activation and chemotaxis of sperm in fish is best represented by work on the Pacific herring. Sperm motility in the herring can be initiated by both diffusible peptides (herring sperm activating factors; HSAPs) and by a chorion protein (sperm motility initiation factor, SMIF) that is located close to the micropyle which the sperm must enter in order to reach the egg surface (Oda et al., 1998; Vines et al., 2002). HSAPs are considered to initiate sperm motility and chemotaxis while SMIF triggers the sperm to swim in circles thereby increasing the probability that it will enter the micropyle.

In amphibians, *Xenopus laevis* and *Xenopus tropicalis* have now taken a commanding lead in the study of sperm chemotaxis in lower vertebrates. Studies for over 40 years have indicated that the egg jelly of amphibians contains factors important for fertilization success (Katagiri, 1987). Once jelly is removed from a frog egg, fertilization plummets from about 95% to 10%; if jelly is added back in the form of jelly glycoproteins solubilized by reducing agents, fertilization is fully restored (Olson and Chandler, 1999). Although the full range of biological activities in jelly is still unknown, it quickly became clear that one such activity was that

TABLE 1

SPERM CHEMOATTRACTANTS ARE CHEMICALLY DIVERSE

Species	Agent	Type	Sperm responses	References
Sea Urchin <i>A. punctulata</i>	Resact	Peptide, 14 residues	Chemotaxis; cGMP signals, flagellar bending, initiates circular paths	Ward et al., 1985; Garbers, 1989; Bohmer et al., 2005
Sea Urchin <i>S. purpuratus</i>	Speract	Peptide, 10 residues	Chemokinesis; chemotaxis, calcium waves, flagellar bending	Shiba et al., 2005; Wood et al., 2005
Starfish	StarTrak	Protein, 13 kD	Chemotaxis	Miller and Vogt, 1996
Starfish	Asterosaps	Peptides with steroidal co-agent	Chemokinesis; Chemotaxis (?)	Nishigaki et al., 1996; Matsumoto et al., 2003
Coral	Dodeca-2,4-diynol (among others)	Low molecular weight lipid	Chemotaxis	Babcock, 1995; Morita et al., 2006
Brown Algae	Finaverrene (among others)	Unsaturated C ₁₁ hydrocarbons	Chemotaxis; mating synchrony	Boland et al., 1995; Muller et al., 1982
Ascidians	Sulfated Steroids	Sulfated Steroid	Initiation of circular swimming paths	Yoshida et al., 2002
Abalone	L-Tryptophan	Amino Acid	Chemotaxis	Riffel et al., 2002, 2004
Jellyfish	Egg/ovarian extracts	Unknown	Chemotaxis; activation of motility	Miller et al., 1997
Cuttlefish	SepSAP	Amidated hexapeptide	Chemotaxis	Zatylny et al., 2002
Fish Herring	HSAPs	Peptides	Chemotaxis	Oda et al., 1998
Frog <i>X. laevis</i> <i>X. tropicalis</i>	Allurin	Protein, 21 kDa	Chemotaxis	Al-Anzi et al., 1998; Olson et al., 2001
Mouse <i>M. musculus</i>	Progesterone, follicular fluid	Steroid, unknown agent	Chemotaxis, calcium waves	Giojalas et al., 1998
Human <i>H. sapiens</i>	Follicular fluid	unknown agent	Chemotaxis	Ralt et al., 1991; Olivera et al., 1999
Human <i>H. sapiens</i>	Progesterone	Steroid	Chemotaxis; hyperactivated motility; calcium signaling; acrosome reaction	Villanueva-Diaz, 1995; Teves et al., 2006; Kirchman-Brown et al., 2002.
Human <i>H. sapiens</i>	Bourgeonal	Aromatic aldehyde	Chemotaxis; calcium waves, cGMP signals	Spehr et al., 2003, 2006

of a sperm chemoattractant, allurin (Al-Anzi and Chandler, 1998). As expected of a sperm chemoattractant, allurin was found to be among the proteins that rapidly diffuse out of the jelly and therefore a rich source of the protein was «egg water,» medium conditioned by incubation with freshly spawned eggs for periods as short as 5 minutes (Olson *et al.*, 2001; Xiang *et al.*, 2005).

Purification, sequencing and molecular cloning of allurin mRNA provided evidence that this protein belongs to the CRISP (Cysteine-Rich Secretory Protein) family, members of which were already known to be mammalian sperm binding proteins (Olson *et al.*, 2001). A rapidly expanding subject of research, Crisp family proteins are now proposed to be involved in numerous steps of sperm maturation and sperm-egg interaction as illustrated in Fig. 1. In the male reproductive tract Crisp 2 is produced by developing spermatids and is thought to mediate Sertoli cell-spermatocyte adhesion during spermatogenesis (Maeda *et al.*, 1998). In addition, Crisp 2 is packaged into the acrosomal granule (Hardy *et al.*, 1988; Foster and Gerton, 1996). During later stages of sperm maturation in the epididymis Crisp 1, Crisp 4, and Crisp 3 (in some species) are produced and interact with the sperm surface (Roberts *et al.*, 2006, 2007). After ejaculation and entry into the female reproductive tract, sperm shed loosely bound Crisp 1 and acquire the ability to capacitate by the time they enter the ampulla of the oviduct (Rochwerger and Cuasnicu, 1992a; Roberts *et al.*, 2003). In *Xenopus*, sperm are guided to the egg by a gradient of the Crisp family protein allurin. Recent data leads us to propose that a similar mechanism may exist in mammals; that as capacitated spermatozoa swim up the oviduct in search of a fertilizable egg, Crisp proteins not unlike allurin (along with other factors such as progesterone) elicit sperm chemoattraction and direct motility of the sperm toward the egg. After arrival at the egg, sperm must next bind to the zona pellucida, penetrate it, bind and fuse with the egg plasma membrane. Crisp 1 has been shown to regulate sperm capacitation, and this protein has also been implicated in sperm-zona pellucida binding and sperm-egg fusion (Cohen *et al.*, 2007; Cuasnicu *et al.*, this issue). At the zona pellucida, Crisp 1, present on the dorsal head of sperm, modulates sperm-zona binding and migrates to the equatorial segment during the acrosome reaction. After migration, sperm-bound Crisp 1 is proposed to be involved in sperm-egg plasma membrane fusion through binding to specific Crisp protein receptors on the egg surface (Cuasnicu *et al.*, this issue). Crisp 2 has also been implicated in sperm-egg plasma membrane fusion events since it is also able to bind to eggs at the same sites as Crisp 1 and is present in the acrosomal granule of sperm (Busso *et al.*, 2007b).

