Allurin, a 21-kDa sperm chemoattractant from *Xenopus* egg jelly, is related to mammalian sperm-binding proteins

John H. Olson*, Xueyu Xiang*, Tillmann Ziegert, Andrew Kittelson, Alan Rawls, Allan L. Bieber, and Douglas E. Chandler†

Molecular and Cellular Biology Program and the Departments of Biology and Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287-1501

Edited by William J. Lennarz, State University of New York, Stony Brook, NY, and approved August 10, 2001 (received for review June 22, 2001)

Previously, we demonstrated that a protein from *Xenopus* egg jelly exhibits sperm chemoattractant activity when assayed by either video microscopy or by sperm passage across a porous filter. Here we describe the isolation and purification of allurin, the protein responsible for this activity. Freshly oviposited jellied eggs were soaked in buffer, and the conditioned medium was loaded onto an anion exchange column and eluted with an NaCl gradient. The active fraction was purified further by RP-HPLC, the chemoattractant protein appearing as a single sharp peak. The amino acid sequence of the protein, determined by direct sequencing and cloning of cDNAs coding for the protein, consisted of 184 amino acids having a molecular mass of 21,073 Da. The protein shares homology with the mammalian cysteine-rich secretory protein (CRISP) family that includes testes-specific spermatocyte protein 1, a cell adhesion protein which links spermatocytes to Seritoli cells, and acidic epididymal glycoproteins that bind to sperm and have been implicated in sperm–egg fusion. Phylogenetic analysis suggests that allurin evolved from the ancestral protein that gave rise to the mammalian CRISP family. Addition of allurin to this family portends that the CRISP family represents a group of ''sperm escort'' proteins, which bind to sperm at various steps in their life history, facilitating passage from one functional stage to the next. Allurin stands out in this regard, representing both the first vertebrate sperm chemoattractant to be purified and sequenced and the first member of the CRISP family to be found in the female reproductive tract.

sperm chemotaxis | fertilization | TPX-1 | AEG | CRISP

Fertilization of both vertebrate and invertebrate eggs requires a series of interactions between sperm and the egg extracellular matrix. The inner extracellular matrix layers, such as the zona pellucida in mammalian eggs and the vitelline envelope in amphibian eggs, have been well studied in regard to their ability to bind sperm and to elicit the acrosome reaction (1–7). The outer matrix layers on vertebrate eggs, in contrast, have received much less attention and until recently had not been characterized with respect to sperm-activating proteins (8). Our studies in the African clawed frog *Xenopus laevis*, however, have demonstrated that the jelly layers of *X. laevis* contain at least two sperm-modifying activities, both small proteins (9, 10). The first, a ''fertilization promoting'' factor, seems to be required for sperm to bind to the vitelline envelope of the egg (9). The second, a sperm chemoattractant, is released from the jelly within minutes of jelly hydration during spawning (10).

Sperm chemoattractant peptides from invertebrate eggs have been well characterized (11, 12). Resact, isolated from the jelly of sea urchin eggs, is a classic example (13). This peptide is highly potent, binds to an identified receptor on the sperm flagellum, and mediates sperm movement toward the egg (14, 15). In contrast, sperm chemoattractant proteins have not been isolated from vertebrates, although motility-initiating proteins have (16). Several laboratories have reported that fluid from human ovarian follicles exhibits sperm chemoattraction activity, which can be correlated to fertilization success (17–20); however, the active factor has not been characterized to date (20).

In this study, we report purification and characterization of allurin, a *Xenopus* egg jelly chemoattractant protein. Jelly proteins, including allurin, are secreted in the oviduct and applied to the egg as it transits from the ovary through the oviduct to the uterus just before spawning. This protein is acidic, 184-aa in length, and bears amino acid sequence homology to the mammalian cysteine-rich secretory protein (CRISP) family, members of which are known to bind to sperm (21–29). The closest homologues of allurin include testis-specific spermatocyte protein (TPX-1), a testis protein thought to mediate adhesion between spermatocytes and Seritoli cells (22–24), and acidic epididymal secretory glycoproteins (AEGs), which are thought to play a potential role in sperm–egg fusion (25–29). The addition of allurin to the CRISP family suggests that this family includes a group of ''sperm escort'' proteins, which bind to sperm and facilitate the passage of these cells from one functional stage to the next.

Materials and Methods

Animals and Materials. Male and female *X. laevis* were obtained commercially and kept on a 12-h/12-h light/dark cycle at 20°C in 10% Holtfreter's solution as described (9). Jellied eggs were obtained by manual stripping of females primed with 1,000 units of human chorionic gonadotropin (hCG; Sigma) 12 h prior. Sperm were obtained from euthanized frogs by maceration of the testes and suspension in 1.5 × oocyte Ringer's medium (OR-2) buffer at 2×10^7 cells per ml as described (10) and were stored on ice until use. The composition of 1.5 × OR-2 buffer in mM/liter is NaCl, 124; KCl, 3.77; $CaCl_2$, 1.5; $MgCl_2$, 1.5; Na_2HPO_4, 1.5; Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 10 (adjusted to pH 7.8). The composition of fertilization buffer (F-1) in mM/liter is NaCl, 41.25; KCl, 1.25; $CaCl_2$, 0.25; $MgCl_2$, 0.06; Na_2HPO_4, 0.5; Hepes, 2.5 (adjusted to pH 7.8). TRIzol reagent, *Taq* polymerase, and reverse transcriptase were obtained from GIBCO Life Technology. HPLC-grade acetonitrile was obtained from Fisher Scientific. All chemicals were reagent-grade unless noted otherwise.

