Jeltraxin, a Frog Egg Jelly Glycoprotein, Has Calcium-Dependent Lectin Properties and Is Related to Human Serum Pentraxins CRP and SAP

Thomas R. Peavy,[‡] Cesar Hernandez,[§] and Edward J. Carroll, Jr.*[,§]

Genetics Program, University of California, Riverside, California 92521, and Department of Biology, California State University, Northridge, California 91330

Received July 24, 2003

ABSTRACT: The egg jelly that encapsulates amphibian eggs is essential for fertilization, but its molecular composition and roles remain largely unknown. We identified a calcium-dependent lectin from the pentraxin superfamily in the egg jelly coat from the South American burrowing frog, *Lepidobatrachus laevis*. This lectin, jeltraxin, was related to the host-response acute phase serum proteins C-reactive P component (CRP) and serum amyloid P component (SAP). The amino acid sequence of jeltraxin is 44% identical to that of *Xenopus laevis* CRP, 31−35% identical to those of mammalian CRP and SAP, and 21−27% identical to those of the large fusion pentraxins. Expression of jeltraxin mRNA was restricted to the oviduct, which distinguishes it as the first serum-related pentraxin not expressed in the liver. Purified jeltraxin was previously shown to exist in an oligomeric complex of ∼250 kDa comprised of self-associating subunits. We have demonstrated by MALDI-TOF that this configuration is due to a decameric complex of 27.7 kDa subunits. Biotinylated jeltraxin bound to the high-molecular mass components of the egg jelly in a calcium-dependent manner with specificity for β-galactose residues. On the basis of homology modeling, we predict that jeltraxin will coordinate two calcium ions. The function of jeltraxin will likely be related to its calcium-dependent lectin properties.

Anuran oocytes accumulate several jelly coat layers after passing through the oviduct, and these extracellular investments are important for fertilization (*1*). Jelly coat layers mediate the initial sperm interactions with the egg, and their molecular configuration promotes sperm penetration and fertilization. Without jelly coat layers, oocytes cannot be fertilized under normal conditions but can be fertilized when solubilized jelly coat components were reintroduced (*2*). Our current understanding of the role of egg jelly during fertilization includes providing diffusible factors for sperm chemoattraction (*3*, *4*), inducing the sperm acrosome reaction (*5*−*8*), and preventing polyspermic fertilization (*9*). In addition, jelly maintains an ionic environment conducive to fertilization, and especially important are divalent cations (*10*).

Anuran egg jelly coat layers are comprised of large fibrillar glycoconjugates that form a scaffold for small diffusible proteins (*11*, *12*). Relatively little is known about the macromolecules that comprise the egg jelly. A few jelly macromolecules have been characterized to date (*13*−*16*), but only a 21 kDa diffusible chemoattractant, allurin, from *Xenopus laevis* egg jelly has been cloned and sequenced (*3*). Allurin was found to be related to the mammalian cysteine-rich secretory proteins (CRISP) that bind sperm at various stages of maturation. Uncovering the molecular identity of other jelly glycoproteins will be crucial to our understanding of the structure and function of egg jelly coats.

We have chosen to study a jelly glycoprotein from the South American burrowing frog, *Lepidobatrachus laevis*, because of its relative abundance within the jelly and its ease of purification from mercaptan-soluble egg jelly (*17*). This jelly glycoprotein has an apparent size of 29.7 kDa as determined by SDS−PAGE, contains 6% carbohydrate, and constitutes ∼60% of the total jelly protein. It was purified as a large self-associating oligomeric complex approximately 250 kDa in size. This glycoprotein is present throughout the two jelly coat layers of oviposited eggs and is found in oviduct extracts (*17*, *18*). Although it is a major component of the *L. laevis* egg jelly, the role of this egg jelly glycoprotein has not been clear. Fertilization was inhibited by only 15% using antisera against the glycoprotein. However, we suspect that the antisera may not have been as potent against the active form of the glycoprotein since the antigen used for antiserum production was a mixture of SDS−PAGE-purified subunits and also the mercaptan-reduced oligomeric complex.