The CRISP Family

The Crisp protein family is the vertebrate branch of the CRISP/Antigen 5/PR-1 (CAP) superfamily which includes proteins from species as diverse as prokaryotes, plants, fungi, insects, reptiles, avians, amphibians, and mammals (Kratzschmar *et al.*, 1996). As shown in the phylogenetic tree of Fig. 2, the CRISP families radiated from CAP ancestral genes that gave rise not only to pathogenesis-related proteins found in numerous plant families (the PR proteins, not shown) and venom antigens in insects such as wasps and bees but also to the complete

variety of Crisps found in snake venoms, amphibian reproductive tissues, and reproductive organs of all commonly studied mammals (Gibbs and O'Bryan, 2007). Studies of plant and venom CAP proteins have contributed significantly to knowledge of Crisp structure and function; notably, the complete tertiary structure of the tomato pathogenesis-related protein P14a has been determined by NMR (Fernandez *et al.*, 1997), the structure of wasp and hookworm venom antigen proteins have been determined by x-ray crystallography (Henriksen *et al.*, 2001; Asojo *et al.*, 2005), and three snake venom Crisp proteins have had their x-ray crystal structures solved as well (Fig. 2; Wang *et al.*, 2004; Wang *et al.*, 2005; Guo *et al.*, 2005; Shikamoto *et al.*, 2005).

All members of the CAP/CRISP family share a homologous N-terminal CAP domain of about 160 amino acids which is also referred to as the pathogenesis-related or PR domain since its amino acid sequence is homologous to and its structure (where determined) is similar to that of pathogenesis-related proteins in plants (see Fig. 3). This domain contains 6 conserved cysteine residues that form 3 disulfide bonds in most family members. The PR domain is linked to the cysteine-rich domain (CRD) found only in the CRISP family. The CRD domain itself is divided into a 26-amino-acid hinge region that contains 4 conserved cysteines involved in 2 disulfide bonds and the ion channel regulatory (ICR) region consisting of approximately 40 amino acids including 6 conserved cysteine residues that also are disulfide bonded.

Conservation of the CRD domain is less widespread than that of the PR domain. Some CAP/CRISP family members have modified C-terminal domains that do not resemble ICR domains, for example, the wasp venom antigen (Henriksen *et al.*, 2001). Alternatively, other CRISP family members such as the allurins and the mammalian GliPR proteins (not shown, Szyperski *et al.*, 1998) have a hinge region but lack the ICR region. One member, a short splice variant of human Crisp 1 completely lacks both the hinge and ICR regions (Hayashi *et al.*, 1996; Kirchoff, 1998). Finally, all three domains have shown up in a set of larger Crisp proteins – the proteins in the LD1 and LD2 families – but interspersed with non-Crisp sequences to produce proteins of about 500 residues. The LD 1/2 family also contains a LCCL domain, function unknown, for which the family is named (based on its presence in *Limulus* factor C) (Clark *et al.*, 2003). Like other Crisp

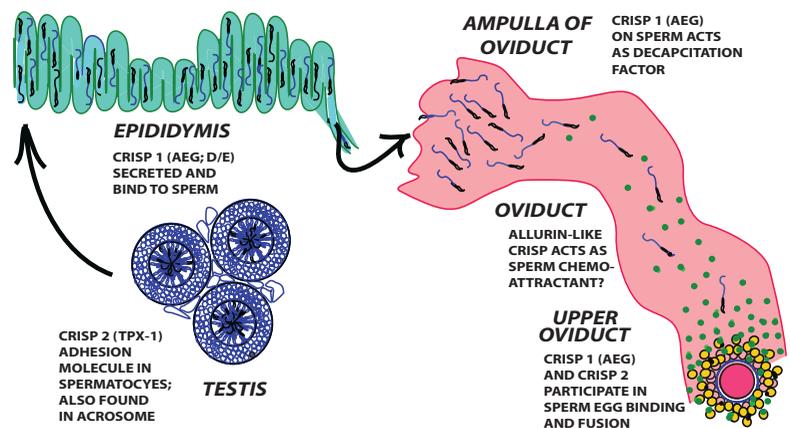


Fig. 1. Diagram of proposed Crisp protein functions in the mammalian reproductive tract.

proteins the Crisp LD 1/2 family is observed in both lower vertebrates such as frogs as well as mammals (see Figs. 2 and 3).

Although the basic structural relationships between the PR, Antigen and CRISP subfamilies are clear, the biological functions of each of the domains involved remain unclear. The function of the CAP/PR domain in both pathogenesis-related plant proteins and in insect venom antigens is completely unknown. In contrast, there is strong evidence that the CAP/PR domain of mammalian Crisps is a sperm binding domain that plays an important role in sperm maturation, capacitation and sperm-egg binding and fusion. For this reason databases for conserved protein domains (e.g. CDD at NCBI) recognize the CAP/PR domain as a «sperm coating protein» (SCP) domain with two highly conserved «signature» sequences that in mouse Crisp 1 are found at residues 120-130 and residues 148-159. As an example, studies with the mammalian sperm binding protein Crisp 1 in rat, mouse and human demonstrate that incubation of this protein with zona-free eggs can prevent sperm-egg fusion (Rochwerger *et al.*, 1992b; Cohen *et al.*, 1996, 2000a, 2001). The activities of overlapping

synthetic peptides indicate that the region of Crisp 1 responsible for this action is the second amino acid signature sequence (148-159) in the CAP/PR domain (Ellerman *et al.*, 2006). However, the functional role of the CAP domain in mammalian Crisp proteins found in non-reproductive tissues remains unknown.

Studies in venom proteins have provided a more general model for CRD domain functionality. The ICR domain of the CRD structurally resembles ion channel regulatory proteins that have been identified in the sea anemone, namely Bgk and Shk (Casteneda *et al.*, 1995; Cotton *et al.*, 1997). Ion channel regulatory activity of Crisp proteins has been examined most thoroughly for heliothermine, a Crisp family venom protein from the Mexican beaded lizard. Heliothermine blocks activity of a number of channels including voltage-gated Ca²⁺ and K⁺ channels as well as ryanodine receptor Ca²⁺ channels (Nobile *et al.*, 1994, 1996; Morrissette *et al.*, 1995). This activity is not unique to heliothermine; other Crisps including, natrin, pseudocin and pseudochetoxin also have Ca²⁺ and K⁺ channel blocking activity (Wang *et al.*, 2005; Brown *et al.*, 1999; Yamazaki, 2002). Other Crisps have been shown to prevent contraction of smooth muscle suggesting that they might have ion channel inhibitory abilities as well.