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This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AEG, acidic epididymal secretory glycoprotein; CRISP, cysteine-rich secretory protein; 12HEW, 12-h egg water; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight; OR-2, oocyte Ringer's medium; TPX-1, testis-specific spermatocyte protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF393653).

*J.H.O. and X.X. contributed equally to this work.

†To whom reprint requests should be addressed at: Department of Biology, Arizona State University, P.O. Box 871501, Tempe, AZ 85287-1501. E-mail: d.chandler@asu.edu.

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Preparation of Egg Jelly Components. Soluble whole-egg jelly was prepared by dejellying freshly oviposited eggs with 25 mM DTT in 1.5 × OR-2 buffer as described (9). Solubilization, monitored through a dissecting microscope, was allowed to continue until most of the egg jelly was removed but the vitelline envelope remained intact. "Diffusible" egg jelly components were prepared by soaking jellied eggs in 1.5 × OR-2 for 12–16 h at 4°C. Then the resulting conditioned medium [referred to as 12-h egg water (12HEW)] was dialyzed and concentrated by centrifugation through a 5-kDa cutoff Centricon centrifugal filter (Millipore). The diffusible component-depleted eggs then were washed in 3 changes of 1.5 × OR-2 buffer and dejellied by use of DTT to produce a solution of "structural" egg jelly components.

Purification of Allurin. The concentrated 12HEW was separated by anion exchange chromatography, using an Amersham Pharmacia-LKB FPLC unit equipped with a 17 cm by 1 cm column packed with Q Hyper D ceramic resin (Life Technologies) and equilibrated with 50 mM Na_2HPO_4 buffer (pH 7.45). A 1-ml aliquot of 12HEW was loaded onto the column and eluted with a linear gradient of 1 M NaCl in equilibration buffer. Fractions were analyzed by SDS/PAGE by use of 4–20% gradient gels (Sigma), a Laemmli buffer system (30), and were assayed for chemotactic activity as described (10). The fraction with greatest activity was purified further by RP-HPLC. The HPLC purification step was carried out on a Bio-Rad HPLC unit equipped with a Zorbax Stable Bond Wide Bore C-3 analytical column and used acetonitrile/0.01 M trifluoroacetic acid as a solvent system.

Determination of Atomic Mass. The atomic mass of the chemotactic protein was determined by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS on a Vestec (Houston) unit equipped with a frequency-tripled Nd:yttrium/aluminum-garnet (YAG) laser or on a Micromass (Manchester, U.K.) Lynx 3.5 mass spectrometer. In both cases, sinapinic acid was used as a matrix.

Protein Assay. Protein was assayed either by absorption at 205 nm (31) or by the bichinconic acid assay (ref. 32; Pierce). In both cases, BSA was used as a standard.

Chemotaxis Assay. Sperm chemotaxis was quantified as described (10) by using Corning-Costar transwell assay plates consisting of two chambers separated by a polycarbonate membrane having 12-μm-diameter pores. Sperm (10^7) in fertilization buffer (F-1) was added to the top chamber, and a 100-μl bolus of chemoattractant was added carefully to the floor of the bottom chamber without mixing to allow a diffusion gradient to form (10). After a 45-min incubation at 24°C, the top chamber was removed and the sperm that had crossed into the bottom chamber were pelleted, resuspended, and counted by hemocytometer. All assay data represent means and standard errors for at least three replicate experiments.

Amino Acid Sequence Determination. Purified allurin was subjected to proteolytic digestion by incubation of the protein (150 μg) with either Sepharose-linked trypsin (Sigma) or V8 protease (Pierce). After removal of the enzyme-linked beads by use of a serum separator, the proteolytic fragments from allurin were separated by RP-HPLC as described above. Isolated peptides (and the amino terminus of the intact protein) were sequenced by Edman degradation on a Porton Instruments (Tarzana, CA) 2090 Integrated Amino Acid Sequencer. These chemically determined sequences (underlined in Fig. 4) were used to design degenerate primers for reverse transcription (RT)-PCR amplification of cDNAs coding for allurin. Total RNA was isolated by TRIzol extraction of pars convoluta oviductal tissue from human chorionic gonadotropin (hCG)-primed *Xenopus* females. Then complementary DNA was generated by reverse transcription of total RNA as described by Wilson-Rawls *et al.* (33). Each of three overlapping cDNAs were amplified and then cloned by insertion into a TOPO TA vector (Invitrogen), transfection of competent *Escherichia coli*, and blue/white screening of colonies. Isolated plasmid DNA was sequenced by using the dideoxynucleotide method on an Applied Biosystems Prism 377 DNA sequencer. The nucleotide sequences obtained were translated by using the Expasy Proteonomic Tools web site (http://www.expasy.ch/tools/).

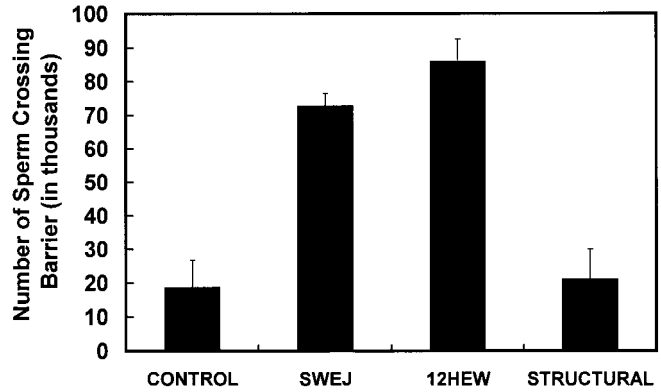

Fig. 1. Sperm chemotaxis activity. Diffusible proteins from *X. laevis* egg jelly exhibit sperm chemoattractant activity. Sperm passage through a porous filter in a two-chamber assay was increased 5-fold over controls by the presence of soluble whole-egg jelly (SWEJ; 60 μg) or diffusible protein extract (12HEW; 30 μg), but not by "structural" (60 μg) egg jelly proteoglycans.