In this paper, we report the cDNA cloning and sequencing of this *L. laevis* egg jelly glycoprotein, establish its relationship as a member of the pentraxin protein superfamily, and demonstrate that it binds jelly ligands in a calcium-dependent manner. Thus, we have named this egg jelly glycoprotein jeltraxin because of its identification as a pentraxin and will use this nomenclature from this point forward.

Pentraxins have been characterized as calcium-dependent lectins of which there are two classes: (1) the classical pentraxins from the serum, CRP[1] and SAP, and (2) the fusion pentraxins (also known as pentaxins) approximately twice the size and containing pentraxin-related carboxy-terminal

* To whom correspondence should be addressed. Telephone: (818) 677-2004. Fax: (818) 677-2044. E-mail: edward.carroll@csun.edu.
[‡] University of California.
[§] California State University.

10.1021/bi035314o CCC: $25.00
Published on Web 10/17/2003

halves and unrelated amino-terminal halves (*19*). CRP and SAP are usually ~25 kDa in size and self-associate to form oligomers that are radially symmetrical pentameric disks, hence the name pentraxins (*20*−*22*). SAP pentamers associate, forming decamers ~250 kDa in size. Synthesized in the liver, serum pentraxins usually function as major acute phase proteins in response to an inflammatory stimulus and are thought to be critical in the host nonspecific defense mechanism (*23*). Besides mammals, serum pentraxins have been identified from the serum of many phylogenetically diverse species such as the anuran *X. laevis* (*24*), several teleost species (*25*, *26*), and the distantly related invertebrate horseshoe crab *Limulus polyphemus* (*27*).

In contrast, much less is known about the structure and function of fusion pentraxins. A fusion pentraxin from the liver of *X. laevis*, XL-PXN1, has been reported but not characterized further (*28*). All other fusion pentraxins that have been described have been from mammalian species and were expressed in a variety of cell types such as neuronal cells (*29*, *30*), endothelial cells (*31*), and most notably spermatocytes (*32*, *33*) and ovarian cumulus granulosa cells (*34*). In guinea pig sperm, a fusion pentraxin termed apexin (p50) was found in the acrosomal matrix that binds other matrix proteins in a calcium-dependent manner and is thought to be important for matrix configuration and release of components. The fusion pentraxin PTX3 is found in the hyaluronan−protein extracellular matrix of follicular mouse eggs secreted by the cumulus granulosa cells. PTX3 plays an important role in ovulation and fertilization as demonstrated by knockout experiments (*34*).

Thus, we report yet another pentraxin found associated with vertebrate gametes that has calcium-dependent binding properties. Unlike apexin and PTX3, jeltraxin was most closely related to the serum pentraxins and has properties similar to those of human SAP.

EXPERIMENTAL PROCEDURES

Animals and Egg Preparations. Adult *L. laevis* frogs originally collected near Filadelphia, Paraguay (*35*), were maintained in tanks under 12 h light−12 h dark conditions. Gravid females were induced to ovulate by subcutaneous injection to the dorsal inguinal area of 100−200 μg/kg of leutenizing hormone-releasing hormone (LH-RH, Bachem, Inc., Torrance, CA) (*17*). After 6−8 h, oviposited eggs were stripped from the females and collected into Ringers solution [110 mM NaCl, 1.9 mM KCl, 0.8 mM $CaCl_2$, and 1.2 mM $NaHCO_3$ (pH 7.8)]. Egg jelly coats were removed by mixing the eggs in a 1:1 ratio with 150 mM β-mercaptoethanol (pH 10) for 2−3 min. The soluble egg jelly was decanted, neutralized, and dialyzed against 0.1 M NaCl, 0.001 M dithiothreitol, and 0.01 M Tris-HCl (pH 8).