Further studies of the CRD domain have been carried out in mammalian Tpx-1/Crisp 2. Synthetic peptides representing the CRD domain of Crisp 2 alone were shown to be capable of blocking ryanodine receptors in smooth muscle *in vitro* and by NMR spectroscopy were shown to have a tertiary structure very similar to the CRD domain of snake venom Crisps (Gibbs *et al.*, 2006). These data suggest a common mechanism of Crisp family members: ion channel regulation, which could affect numerous processes in different species and tissues.

In mammals a number of Crisp family proteins have been identified and characterized, primarily in the male reproductive tract. Six Crisp genes have been identified in mouse of which four are well studied and considered to be paralogs: Crisp 1, Crisp 2, Crisp 3 and Crisp 4. Likewise, three paralogous genes have been identified in rat (Crisp 1, Crisp 2, and Crisp 4) and three in human (Crisp 1, Crisp 2, and Crisp 3) (see Fig. 2). In most cases these Crisp family proteins are expressed in the male reproductive tract in an androgen-dependent manner and are thought to play a role in sperm maturation and sperm-egg interactions (Dacheux *et al.*, 2006; Udby *et al.*, 2005; Jalkanen *et al.*, 2005; Eberspracher *et al.*, 1995; Schwidetzky *et al.*, 1995; Kratzschmar *et al.*, 1996).

As summarized in Table 2, the mammalian Crisp proteins have been implicated in a number of fertilization events. Crisp 1, well-studied in rodents and also known as protein DE and acidic epididymal glycoprotein (AEG), is secreted in the epididymis and binds to the sperm surface during epididymal transit (Rochwerger and Cuasnicu, 1992a; Roberts *et al.*, 2001, 2002; Tubbs *et al.*, 2002). Crisp 1/AEG/Protein DE is present in two forms that are thought to be identical in amino acid sequence but immunologically distinguishable (Roberts *et al.*, 2002, 2006). The first form, protein D, is the major species of Crisp 1 present in epididymal fluid (>95% of total) and the second form, protein E, is thought to differ from D by the addition of an O-linked oligosaccharide having at least one N-acetyl-glucosamine (Roberts *et al.*, 2006). Proteins D and E exhibit differential expression; Protein D is synthesized more proximally in the epididymis than protein E (Roberts *et al.*, 2002). These proteins also exhibit different sperm-binding abilities. Protein D appears to bind to the sperm surface in a transient

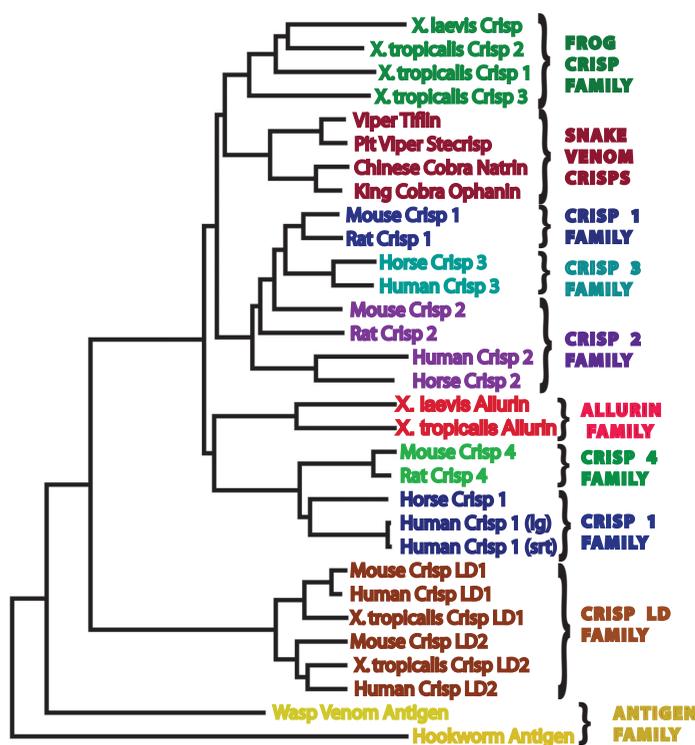


Fig. 2. Phylogenetic relationships between Crisp proteins. The tree was constructed using amino acid sequences, default parameters for Clustal W alignment and nearest-neighbor joining methods in MEGA 4 (Tamura *et al.*, 2007). The GenBank Identification numbers for the sequences used (from top to bottom) are: 147901846, 56118479, 77621326, 45361474, 21305552, 60593434, 32492058, 28972960, 31981913, 12408313, 126352541, 5174674, 6678422, 13676848, 4507670, 126352425, 15987512 (*X. laevis* allurin), 73788810, 88595370, 86476049, 126352648, 25121981, 25121983, 123173745, 52694670, scaffold_260:1567801-1600106 (*X. tropicalis* Crisp LD1, putative), 60592999, 73853835, 142374443, 162550, 3396069.

TABLE 2

REPRODUCTIVE FUNCTIONS OF CRISP FAMILY PROTEINS IN MAMMALS

Process	Proteins Involved	Location of Action	Specific Functions	References
Gametogenesis	Crisp 2	Testes	Sertoli cell adhesion; acrosome biogenesis	O'Bryan et al., 2001; Maeda et al., 1998; Hardy et al., 1988
Epididymal Maturation	Crisp 1 Crisp 4 Crisp 3	Epididymis	Coat sperm surface (Crisp 4 in proximal epididymis, Crisp 1 in distal epididymis); Seminal plasma components (Crisp 1, 4 and 3 (humans and horse))	Rochwerger and Cuasnicu, 1992; Roberts et al., 2001, 2002; Schambony et al., 1998a,b
Capacitation	Crisp 1	Oviduct Ampulla	Decapacitation factor; unbinding/ migration at sperm surface to allow capacitation to proceed	Roberts et al., 2003, 2007; Rochwerger and Cuasnicu, 1992
Chemoattraction	Truncated Crisp 1? Allurin?	Oviduct	Sperm chemoattraction?	Proposed here
Zona-Pellucida Interaction	Crisp 1	Zona Pellucida	Zona penetration	Busso et al., 2007
Gamete Fusion	Crisp 1 Crisp 2	Egg plasma membrane	Crisp 1 (equatorial region/ fusogenic region) Crisp 2 (acrosomal matrix)	Cuasnicu, this issue; Ellerman et al., 2006; Busso et al., 2005, 2007; Cohen et al., 2001; Da Ros et al., 2007

and reversible manner whereas Protein E binds essentially irreversibly (Roberts *et al.*, 2007). In addition, protein D has been shown to bind principally to the head of rat sperm; in contrast, protein E has been shown to bind almost exclusively to the tail by immunocytochemistry with protein E specific antibodies.