Results

Extracts of diffusible jelly proteins (12HEW) stimulated a 5-fold increase in movement of sperm into the chemoattractant chamber over controls by using only buffer (see Fig. 1). This sperm chemoattractant activity was comparable to that of soluble whole-egg jelly. In contrast, the activity was not observed with structural proteoglycans. Therefore, 12HEW, the highly active but less complex extract, was used as starting material for purification of the chemoattractant protein.

Freshly prepared 12HEW, 650 ml obtained from 60 grams of jellied eggs, was concentrated 145-fold by centrifugation, using a Centricon 5-kDa cutoff device to yield 11.2 mg of protein in a 4.5-ml volume of 50 mM Na_2PO_4 buffer (pH 7.45). Ion exchange separation of 1 ml of this sample resulted in the detection of 7 major peaks (see Fig. 2*A*). Measurement of sperm chemotaxis activity in each peak demonstrated that peak 3 contained the greatest total activity at a specific activity 4 times that of the starting material (Fig. 2*B*). SDS/PAGE analysis of all fractions demonstrated the presence of a prominent band at M_r = 23 kDa in peaks 3 (arrows, Fig. 2*C*) and 7. This band correlated with the presence of sperm chemoattractant activity in both peaks, and as measured by assay, represented 88% of the protein in peak 3, making this protein a prime candidate for further purification. Therefore, peak 3 material was concentrated 30-fold by centrifugation, using a Centricon 5-kDa cutoff device, diluted into 5% acetonitrile/95% 0.01 M trifluoracetate, and 100 μg of protein loaded onto a C3 RP-HPLC column. Protein was eluted by using a linear gradient of acetonitrile, resulting in the appearance of a single sharp peak at 60% acetonitrile (see Fig. 3*A*). This peak, when subjected to MALDI-TOF MS, exhibited single-, double-, and triple-charged molecular ions having mass/charge ratios of 21053, 10534, and 7023 Da per electron unit, respectively (see Fig. 3*B*). The peak appeared to consist of a single protein

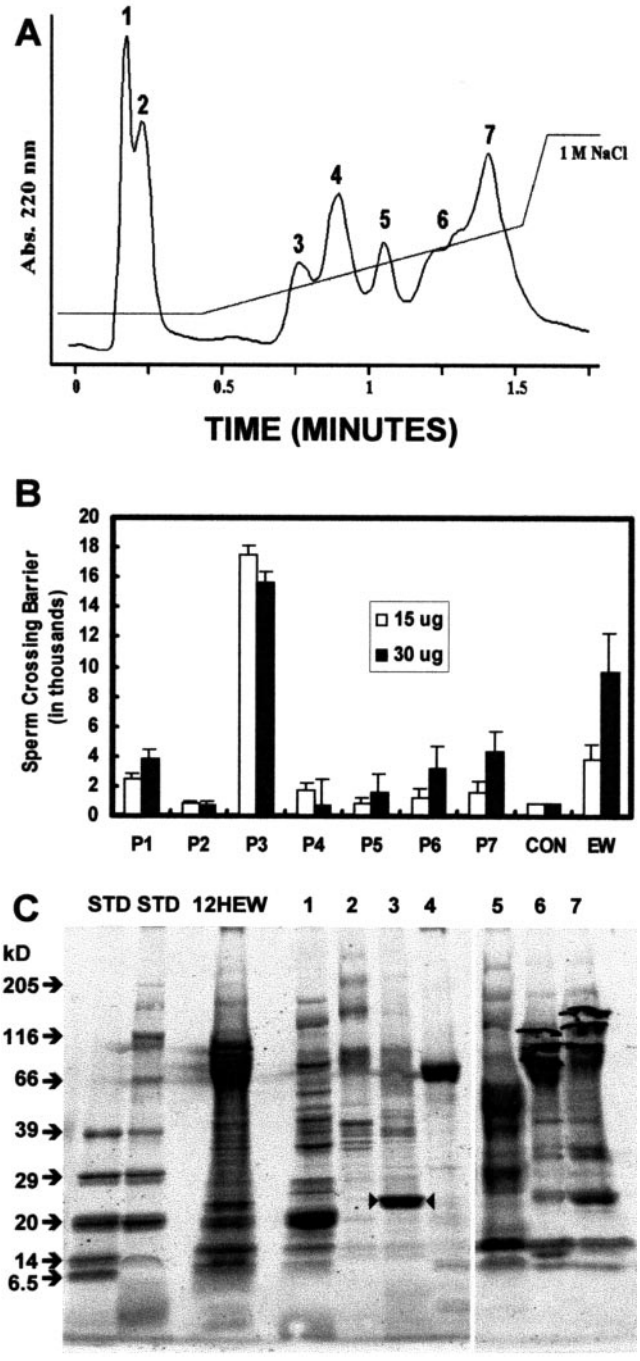

Fig. 2. FPLC purification of allurin. (*A*) Elution profile of 12HEW applied to a FPLC anion exchange column and eluted with 50 mM phosphate buffer (pH 7.45) and a linear gradient of NaCl. (*B*) Sperm chemotaxis activity. Sperm chemotaxis assays of each FPLC fraction indicate that high activity is found in fraction 3 with lower activity found in fraction 7. (*C*) SDS/PAGE analysis of each of the numbered fractions in *A*. Allurin is seen as a major band at M_r = 23 kDa (arrows) in fractions 3 and 7.