Purification of Jeltraxin from Jelly. Soluble egg jelly was dialyzed against 0.025 M Tris-HCl (pH 8.1) and applied to a Bio-Rad Uno-Q1 anion exchange column (bed volume of 1.3 mL). The column was washed with several column volumes of the initial buffer, and a salt gradient from 0 to 1 M NaCl was applied. Collected fractions were dialyzed against 1 mM dithiothreitol (pH 7.2) and analyzed by SDS−PAGE. The peak that eluted at 0.25 M NaCl contained the 29.7 kDa glycoprotein (jeltraxin). After dialysis against phosphate-buffered saline, the protein concentration was measured with the bicinchioninic acid assay (*36*) using bovine serum albumin as a reference.

Biotinylation of Jeltraxin. Purified jeltraxin (1 mg/mL) was biotinylated by addition of 50 μg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 2 h on ice. The resultant solution was dialyzed extensively against Tris-buffered saline (TBS) to remove excess reagent.

Electrophoresis and Blotting. Soluble egg jelly was fractionated by 12% SDS−PAGE under reducing conditions. Gels were stained in Coomassie brilliant blue or the proteins transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). Following the transfer, the membrane was cut into strips to separate sample lanes, blocked with 3% BSA (fraction V, Sigma, St. Louis, MO) in TBS for 2 h, incubated with 0.25 mg/mL biotinylated jeltraxin in TBS and Tween 20 (0.01%, v/v) containing either 10 mM $CaCl_2$ or 10 mM EGTA overnight at 4 °C, washed in TBS and Tween, and then treated with 1 μg/mL streptavidin−horseradish peroxidase conjugate (Sigma) for 1 h. Complexes were detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Plate Binding Assay for Binding of Biotinylated Jeltraxin to Egg Jelly. Soluble egg jelly was diluted to a concentration of 0.1 mg/mL in 100 mM sodium carbonate (pH 9.5) and 50 μL added to microtiter plate wells (Nunc-Immuno Plate, Nalge Nunc International, Rochester, NY) to bind at 4 °C overnight. After blocking with 3% BSA in TBS had been carried out, 50 μL of biotinylated jeltraxin (30 μg/mL) in TBS and Tween was added to the wells in the presence of either calcium (0.1−10 mM), EGTA (1−10 mM), or calcium (10 mM) containing either monosaccharides or disaccharides (0.1 M) for 4 h at room temperature. Glucose, galactose, fucose, mannose, glucosamine, galactosamine, melibiose, and lactose were purchased from Sigma, whereas galactose 6-*O*-sulfate, glucosamine 6-*O*-sulfate, and galactosamine 6-*O*-sulfate were purchased from V-labs (Covington, LA). After being washed with TBS and Tween diluent buffers (i.e., $CaCl_2$ or EGTA), wells were treated with 50 μL of the streptavidin−horseradish peroxidase conjugate (5 μg/mL) for 1 h. The amount of jeltraxin bound was quantified chromogenically for all wells by adding substrate [0.6 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), 0.2% (v/v) H_2O_2 in 50 mM citric acid (pH 4.0)] and kinetically monitoring product formation at 405 nm over the first 5 min. Experiments were repeated in triplicate.

In-Gel Trypsin Digestion and Peptide Sequencing. SDS−PAGE-separated jeltraxin was sliced out of the gel, vacuum-dried, and sent to the Protein and Nucleic Acid Facility at Stanford University Medical Center (Palo Alto, CA) for in-gel trypsin digestion and peptide sequencing.

RNA Isolation. Tissues were removed from LH-RH-stimulated or nonstimulated animals after anesthetization in a 0.5% *m*-aminobenzoic acid ethyl ester methanesulfonate salt solution (MS-222, Sigma). Total RNA was isolated from the tissues by the guanidinium thiocyanate/phenol/chloroform method (*37*), and mRNA was purified with the FastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA).

[1] Abbreviations: CRP, C-reactive P component; SAP, serum amyloid P component; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LH-RH, leutenizing hormone-releasing hormone; TBS, Tris-buffered saline; PR, pars recta; PC, pars convoluta.