Both Protein D and E are thought to be present in full-length and proteolytically processed forms; proteolytic processing is proposed to occur after secretion resulting in a heterogeneous protein D/E population composed of full-length, high molecular weight (about 32 kDa) forms as well as lower molecular weight forms. The lowest molecular weight form of Protein E (approximately 22 kDa) is most strongly associated with the sperm surface. Based on the loss of cross-reactivity with an antibody that recognizes part of the CRD domain, this form of Crisp 1 is presumed to undergo a C-terminal cleavage event resulting in a truncated form of this protein lacking the CRD domain (Roberts *et al.*, 2007).

After ejaculation, in the female reproductive tract, Crisp 1 is shed from the surface of sperm concomitant with capacitation. Indeed, Crisp 1 in the epididymis is thought to prevent sperm capacitation presumably by an unknown ion channel regulatory mechanism; incubation of sperm with exogenous full-length Crisp 1 results in inhibition of the tyrosine phosphorylation usually seen during capacitation and the ability of the sperm to undergo progesterone-induced acrosome reaction (Roberts *et al.*, 2003). This inhibition or "decapacitation" can be rescued by removing the sperm from exogenous Crisp 1-containing media; the degree of sperm recovery is inversely correlated with the amount of Protein D remaining bound to the sperm surface. Thus, as ambient Crisp 1 levels decrease in the female tract, loosely bound Crisp 1 (presumably mostly protein D) dissociates from the sperm surface thereby allowing the sperm to capacitate (Roberts *et al.*, 2007).

Dissociation of protein D from the sperm surface to permit capacitation appears to be partial; immunocytochemistry demonstrates that a por-

tion of Crisp 1 remains bound to the dorsal region of the sperm head after capacitation (Rochwerger and Cuasnicu, 1992a). Subsequently, during the acrosome reaction, this Crisp 1 migrates to the equatorial region, a location considered vital for subsequent sperm-egg fusion (Da Ros *et al.*, 2004). Crisp 1 migration is bicarbonate dependent (like capacitation) and some sperm show evidence of early migration even during capacitation (Rochwerger and Cuasnicu, 1992a; Da Ros *et al.*, 2004). Thus, Crisp 1, before migration, is in position to influence sperm-zona pellucida binding while after migration is positioned to play a role in sperm-egg plasma membrane binding (Cohen *et al.*, 2007)

Indeed, recent studies indicate that purified Crisp 1 can bind to both the zona pellucida and the egg plasma membrane of rodent eggs. In fact, incubation of zona-intact eggs with either anti-Crisp 1 antibodies or exogenous Crisp 1 results in a decrease in number of sperm bound to the zona pellucida (Busso *et al.*, 2007a). In addition to its possible role in sperm-zona interactions, Crisp 1 has been directly implicated in regulation of sperm-egg fusion at the plasma membrane. As discussed previously, Crisp 1 can bind to the egg plasma membrane of zona-free eggs through a conserved signature region and regulate or mediate sperm-egg fusion (Ellerman *et al.*, 2006; Cohen *et al.*, 1996, 2000a, 2007; Da Ros *et al.*, 2006, 2007a). Zona-free eggs incubated with exog-

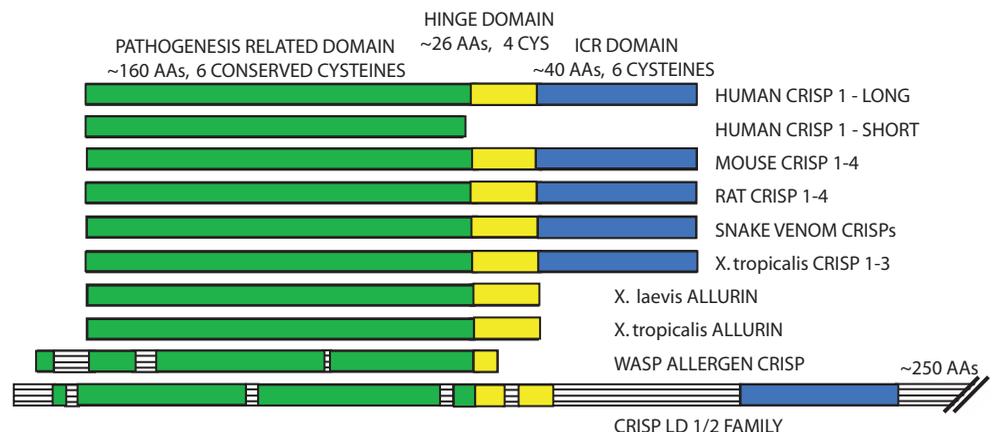


Fig. 3. Domain organization of selected Crisp proteins. Amino acid sequences characteristic of the pathogenesis-related (PR), hinge and ion channel regulatory (ICR) domains are represented by green, yellow and blue bars respectively. Regions of non-Crisp amino acid sequences are indicated by hatched bars. Bar lengths are not strictly to scale.

enous purified Crisp 1 or a synthetic peptide representing the egg binding domain of the protein results in a decreased number of gamete fusion events without decrease in the number of sperm binding to the egg plasma membrane (Da Ros *et al.*, 2006). These data suggest that some form of Crisp 1 may be involved in events leading to sperm-egg fusion but that these events must come after initial sperm-egg binding (Cohen *et al.*, 2007; Da Ros *et al.*, 2006). Parallel studies have shown that incubation of sperm with Crisp 1 antibodies prevents fertilization and that an immunological approach may have uses in contraception (Ellerman *et al.*, 1998, 2007). A detailed account of these studies may be found in the article by Cuasnicu *et al.*, this issue.

Although other Crisp proteins play a role in sperm physiology their roles in sperm-egg communication are not as well defined. Crisp 2 has been shown to bind to the mouse egg plasma membrane and by competition studies is thought to bind the same complementary sites on the egg's surface as Crisp 1. Crisp 2 is presumed to bind to the egg via the same signature sequence as does Crisp 1 since this sequence in Crisp 2 differs from that in Crisp 1 by only two amino acids (Busso *et al.*, 2005, 2007b). Crisp 2 is not found on the surface of mature sperm, but is present intracrosomally (Hardy *et al.*, 1988; Foster and Gerton, 1996; O'Bryan *et al.*, 2001), suggesting that its exposure during the acrosome reaction may position it for involvement in sperm-egg fusion events (Busso *et al.*, 2005). Crisp 2 is also found in the testes during spermatogenesis; it is produced by developing spermatids and associates with the sperm plasma membrane to mediate sperm adhesion to Sertoli cells (Maeda *et al.*, 1998). As mentioned previously, the CRD domain of this Crisp family member has been shown to block ryanodine receptors *in vitro* suggesting a possible biochemical mechanism for the biological roles of Crisp 2 (Gibbs *et al.*, 2006).