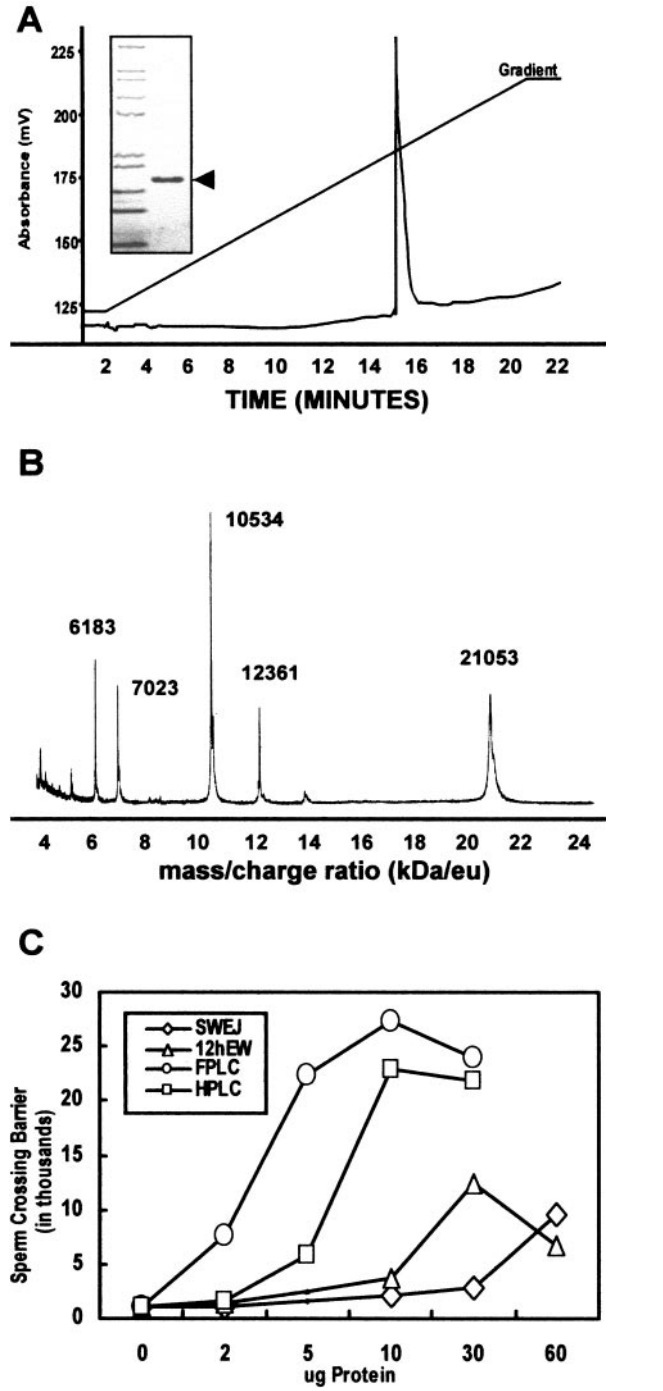

Fig. 3. HPLC purification of allurin. (*A*) HPLC purification of FPLC fraction 3. Elution profile of fraction 3 applied to a C-3 RP-HPLC column and eluted with acetonitrile. Allurin elutes as a sharp peak at 60% acetonitrile. SDS/PAGE analysis of HPLC-purified allurin is shown in the *Inset* (arrowhead). Protein standards at 200, 116, 97, 66, 55, 36, 31, 21, 14, and 6 kDa are shown in the lane to the left. (*B*) MALDI-TOF spectrum of HPLC peak. MALDI-TOF MS of HPLC-purified allurin. Single-, double-, and triple-charged molecular ions are seen at 21,053, 10,534, and 7,023 Da per electron unit, respectively. Cytochrome C, used as an internal standard, exhibits single- and double-charged molecular ions at 12,361 and 6,183 Da per electron unit, respectively. Because of matrix adduct-induced broadening of the single-charged molecular ion peak, the most accurate estimate of molecular mass is considered to be 21,068 Da, which is derived from the double-charged molecular ion peak. (*C*) Chemotaxis activity during purification. Dose-response curves for sperm chemoattractant activity in whole egg jelly (◇), 12HEW (△), FPLC fraction 3 (○), and HPLC-purified allurin (□). Data points represent means of 3 replicate experiments. All standard errors are less than 10% of the mean.

purified to near-homogeneity as indicated by both the MS data and SDS/PAGE analysis (arrowhead, *Inset*, Fig. 3*A*).