Cloning and Sequencing of Jeltraxin cDNA. An oviduct cDNA library was constructed using oviduct mRNA isolated from a post-4 h LH-RH-stimulated female and the unidirectional ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). The oviduct cDNA library was packaged into viral coats using Gigapack II Gold packaging extracts (Stratagene) and amplified using *Escherichia coli* XL1-Blue cells. The amino-terminal portion of the jeltraxin cDNA was PCR amplified (35 cycles at 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 150 s) using degenerate primers designed from the first peptide underlined (N-terminus) in Figure 1A (5′-ACNG-GNAARACNATHATGYTNTT-3′) and the second peptide underlined (5′-RTTNGGNGGRTANGGRTADAT-3′). PCR products were cloned into the PCR II vector (Invitrogen) and sequenced using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH). Plaque lift hybridization screening of the oviduct cDNA was performed using a fluorescein-labeled probe (Renaissance random primer labeling kit; DuPont NEN, Boston, MA) generated from the amino-terminal PCR product and chemiluminescent detection (DuPont NEN). Positively hybridizing phagemids were purified, plasmids excised, and their cDNAs sequenced commercially by the University of California Berkeley DNA Sequencing Facility (Berkeley, CA). The rest of the 5′ end cDNA sequence was obtained by PCR amplification using primers matching the cDNA sequence and the vector's M13 reverse primer. PCR products were cloned and sequenced commercially.

Northern Blot Analysis. Fifteen micrograms of total RNA preparations was denatured in formamide buffer, electrophoretically separated in 1.4% formaldehyde gels, and transferred to charged nylon membranes (Genescreen Plus, DuPont NEN) (*38*). Membranes were prehybridized in ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA) at 68 °C for 3 h. Membranes were then incubated in fresh hybridization solution containing heat-denatured α-^{32}P-labeled jeltraxin cDNA generated by random primer incorporation for 1 h at 68 °C. After being washed twice in high-stringency buffer (0.1× SSC and 0.1% SDS) for 40 min at 50 °C, membranes were exposed to autoradiographic film.

Homology Modeling. The Web-based comparative protein modeling program Swiss-Model (version 3.5, http://www.expasy.ch/swissmod/SWISS-MODEL.html) was used to predict the three-dimensional structure of jeltraxin using the Brookhaven Protein Data Bank (PDB) structures for human CRP (entries 1GNH and 1B09) and SAP (entries 1LGN and 1SAC). The atomic coordinates were optimized using the energy minimization program Grosmos96 and visualized with SwissPDBviewer and POV-ray programs.

MALDI-TOF Analysis. MALDI mass spectra were acquired using a Bruker Proflex III linear time-of-flight instrument (Bruker-Daltonics, Billerica, MA). The spectra that were obtained were an average of 200 laser shots (nitrogen laser, 337 nm). All spectra were recorded in the positive ion mode at an accelerating voltage of 20 kV. The matrix solution was prepared by adding ca. 10 mg of sinapinic acid (solid) to ca. 0.5 mL of 0.1% trifluoroacetic acid in a 2:1 water/acetonitrile mixture. The purified jeltraxin solution (2 mg/mL) was dialyzed against dH_2O and 2 μL mixed with 2 μL of matrix solution. A 1 μL aliquot of this mixture was deposited on the MALDI target and dried in ambient air.

RESULTS

Cloning and Sequencing of Jeltraxin cDNA. SDS−PAGE-fractionated jeltraxin was submitted for trypsin in-gel digestion, and three of the resulting peptides were sequenced. Degenerate oligonucleotide primers were designed from one of the peptides and also from the amino-terminal peptide sequence previously determined (*17*), and used to PCR amplify a 201 bp product from an oviduct cDNA library. Approximately 1.5×10^5 recombinant plaques were screened by hybridization with this partial cDNA, resulting in the identification of six positive clones. The two largest clones were bidirectionally sequenced, yielding the sequence shown in Figure 1A.