Crisp 3 has a wider tissue distribution and has not been found in the male reproductive tract of mouse; however, the equine Crisp 3 is a major protein component of horse seminal plasma (Topfer-Peterson *et al.*, 2005). In horse, this protein is associated with the sperm surface and is correlated with fertility in stallions (Schambony *et al.*, 1998a,b; Hamann *et al.*, 2007). In addition, there are two molecular weight forms (29 and 31kDa) of Crisp 3 found in human secretory epithelia (epididymal and prostatic) suggesting a possible role for this protein in reproductive function (Udby *et al.*, 2005; Bjartell *et al.*, 2006). In addition, Crisp 3 is also found in other tissues including neutrophils and exocrine secretions and is hypothesized to play a role in innate immunity (Haendler *et al.*, 1993; Udby *et al.*, 2002a,b).

Crisp 4 is expressed in multiple regions of the epididymis and has been shown to interact with sperm; however, the role of this protein in sperm function/maturation has yet to be elucidated (Jalkanen *et al.*, 2005). Sperm in mouse and rat are exposed to Crisp 4 in the epididymis prior to exposure to Crisp 1 based on differential expression patterns of these genes. The rat and mouse Crisp 4 genes are orthologous to each other as well as orthologs of human Crisp 1 (Nolan *et al.*, 2006). Further studies of Crisp 1 and Crisp 4 will be necessary to determine the functional relationships of these gene products.

In non-mammalian vertebrates, additional Crisps have been identified and implicated in reproductive and developmental functions. X-Crisp is a full-length Crisp family protein that is involved in degradation of the vitelline envelope resulting in hatching in

Xenopus laevis embryos (Schambony *et al.*, 2003). The CRD domain of this protein is required for its functionality. In contrast, allurin, also identified in *X. laevis*, lacks all but four residues of the ICR portion of the CRD domain. As indicated previously, allurin is homologous to mammalian sperm binding Crisps and elicits chemotaxis in *X. laevis* sperm (Olson *et al.*, 2001). More recently Xt allurin, the ortholog of allurin in *X. tropicalis* has been identified (Burnett *et al.*, 2008). This protein also lacks an ICR domain, but can elicit chemotaxis in *X. tropicalis* sperm indicating that this activity must be restricted to the PR domain or hinge region of this protein. Intriguingly, sperm chemotaxis represents another functional role of Crisp family proteins in sperm maturation and sperm-egg interactions suggesting that sperm may be shepherded and guided by Crisp family proteins through many functional stages required to accomplish fertilization (summarized in Fig. 1).

Amphibian crisps and chemoattraction

In 2001, we purified and sequenced allurin from *X. laevis* egg jelly. Interestingly, this 21kD chemoattractant protein was also the first Crisp family member identified as being produced in the female reproductive tract. Allurin is dissimilar to most other known chemoattractants in that it is a protein of substantial size rather than a small molecular weight compound, but its homology to mammalian sperm binding proteins has made it an obvious target for further study.

Subsequently, we used DNA probe hybridization, RT-PCR amplification and immunocytochemistry to determine the site of allurin expression and the means by which this protein is applied to eggs as they pass down the frog oviduct (Xiang *et al.*, 2004). Dot and western blots showed that the oviduct is the only organ to express allurin and that both allurin mRNA levels and protein levels rise substantially (3 and 7 fold, respectively) over a 12 hour period after human chorionic gonadotropin (hCG) priming of the frog. Most vertebrates ovulate in response to luteinizing hormone-like hormones such as hCG but frogs, in addition, increase production of both egg jelly and allurin in the oviduct. Thus, allurin also represents the first Crisp family member known to be expressed in response to hormonal stimulation in females. In the male, CRISP family members show androgen-dependent expression and in some cases androgen response elements (AREs) have been identified in CRISP gene regulatory loci. This suggests common themes in vertebrate Crisp proteins of amino acid sequence homology, function (sperm-binding), and hormone-induced expression.

Immunocytochemistry showed that allurin secretion and application to the egg does not coincide with the application of other jelly components (Xiang *et al.*, 2004). The luminal surface of the frog oviduct exhibits a pattern of protrusions which when seen in section consists of a series of cellular arches that cover deeper tubular glands (see Fig. 4A). At higher magnification, one can see that the cells making up the tubular glands are filled with large, basophilic granules containing high molecular weight jelly glycoconjugates (Fig. 4B). The arches themselves are covered with epithelial cells whose dense array of cilia aide propulsion of the eggs, single file, down the oviduct from ovary to uterus, a journey that takes about 3 to 4 hours. In electron micrographs, these cilia are seen to have the usual 9+2 arrangement of microtubules and are well anchored by basal bodies in the