Dose-response curves for the sperm chemoattractant activity of the soluble whole-egg jelly, 12HEW, and for the FPLC- (peak 3) and HPLC-purified material (see Fig. 3*C*), demonstrate half-maximal activities of these preparations at 45, 15, 3, and 7 μg, respectively. By using these data, one can determine that the FPLC-purified material is purified 15-fold over the activity

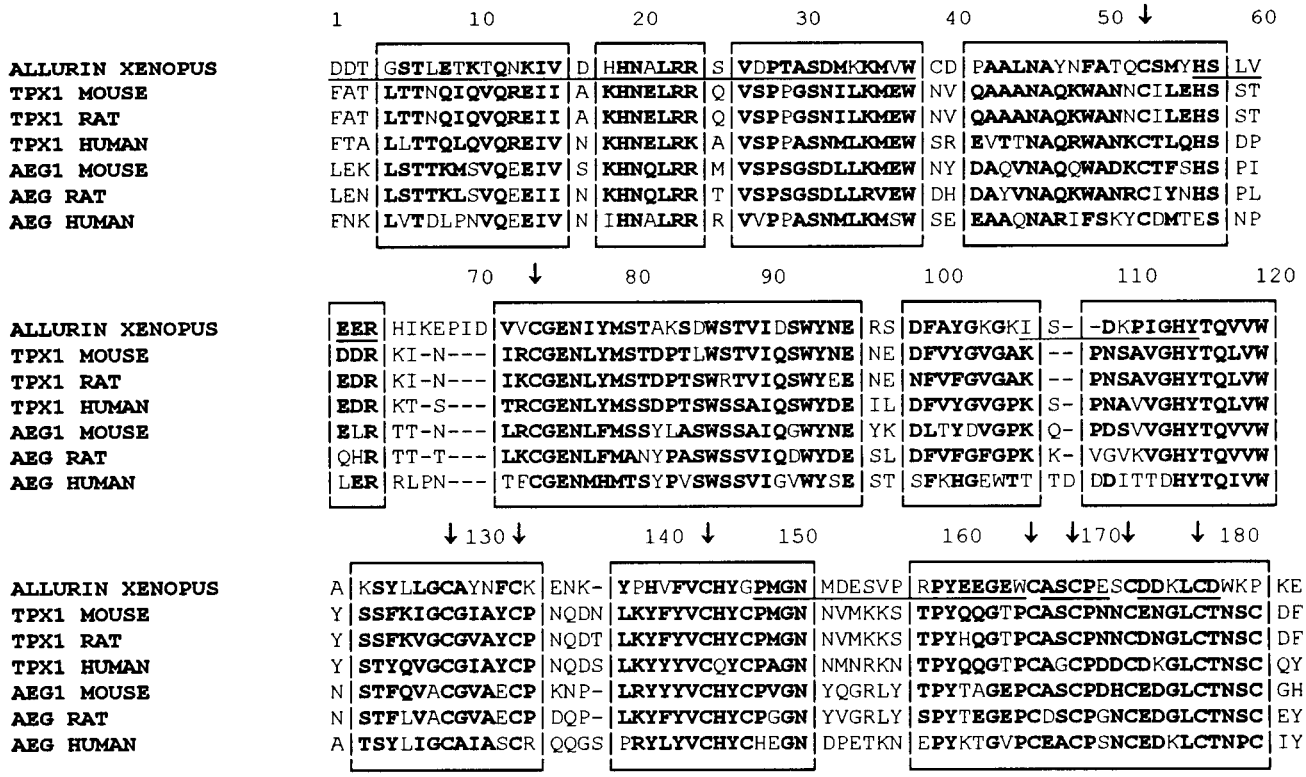

Fig. 4. Complete amino acid sequence of allurin and its alignment with mammalian TPX-1 and AEG sequences. Amino acid identity or conservative substitution at each position is shown in bold letters. Boxes enclose regions of high homology (>70%). Arrows point to positions of conserved cysteines. Regions of the allurin sequence that have been determined by chemical means (Edman degradation) are underlined, whereas those determined by molecular cloning are not. Only partial sequences for TPX-1 and AEG are shown. Sequence alignment was carried out by using CLUSTAL W for GenBank or EMBL/SwissProt accession nos. (top to bottom, respectively): ALLURIN; AAA40472.1/P16563; AAD48090.1/Q9R1L4; AAA61220.1/P16562; AAA37185.1/Q03401; AAB59716.1/P12020; and CAA64524.1/P54107.

found in whole-egg jelly at a yield of 22% of the total sperm chemoattractant activity in whole-egg jelly. Because the HPLC-purified material has a lower specific activity than the FPLC-purified material (likely the result of exposure to harsh solvents), our estimate of fold purification represents a minimum value.

HPLC-purified allurin was subjected to trypsin and V8 protease digestion. The fragments were purified by RP-HPLC, and the amino acid sequence of well separated fragments and the amino terminus of the intact protein were determined by Edman degradation (underlined sequences, Fig. 4). The amino acid sequence was completed by reverse transcription (RT)-PCR amplification, cloning, and sequencing of three cDNAs coding for overlapping regions of the chemoattractant protein. The complete amino acid sequence (Fig. 4) showed allurin to consist of a single 184-aa chain whose calculated average molecular mass of 21,073 Da is nearly identical to the experimental molecular mass of 21,068 Da determined by MALDI-TOF MS (see Fig. 3*B* and legend). The experimental isoelectric point of 5.35 determined by isoelectric focusing is close to the value of 5.2 predicted for this sequence. In addition, an experimental array of peptides resulting from a trypsin digest of the HPLC-purified protein was detected by MALDI-TOF MS. This peptide array, representing the entire allurin sequence, was entirely consistent with the peptide masses predicted by PAWS software (ProteoMetrics, New York; data not shown).

By using the National Center for Biotechnology Information's BLAST to search GenBank (34), we find that the amino acid sequence for allurin shows a high level of homology to sperm-binding proteins in mammals such as TPX-1 and the epididymal protein AEG. As shown in Fig. 4, the complete allurin sequence demonstrates 61% homology to these proteins (bold letters, Fig. 4), and large portions of the sequence are homologous at levels of 70–90% (boxes, Fig. 4). Also notable are a series of nine conserved cysteines that are hallmarks of the CRISP family of proteins (arrows, Fig. 4). Not shown are signal peptide sequences for TPX-1 and AEG and a 60-aa sequence at the C-terminal of TPX-1 and AEG that contains additional conserved cysteines but which is not found in allurin. Because allurin is a secreted protein, it too must have an N-terminal signal peptide that is not found in the processed functional amino acid sequence shown in Fig. 4.