Sequence Analysis. The full-length cDNA sequence was 967 bp in length, encoding a 225-amino acid open reading frame (Figure 1A). The amino-terminal and tryptic peptide sequences obtained from the protein were located in the deduced sequence. The first 19 amino acids comprise the signal peptide as predicted by the web-based program SignalP (http://www.cbs.dtu.dk/services/SignalP/) (*39*). Without the signal peptide, the molecular mass calculated for the mature protein was 23.6 kDa and the theoretical pI was 5.6 (*40*). Two potential N-linked glycosylation sites were identified at amino acid residues 87−89 and 207−209. In addition, a pentaxin signature peptide (H-x-C-x-[S/T]-W-x-[S/T]; prosite accession no. PS00289) was located at residues 110−117. Although the first residue did not match, it was a conservative substitution (R instead of H). It should be noted that the first residue of the salmonid SAP pentaxin peptide is a nonconservative substitution.

Relationship to Pentraxins. A search of the protein family database Pfam with the jeltraxin sequence resulted in its unequivocal identification as a pentraxin gene family member (*E*-value score of 4.8×10^{-70}). A Blast search did not identify any other significant gene family relationships. A sequence alignment and a gene tree are presented in Figure 1B and Figure 2, respectively. Jeltraxin was most closely related to the serum pentraxins CRP and SAP rather than to the large fusion pentraxins. The amino acid sequence of jeltraxin was 44% identical with that of *X. laevis* CRP, 31−35% identical with those of mammalian CRP and SAP, 24% identical with that of horseshoe crab CRP, and 21−27% identical with those of fusion pentraxins. In addition, the protein size of jeltraxin was comparable to those of the serum pentraxins which is consistent with this relationship. The residues known to coordinate calcium binding in human SAP and CRP were conserved in jeltraxin as well as the pair of cysteine residues that form a disulfide bridge (*22*, *41*). The conservation of these cysteine residues in all pentraxins indicates that it is an important structural feature.

mRNA Expression. The expression of jeltraxin mRNA within the morphologically and histochemically distinct regions of the oviduct (*18*) was examined by Northern blotting (Figure 3). A single 1 kb band hybridized to radiolabeled jeltraxin cDNA and was found predominantly in pars convoluta regions PC2−4 of the oviduct. The initial portions of the oviduct (PR and PC1) expressed 10−20-fold less jeltraxin by comparison. Jeltraxin was not detected in