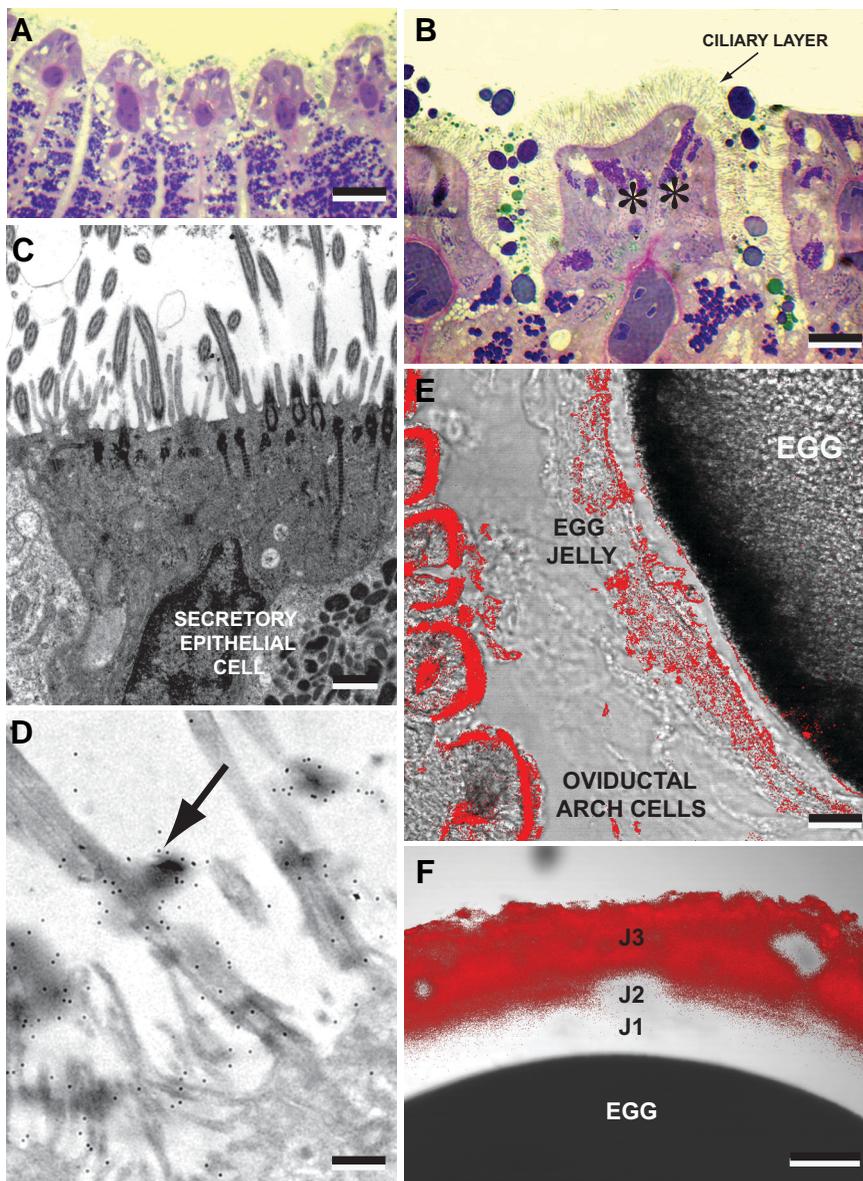


Fig. 4. Secretion of allurin in the frog oviduct and its application to the egg. (A) The *X. laevis* oviduct consists of tubular jelly secreting glands capped by arches of epithelial cells that face the oviduct lumen. Below the arches, cells that synthesize and secrete jelly glycoconjugates are filled with large, strongly basophilic granules. The cells of each tubular gland fan out from a central duct that empties into the lumen at the crevices between the epithelial arches. Bar, 25 μm . (B) Each arch contains at its center a capillary loop filled with blood cells. Covering the capillary is a single layer of ciliated epithelial cells and interspersed among these cells are occasional secretory cells that have clusters of lightly stained granules (asterisks). These are thought to be the allurin producing cells. Bar, 10 μm . From Chandler and Roberson, 2008 with copyright permission from Jones and Bartlett Publishers (C) Electron micrograph of cilia emerging at the apical plasma membrane of arch epithelial cells. Bar, 1 μm . (D) Immunocytochemical localization of allurin at the ultrastructural level. Arrows point to colloidal gold beads marking the presence of allurin on the extracellular surface of the cilia. Bar, 0.4 μm . (E) Immunocytochemical localization of allurin as it is applied to an egg passing down the oviduct. The secondary antibody signal (red) is superimposed on a transmitted light image of the egg and oviductal epithelium. Heavy deposits of allurin on the ciliary border are mixed with jelly and «brushed» onto the egg. Bar, 10 μm . (F) In the freshly spawned egg, allurin has become concentrated in the outmost (J3) jelly layer poised to be released into the medium. Bar, 100 μm . From Xiang *et al.*, *Dev. Biol.* 275:343-355 (2004) with copyright permission from Elsevier Science.

epithelial cells (Fig. 4C).

Localization of anti-allurin antibodies in the oviduct (red signal, Fig. 4E) shows that massive amounts of this protein are present in the ciliary layer of the arch cells and that this protein is “painted” onto the egg as it passes. Immunocytochemistry at the electron microscopy level confirms that allurin is present on the extracellular surfaces of these microvilli (arrow, Fig. 4D). These cilia therefore, might literally be considered a “brush” border. In contrast, the deeper simple tubular glands of the oviduct that produce jelly show no staining for allurin nor is there allurin present in the secretory ducts associated with these glands. Instead, allurin appears to be released from secretory cells in oviductal epithelium that are nestled between the more numerous ciliated cells (asterisks, Fig. 4B). Once the ciliary “brush” is loaded by these cells, allurin and high molecular weight jelly glycoconjugates from the tubular glands are mixed as they are applied to the passing egg. The bulk of the allurin is applied to the egg with the innermost jelly layer (J1) in the upper portion of the oviduct. Following application, allurin diffuses from J1 and ultimately to J3, the outermost layer, during subsequent jelly additions (see Fig. 4F). This change in localization readies allurin for rapid diffusion from the egg jelly upon spawning (Xiang *et al.*, 2005).

Although allurin is a relatively large chemoattractant it is effectively and rapidly released into the medium resulting in timely formation of an allurin gradient. Western blotting confirmed the appearance of allurin in the medium within 1.5 minutes and nearly half of the allurin present in the jelly layers is released within 5 minutes after spawning. Computer modeling showed that both diffusion and medium mixing at the jelly surface during spawning are both needed to account for this rapid release (see Fig. 5A). Release is likely accomplished by swelling and ion exchange of the medium with J3 components. J2, a compact and less permeable layer underneath may act to prevent diffusion of allurin into J1 thereby redirecting it back into the surrounding media (Xiang *et al.*, 2005).

The allurin gradient, rapidly established after spawning, must be detected and responded to by sperm to result in effective chemoattraction. Our current goal is to characterize the binding of allurin to the sperm surface and the behavioral changes that sperm exhibit in response to this protein. One approach is to conjugate Oregon Green 488 to allurin using an amine-reactive derivative. Fluorescent allurin is then used to assess binding of allurin to individual sperm by fluorescence microscopy and to sperm populations by flow cytometry. As shown in Fig. 5B,