Further analysis of the allurin sequence by using nearest-neighbor joining methods in MEGA 2, a phylogenetic tree-building program (35), revealed that this protein is part of a large family which includes pathogenesis-related proteins in plants, and venom proteins in insects as well as the CRISP family of mammalian proteins (see Fig. 5). The position of allurin within this tree suggests its sequence is related to an ancestral protein that gave rise to this mammalian protein family.

Discussion

Xenopus egg jelly is known to be composed of high molecular weight glycoconjugates that form a structural network (36–38) and low molecular weight proteins that are capable of diffusing out of the jelly matrix during hydration of these layers at spawning (36). A diffusible protein, allurin, has been purified to homogeneity and has been shown to exhibit sperm chemoattractant activity by use of a quantitative two-chamber assay. This assay has been demonstrated to measure directed sperm move-

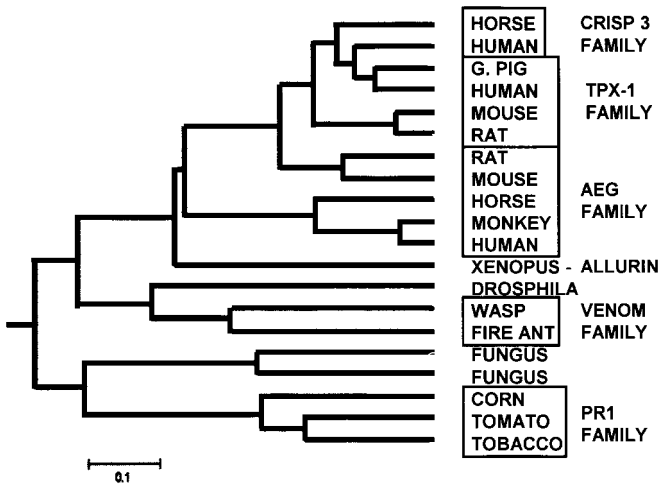

Fig. 5. Phylogenetic analysis suggesting allurin is closely related to a vertebrate ancestral protein that gave rise to the mammalian CRISP family. The phylogenetic tree was constructed by using the nearest-neighbor joining method with MEGA 2 software (35) and the sequences of GenBank or EMBL/SwissProt accession nos. (top to bottom, respectively): CAA04729.1/O19010; CAA64527.1/P54108; AAC52616.1/Q60477; AAA61220.1/P16562; AAA40472.1/P16563; AAD48090.1/Q9R1L4; AAB59716.1/P12020; AAA37185.1/Q03401; CAA07160.1/NONE; AAD27611.1/Q9XSD3; CAA64524.1/P54107; ALLURIN; AAF54913.1/Q9VFY2; AAA30333.1/P35786; AAB65434.1/O16135; AAA16207.1/P35794; AAA16208.1/P35795; AAC25629/O82086; AAA03616.1/P04284; and CAA32228.1/P11670.

ment in response to a chemical gradient of chemoattractant (10). If a chemoattractant such as allurin or 12HEW is pipetted carefully onto the floor of the bottom chamber at its edge, sperm movement through the membrane into the chamber is stimulated (see Fig. 1). In contrast, sperm passage is not stimulated if the chemoattractant is mixed uniformly with the bottom chamber buffer in advance (10). We interpret these data to suggest that focal addition of chemoattractant followed by diffusion of the active agent (allurin) sets up a concentration gradient within the bottom chamber and membrane barrier which is detected by sperm in the upper chamber. This interpretation is in agreement with the observation that focal addition of a fluorescent dye (Lucifer yellow) to the bottom chamber results in a diffusion gradient of the dye being set up which extends to the membrane barrier within 5–10 min (B. Al-Anzi and D.C., unpublished observations).

How vertebrate sperm detect such a concentration gradient *in vivo* is unknown. Studies that have used atrial natriuretic peptide as a chemoattractant for human sperm have suggested the involvement of a guanylate cyclase receptor (39, 40) similar to the resact receptor of sea urchin sperm (41). However, mechanistic information for naturally occurring chemoattractants in vertebrates such as follicular fluid is not available. Studies in other cell types exhibiting chemotactic behavior, such as neutrophils and Dictyostelium slugs, indicate that variations as little as 10% in chemotactic receptor occupancy from one side of a cell to the other can stimulate focal phospholipid metabolism and thereby trigger pseudopod extension (42, 43).

Although it is not yet clear whether allurin is required for successful fertilization in *Xenopus*, the protein is at the right time and place to play an important role. Based on measurements of protein efflux from jelly (36) and on measurements of chemoattractant activity in the medium (J.O., unpublished observations), we estimate that 50% of the allurin present in the egg jelly layers is released into the medium within 5 min after spawning. Thus, *Xenopus* sperm deposited onto the egg mass are exposed immediately to this chemoattractant, a mechanism that may serve to direct sperm to the egg surface at the beginning of a relatively short (15 min) lifespan (44, 45).