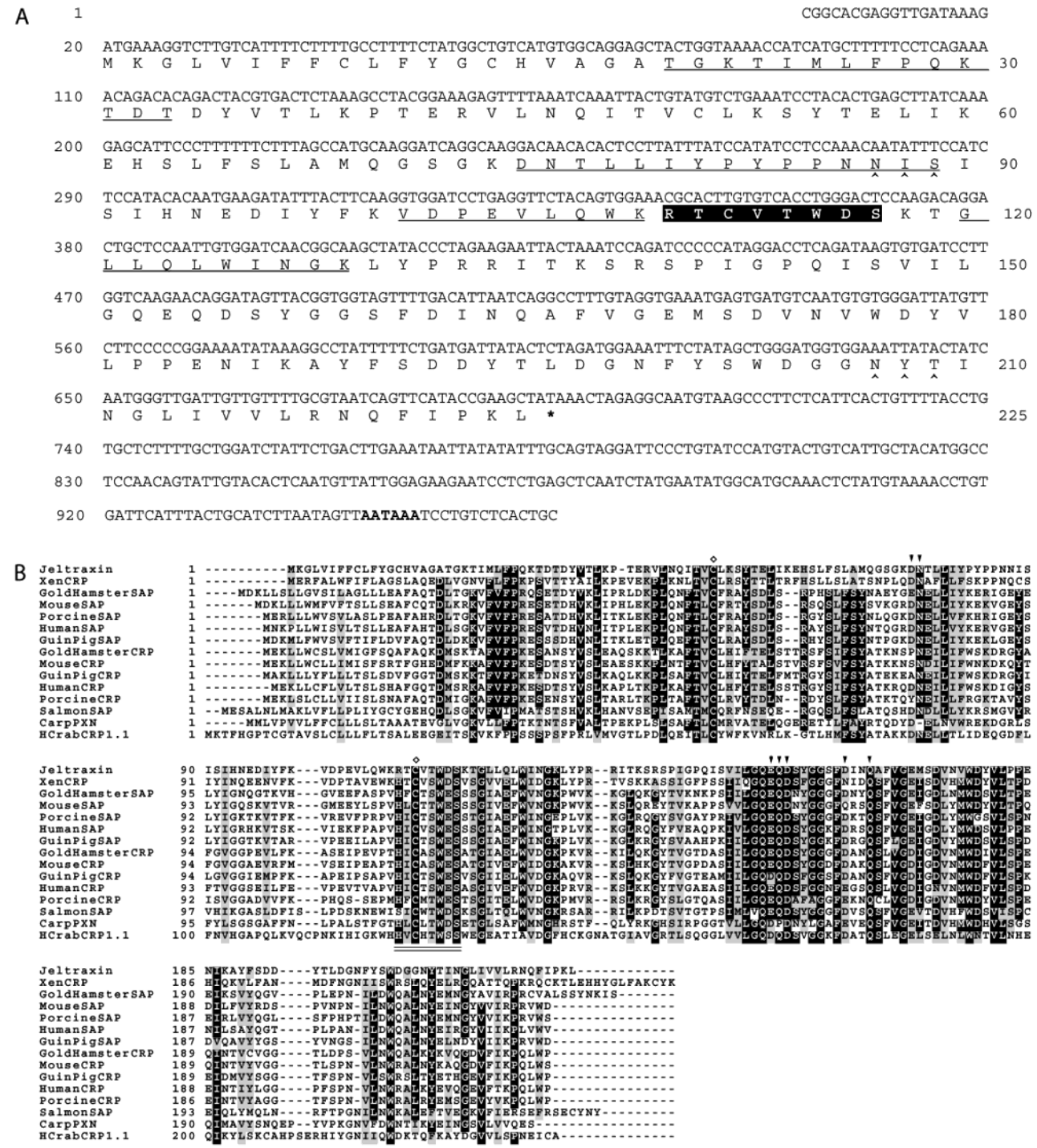

FIGURE 1: Jeltraxin cDNA sequence and alignment with pentraxins. (A) Jeltraxin cDNA sequence (GenBank entry AF047712) and predicted amino acid sequence. Symbols are as follows: underlined amino acids for peptides that were sequenced with the first being the amino terminus, black highlighting for the pentaxin consensus sequence (HxCx[S/T]WxS), arrowheads for potential N-linked glycosylation sites, asterisks for the stop codon, and bold type for the polyadenylation consensus site. (B) ClustalW (version 1.8) amino acid sequence alignment of jeltraxin with pentraxin gene family members *X. laevis* CRP (AAA49692), golden hamster SAP (P07629), mouse SAP (AAA40092), porcine SAP (BAA21474), human SAP (BAA00060), guinea pig SAP (AAC60661), golden hamster CRP (P49262), mouse CRP (CAA35531), guinea pig CRP (AAC60662), human CRP (AAB59526), porcine CRP (BAA21473), salmon SAP (CAA67765), carp pentraxin (BAB69039), and horseshoe crab CRP 1.1 (P06205). Black highlighting was used when more than 11 of the 15 aligned amino acids were identical, and gray highlighting was used likewise for conservative amino acid substitutions. Diamonds mark the two conserved cysteine residue sites. The doubly underlined sequence is the pentaxin consensus sequence. Arrowheads point to the residues known to coordinate calcium ions in human SAP and CRP by crystallographic analyses.

the pars uterina region where eggs are oviposited and no secretory glands are found. In addition, jeltraxin was not detected in a mixture of oviduct mRNA from an animal not hormonally stimulated, although a faint band was observed with a longer exposure time. Other tissues were examined for expression (ovary, testis, liver, fat bodies, stomach, heart, lung, and spleen) but were negative (data not shown).