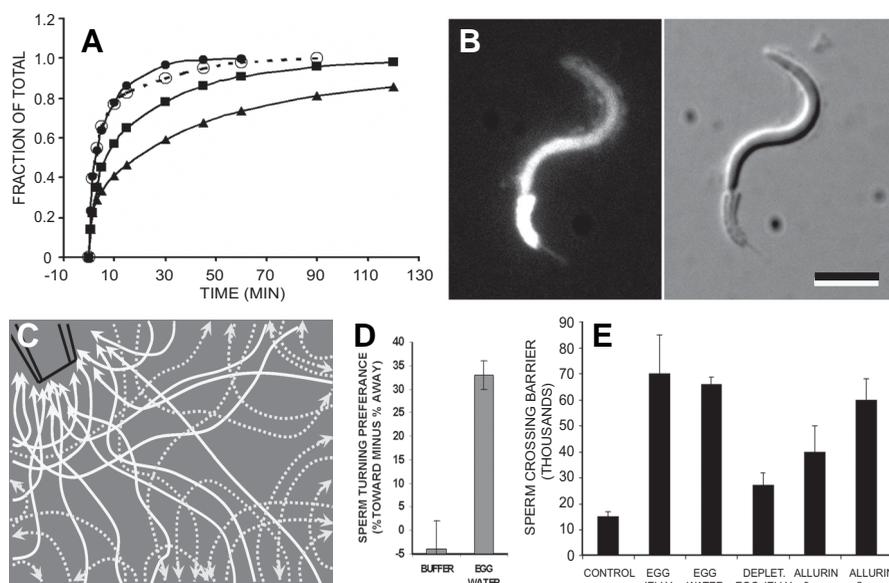


Fig. 5. Sperm responses to allurin. (A) Release of allurin from the egg into the surrounding medium occurs rapidly with a half time of 4 minutes (open circles). Release appears to involve both diffusion and convection. Based on computer modeling, diffusion alone is slower (solid triangles; half time 20 minutes), intermittent convection faster (solid squares, half time 10 minutes) and continuous mixing best approximating allurin release (solid circles, half time 4 minutes). From Xiang et al., *Mol. Reprod. Dev.* 70:344-60 (2005) with copyright permission from John Wiley and Sons. (B) Binding of Oregon Green-conjugated allurin to the midpiece and head of *X. laevis* sperm (left). Very little binding is observed on the flagellum. A phase contrast image of the same sperm is shown on the right. Bar, 5 µm. (C,D) Trajectories of *X. laevis* sperm surrounding a capillary filled with concentrated egg water. Sperm turning toward the pipette (solid lines) outnumber those turning away (dashed lines) as shown quantitatively in panel (D). From Al-

Anzi and Chandler, *Dev. Biol.* 198:366-375. (1998) with copyright permission from Elsevier Science. (E) Allurin-mediated chemotaxis by *X. laevis* sperm as measured in a two-chamber assay. Both solubilized egg jelly and egg water elicit ~5 fold greater sperm passage across a porous membrane than do controls. Purified *X. laevis* allurin stimulates sperm passage to a similar extent while jelly depleted of allurin exhibits reduced activity.

microscopic examination indicates that allurin binds to the midpiece and the head of *Xenopus* sperm with the midpiece exhibiting the highest intensity. In flow cytometry, an intense allurin signal is detected that is an order of magnitude above background despite the fact that the surrounding solution contained free allurin in order to keep bound allurin from dissociating from its receptors on the sperm surface (L. Burnett, unpublished data).

Sperm behavioral responses to allurin have been assayed in three ways (Sugiyama et al., 2005). First, tracking of sperm in the vicinity of a capillary filled with concentrated egg water showed that many sperm turned toward and swam to the source of chemoattractant (solid arrows, Fig. 5C) while in the absence of chemoattractant no turning preference could be detected (Fig. 5D). Second, we made quantitative comparisons using a "two-chamber" assay in which sperm cross a polycarbonate membrane having pores of defined size in response to a chemotactic gradient and are then counted in the receiving chamber. Using such an assay, one finds that placement of a small drop of solubilized egg jelly in the receiving chamber results in passage of over 5 times as many sperm through the membrane as in controls with buffer alone (see Fig. 5E). Similar sperm attractant activity is seen in egg water as expected due to rapid diffusion of allurin from jelly into medium (3rd bar, Fig. 5E). Solubilized jelly from eggs depleted of allurin in this manner show a considerably reduced activity (fourth bar, Fig. 5E). Allurin purified to homogeneity exhibits similar activity in these assays at lower doses as expected (5th and 6th bars, Fig. 5E).

Although the two-chamber assay provides useful information about sperm movement as a population we wanted to further investigate sperm behavior at the microscopic level. To do this we employed a chamber designed by Zigmond for use with neutrophils, but later applied successfully to mammalian sperm chemotaxis by Giojalas and colleagues (Fabro et al., 2002). The chamber contains two troughs, one acting as a sperm reservoir, the other as a chemoattractant reservoir, separated by a 1 mm-

wide bridge. The movement of sperm in response to the chemoattractant gradient formed is monitored in the bridge area by video microscopy. Sperm can then be tracked to acquire quantitative data for velocity and direction. Using this assay we have determined that allurin is chemotactic and not chemokinetic. Sperm incubated with egg water showed no increase in average velocity compared to sperm incubated with buffer alone; however, in tracking 50 individual sperm, a 3.5-fold increase in distance traveled toward the chemoattractant trough was seen in the presence of egg water as compared to buffer even though total distance traveled remained the same (Burnett, unpublished observations). These data indicate that allurin elicits a change in direction rather than a change in motility from *Xenopus* sperm.

Since species specificity and mechanisms of chemotaxis can vary dramatically, we asked whether allurin-like chemotaxis systems might be present in other species. Indeed, we find that egg water from a closely related species, *Xenopus tropicalis*, exhibits sperm chemoattractant activity and contains an anti-allurin cross-reactive protein that is approximately 20kD and is fully active upon purification (Burnett et al., 2008). Expressed sequence tag databases indicate that transcripts coding for a protein very similar to allurin are produced in the oviduct. Indeed, subsequent RT-PCR showed that this transcript is present and we cloned the full-length transcript from oviductal cDNA. Recombinant forms of this protein synthesized in mammalian cell culture demonstrate the same molecular weight and cross-reactivity as the protein found in *Xenopus tropicalis* egg water. Blotting of this sequence against the *X. tropicalis* genome indicates that the transcript encoding this protein corresponds to a previously unannotated region of the genome. Therefore, we designate this protein as Xt allurin and its newly discovered gene as Crisp A.