Sequence homology of allurin to the mammalian CRISP family of proteins suggests that allurin is a descendant of the ancestral gene that gave rise to this mammalian radiation of sperm-binding proteins. Crisp-1 or AEG is known to be secreted by the epididymis of a number of mammalian species (see Fig. 5) and to bind to the sperm surface at the postacrosomal region (46). During capacitation and subsequent induction of the acrosome reaction in rodent sperm, the bound AEG migrates to the equatorial segment where it could potentially participate in sperm–egg fusion (46, 47). Indeed, AEG has been demonstrated to bind to the egg surface, and the presence of an excess of exogenous AEG can actually block sperm–egg binding (47). How these data relate to the substantial evidence indicating that mammalian sperm–egg fusion is mediated by proteins of the disintegrin/metalloprotease family on the sperm surface and $\alpha 6\beta 1$ integrins on the egg surface (48–52) has yet to be elucidated.

Crisp-2 or TPX-1 was first purified from the acrosomal contents of guinea pig sperm and determined to be a major autoantigen in orchiectomized guinea pigs (22, 23). More recently it has been shown to be expressed and secreted from spermatocytes and in functional assays seems to mediate enhanced binding of spermatocytes to Seritoli cells (24). Crisp-3, found in the seminal vesicles, specific granules of neutrophils, and salivary glands (21), is not known to be a sperm-binding protein.

Members of the CRISP family, including allurin, also share homology with a family of vertebrate and invertebrate venom proteins and with a family of pathogenesis-related proteins in plants. Although the functions of these latter protein families are not known, these homologies are important from the standpoint of protein structure prediction. NMR determination of the tertiary structure of the pathogenesis-related protein 14a in tomato suggests that this family contains a core of antiparallel β-pleated sheets encircled by 3–5 α-helices (53, 54). A series of disulfide bonds between highly conserved cysteine residues (see arrows, Fig. 4) is thought to stabilize this tertiary structure and thereby contribute to the relative heat and solvent stability of allurin and other members of the CRISP family (10).

Addition of allurin to the CRISP family emphasizes the importance of this family in acting as sperm escort proteins. Not only is allurin the first amphibian protein to be added to the family but also is the first family member to be found in the female reproductive tract. Thus, as sperm proceed through their life history, these cells bind secretory proteins of the CRISP family that help mediate their maturation from one functional stage to the next. Their first contact with CRISPs is at the spermatocyte stage where TPX-1 assists spermatocyte–Seritoli cell adhesion. At a subsequent stage, sperm undergoing maturation in the epididymis bind AEGs that seem to aid sperm in becoming fertilization-capable. Next, we hypothesize that mature sperm, in the female tract, interact with female-derived CRISP proteins, some of which may act as chemoattractants, as does allurin. Our hypothesis predicts that allurin binds to a specific receptor on the sperm surface and that similar allurin-like members of CRISP family will be found in the female reproductive tract of mammals.

We thank Bader Al-Anzi, Joshua Johnson, Kristine Stone, and Deetu Simh for technical help and valuable discussions during the course of this study. We also thank Dr. Daniel Brune in the Protein Analysis Laboratory for amino acid sequencing and MALDI-TOF mass spectrometric analysis, Dr. Scott Bingham in the Nucleotide Analysis Laboratory for nucleotide sequencing, Dr. Randall Nelson at Intrinsic Bioprobes, Inc.,

for MALDI-TOF MS, and Dr. Sudhir Kumar for his assistance with use of MEGA 2 software for phylogenetic analysis of allurin, TPX-1, and AEG sequences. We also thank the W. M. Keck Bioimaging Laboratory for use of their Molecular Dynamics Storm PhosphorImager unit. This study was supported by National Science Foundation Grant IBN-9807862, a Research Incentive Award from Arizona State University, and by undergraduate research support for A.K. from the Howard Hughes and Beckman foundations.

1. Wassarman, P. M., Jovine, L. & Litscher, E. S. (2001) *Nat. Cell Biol.* **3,** 9–64.
2. Litscher, E. S., Liu, C., Echelard, Y. & Wassarman, P. M. (1999) *J. Reprod. Fertil.* **116,** 211–216.
3. Tian, J.-D., Gong, H., Thomsen, G. H. & Lennarz, W. J. (1997) *J. Cell Biol.* **136,** 1099–1108.
4. Tian, J.-D., Gong, H., Thomsen, G. H. & Lennarz, W. J. (1997) *Dev. Biol.* **187,** 143–153.
5. Tian, J., Gong, H. & Lennarz, W. J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 829–834.
6. Vo, L. H. & Hedrick, J. L. (2000) *Biol. Reprod.* **62,** 766–774.
7. Hedrick, J. L. & Nishihara, T. (1991) *J. Electron Microsc. Technol.* **17,** 319–335.
8. Katigiri, C. (1987) *Zool. Sci.* **4,** 348–359.
9. Olson, J. H. & Chandler, D. E. (1999) *Dev. Biol.* **210,** 401–410.
10. Al-Anzi, B. & Chandler, D. E. (1998) *Dev. Biol.* **198,** 366–375.
11. Suzuki, N. (1995) *Zool. Sci.* **12,** 13–27.
12. Miller, R. (1985) in *Biology of Fertilization*, eds. Metz, C. & Monroy, A. (Academic, New York), Vol. 2, pp. 275–337.
13. Ward, G. E., Brokaw, C. J., Garbers, D. L. & Vacquier, V. D. (1985) *J. Cell Biol.* **101,** 2324–2329.
14. Shimomura, H., Dangott, D. & Garbers, D. (1986) *J. Biol. Chem.* **261,** 15778–15782.
15. Singh, S., Lowe, D., Thorpe, D., Rodriguez, H., Kuang, W., Dangott, L., Chinkers, M., Goeddel, D. & Garbers, D. (1988) *Nature (London)* **334,** 708–712.
16. Pillai, M., Schields, T., Yanagimachi, R. & Cherr, G. (1993) *J. Exp. Zool.* **265,** 336–342.
17. Ralt, D., Goldenberg, M., Fetterolf, P., Thompson, D., Dor, J., Mashiach, S., Garbers, D. & Eisenbach, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2840–2844.
18. Villanueva-Diaz, C., Arias-Martinez, J., Bustos-Lopez, H. & Vadillo-Ortega, F. (1992) *Fertil. Steril.* **58,** 392–395.
19. Oliveira, R. G., Tomasi, L., Rovasio, R. A. & Giojalas, L. C. (1999) *J. Reprod. Fertil.* **115,** 23–27.
20. Eisenbach, M. & Tur-Kaspa, I. (1999) *BioEssays* **21,** 203–210.
21. Sambony, A., Gentzel, M. Wolfes, H., Raida, M., Neumann, U. & Topfer-Peterson, E. (1998) *Biochim. Biophys. Acta* **1387,** 206–216.
22. Hardy, D. M., Huang, T. T., Driscoll, W. J., Tung, K. K. & Wild, G. C. (1988) *Biol. Reprod.* **38,** 423–437.
23. Foster, J. A. & Gerton, G. A. (1996) *Mol. Reprod. Dev.* **44,** 221–229.
24. Maeda, T., Sakashita, M., Ohba, Y. & Nakanishi, Y. (1998) *Biochem. Biophys. Res. Commun.* **248,** 140–146.
25. Cohen, D. J., Ellerman, D. A. & Causnicu, P. S. (2000) *Biol. Reprod.* **63,** 462–468.
26. Xu, W. & Hamilton, D. W. (1996) *Mol. Reprod. Dev.* **43,** 347–357.
27. Brooks, D. E., Means, A. R., Wright, E. J., Singh, S. P. & Tiver, K. K. (1986) *Eur. J. Biochem.* **161,** 13–18.
28. Charerst, N. J., Joseph, D. R., Wilson, E. M. & French, F. S. (1988) *Mol. Endocrinol.* **2,** 999–1004.
29. Hayahi, M., Fujimoto, S., Takano, H., Ushiki, T., Abe, K., Ishikura, A., Yoshida, M., Kirchoff, C., Ishibashi, T. & Kasahara, M. (1996) *Genomics* **32,** 367–374.
30. Lemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
31. Scopes, R. K. (1974) *Anal. Biochem.* **59,** 277–282.
32. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
33. Wilson-Rawls, J., Hurt, C. R., Parsons, S. M. & Rawls, A. (1999) *Development (Cambridge, U.K.)* **126,** 5217–5229.
34. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402.
35. Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001) *Bioinformatics (Oxford)*, in press.
36. Bonnell, B. S., Reinhart, D. & Chandler, D. E. (1996) *Dev. Biol.* **174,** 32–42.
37. Bonnell, B. S. & Chandler, D. E. (1996) *Mol. Reprod. Dev.* **44,** 212–220.
38. Bonnell, B. S., Smith, S. G. & Hedrick, J. L. (1999) *Anal. Biochem.* **27,** 91–93.
39. Zamir, N., Riven-Kreitman, R., Manor, M., Markler, A., Blumberg, S., Ralt, D. & Eisenbach, M. (1993) *Biochem. Biophys. Res. Commun.* **197,** 116–122.
40. Anderson, R. A., Feathergill, K. A., Rawlins, R. G., Mack, S. R. & Zaneveld, L. J. D. (1995) *Mol. Reprod. Dev.* **40,** 371–378.
41. Garbers, D. (1989) *Annu. Rev. Biochem.* **58,** 719–742.
42. Parent, C. A. & Devreotes, P. N. (1999) *Science* **284,** 765–770.
43. Janetopoulos, C., Jin, T. & Devreotes, P. (2001) *Science* **291,** 2408–2411.
44. Reinhart, D., Ridgeway, J. & Chandler, D. E. (1998) *Zygote* **6,** 173–182.
45. Bernardini, G., Andreitti, F., Camatini, M. & Cosson, M.-P. (1988) *Gamete Res.* **20,** 165–175.
46. Rochwerger, L. & Causnicu, P. S. (1992) *Mol. Reprod. Dev.* **31,** 34–41.
47. Cohen, D. J., Munuce, M. J. & Causnicu, P. S. (1992) *Dev. Biol.* **153,** 83–90.
48. Blobel, C. P., Myles, D., Primakoff, P. & White, J. M. (1990) *J. Cell Biol.* **111,** 69–78.
49. Wolfsberg, T. G., Primakoff, P., Myles, D. G. & White, J. M. (1995) *J. Cell Biol.* **131,** 275–278.
50. Almeida, E., Huovila, A.-P., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G. & White, J. M. (1995) *Cell* **81,** 1095–1104.
51. Myles, D. G., Kimmel, L. H., Blobel, C. P., White, J. M. & Primikoff, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4195–4198.
52. Evans, J. P., Kopf, G. S. & Schultz, R. M. (1997) *Dev. Biol.* **187,** 73–93.
53. Tornero, P., Rodrigo, T., Conejero, V. & Vera, P. (1993) *Plant Physiol.* **102,** 325–325.
54. Fernandez, C., Szyperski, T., Bruyere, T., Ramage, P., Moesinger, E. & Wuethrich, K. (1997) *J. Mol. Biol.* **266,** 576–593.