In addition to Crisp A, other CRISP family members have been identified in the *Xenopus tropicalis* genome, two of which have been annotated: Crisp 2 and Crisp 3. Other Crisp family members with mammalian homologs can be identified including the high

molecular weight members Crisp LD1 and Crisp LD2. We have examined expression of these genes in male and female reproductive tissues including testis, ovary, oviduct and eggs of *X. tropicalis* and our results are summarized in Table 3. Crisp A is the only Crisp gene expressed in the oviduct while Crisp LD1 and Crisp LD2 are expressed in the ovary. As anticipated, no Crisp genes are expressed in the *Xenopus* egg confirming the fact that allurin is not egg-derived but rather oviduct-derived. Interestingly, Crisp family members are also expressed in the testes; in fact, Crisp 2, Crisp 3, Crisp LD1, and Crisp LD2 are all expressed in this tissue. This suggests a possible conservation of Crisp family protein roles in the male reproductive tract of frog since these proteins are known to participate in sperm gametogenesis and gamete interactions in mammals.

Our findings suggest an additional conservation of function among allurins. Despite the fact that allurin from *X. laevis* and allurin from *X. tropicalis* exhibit only 62% sequence identity and these proteins differ in the position of two of their 10 conserved cysteines, both proteins actually show a high interspecies chemotactic activity. Egg water and purified allurin from each species typically elicits a response from heterospecific sperm that is a minimum of 70% of that achieved with sperm of the same species (Burnett *et al.*, 2008). This suggests a relative promiscuity of chemoattractants between species and portends a possibly broader evolutionary conservation of an allurin-mediated mechanism of sperm chemoattraction.

Mammalian crisps and chemoattraction

Allurin's ability to elicit interspecies chemotaxis is reminiscent of the ability of follicular fluid to induce cross-species chemotaxis in mammals. Furthermore, we have recently accumulated a growing body of evidence that Crisp proteins are expressed in the female reproductive tract of mammals and that allurin-mediated chemotaxis may occur in mammals as well as amphibians (Burnett *et al.*, 2006).

First, two-chamber *in vitro* assays demonstrate that allurin can elicit chemotaxis in mouse sperm. The number of sperm passing into the bottom chamber was on average three times greater in the presence of allurin compared to buffer alone (Burnett, unpublished observations). This assay cannot accurately assess what specific changes in sperm behavior are occurring and could include both chemotactic and chemokinetic components. Currently we are using a sperm tracking assay to assess specific sperm behaviors in the presence of allurin.

As one would predict, allurin conjugated to Oregon Green 488 is able to bind to mouse sperm. Binding is seen specifically in the post acrosomal region of the head and the midpiece. This labeling is unique compared to that of Crisp 1 and 2 binding to rat sperm which localizes to the head, tail, and acrosome (Rochwerger and Cuasnicu, 1992). These data indicate that allurin can bind to the mouse sperm surface and elicit chemotaxis *in vitro* suggesting that allurin-mediated chemotactic behavior may be possible in mammalian systems. Whether chemotaxis of mammalian sperm to allurin-like proteins occurs *in vivo* remains to be determined.

Follicular fluid has been identified consistently as a sperm chemoattractant for mammalian sperm and this fluid is thought to be produced by cells derived from the ovary. Eisenbach and colleagues have found that human cumulus cell-culture media can elicit chemotaxis in human sperm (Sun *et al.*, 2005). This finding suggests that cumulus cells are likely to produce and secrete a mammalian sperm chemoattractant. With this in mind we have investigated expression of mammalian Crisp genes in female reproductive tissues of mice using semi-quantitative RT-PCR. We find that a number of Crisp family genes are expressed in the female reproductive tract of mice; Crisp 1 and Crisp LD 2 are both expressed in high levels in the ovary as well as in cumulus oophorus cells. Crisp LD1 appears to be expressed exclusively in the ovary. Crisp 2, Crisp 3, and Crisp 4 show little to no expression in female reproductive tissues. Further studies using immunocytochemistry demonstrate that anti-allurin antibodies (which cross-react with other Crisp proteins) label both mural and oophorus cumulus cells in early antral follicles (L. Burnett, unpublished observations).

These findings lay groundwork for the possibility that Crisp family proteins may play critical roles in female gametogenesis in addition to their involvement in male gametogenesis. Although Crisp 1 and Crisp LD2 are both predicted to have significantly higher molecular weights than allurin it is clear from previous studies of Crisp 1 that this protein can be processed into multiple different forms including a truncated form lacking the CRD domain that would be structurally similar to amphibian allurins. Currently we are examining whether allurin-like proteins can be found and identified in mouse follicular fluid to support conservation of an allurin-mediated chemotaxis model in mouse.

Thus, our studies and those of other laboratories lead to several well defined and testable hypotheses. First, the mammalian follicle (likely the cumulus cells) produces Crisp proteins that not only bathe the developing oocyte but also accumulate in the follicular fluid. Second, Crisp proteins or their truncated products

TABLE 3

EXPRESSION PATTERNS OF CRISP PROTEINS IN THE MALE AND FEMALE REPRODUCTIVE TRACTS

Animal	Testis	Epididymis	Male accessory glands	Ovary	Oviduct	Egg / Cumulus complex
<i>X. laevis</i>	ND	Not applicable	Not applicable	Allurin? (low levels)	Allurin (presumably Crisp A)	ND
<i>X. tropicalis</i>	Crisp 2,3, LD1, LD3	Not applicable	Not applicable	Crisp 1, LD2	Crisp A	None
<i>M. musculus</i>	Crisp 2	Crisp 1, 4	ND	Crisp 1, LD1, LD2 Crisp 3 (low levels)	Crisp 1, 2, LD1, LD2, 3 (low levels)	Crisp 1, LD2 Crisp 3 (low levels)
<i>R. norvegicus</i>	Crisp 2	Crisp 1,4	ND	ND	ND	ND
<i>E. caballus</i>	Crisp 2	Crisp 1	Crisp 3 (seminal plasma)	ND	ND	ND
<i>H. sapiens</i>	Crisp 2	Crisp 1 long, Crisp 1 short, Crisp 3 Crisp 4	Crisp 3 (prostate)	ND	ND	ND

ND = Not Determined

in the follicular fluid are released at ovulation and are disseminated as the ovum proceeds down the oviduct. Third, sperm sensing these Crisp products respond by chemotaxis toward the egg. Fourth, Crisp protein products either from the sperm or from those cached in the ovum play a role in sperm-egg binding and fusion.

Future studies in Crisp family proteins may elucidate additional events involved in sperm and egg maturation, mammalian sperm chemotaxis, and sperm-egg interaction providing us with a broader understanding of multiple fertilization mechanisms.

Acknowledgements

We thank the W. M. Keck Bioimaging Laboratory for use of their Leica NTS laser scanning confocal microscopy unit and their video microscopy equipment. We would also like to thank the Life Sciences Electron Microscopy Facility for use of their Phillips CM12S transmission electron microscope. These studies were supported by grants from the National Science Foundation, most recently IOS 0615435.

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