Homology Modeling. The atomic coordinates from the PDB for the human CRP and SAP crystallographic structures were used to build a theoretical model of jeltraxin (Figure

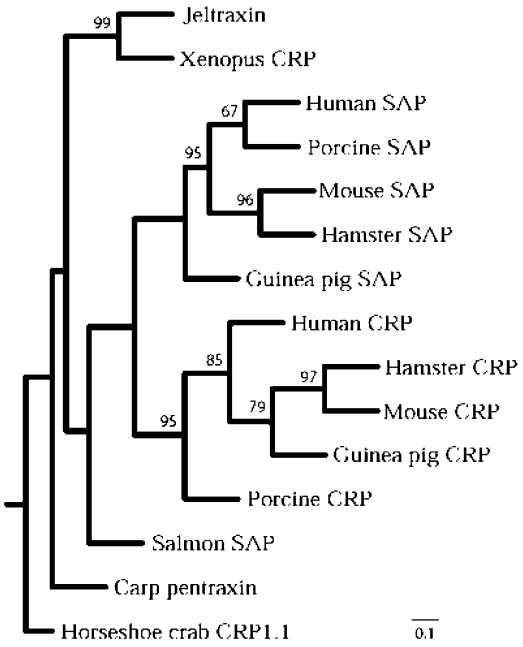

FIGURE 2: Phylogenetic relationship of jeltraxin with pentraxins. The maximum-likelihood method for protein sequences within the Phylip phylogeny inference software package (version 3.6) was used to estimate the relationship of pentraxin family members based on the alignment presented in Figure 1B. Multiple data sets were generated by resampling the alignment data 1000 times to generate the confidence levels that are presented next to branch bifurcations (if >50%). The scale bar represents the number of substitutions per site for a unit branch length.

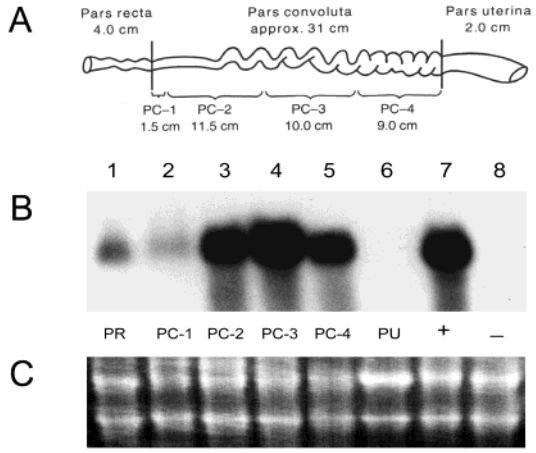

FIGURE 3: Regional expression of jeltraxin in the oviduct by Northern blotting analysis. (A) Illustration of the *L. laevis* oviduct demonstrating the various segments based on histochemical staining properties (*18*). (B) Northern blot of oviduct RNA (15 μg) using a ^{32}P-labeled jeltraxin cDNA probe. Lanes 1−7 were loaded with RNA preparations isolated from hormonally stimulated (LH-RH) animals in the following order: pars recta (PR), pars convoluta segment 1 (PC-1), pars convoluta segment 2 (PC-2), pars convoluta segment 3 (PC-3), pars convoluta segment 4 (PC-4), the pars uterina (PU), and whole oviduct (+). Lane 8 was loaded with a RNA preparation from the whole oviduct from an animal not stimulated with hormones (−). (C) The 28S and 18S rRNA bands of the ethidium bromide-stained agarose gel used in the Northern blot analysis for loading comparison.

4). Jeltraxin was predicted to be a compact globular glycoprotein with a folding pattern equivalent to that of CRP and SAP. The structures consist of a single α-helical domain and antiparallel β-sheet domains arranged in a jellyroll topology with one intrachain disulfide bond between cysteines from a pair of sheets. The side chains that coordinate calcium in CRP are shown in Figure 4, and the corresponding side chains from aligned residues are displayed in the jeltraxin model. Superimposing the positions for the two calcium ions from CRP onto jeltraxin reveals that the corresponding jeltraxin side chains are likely able to coordinate calcium also.

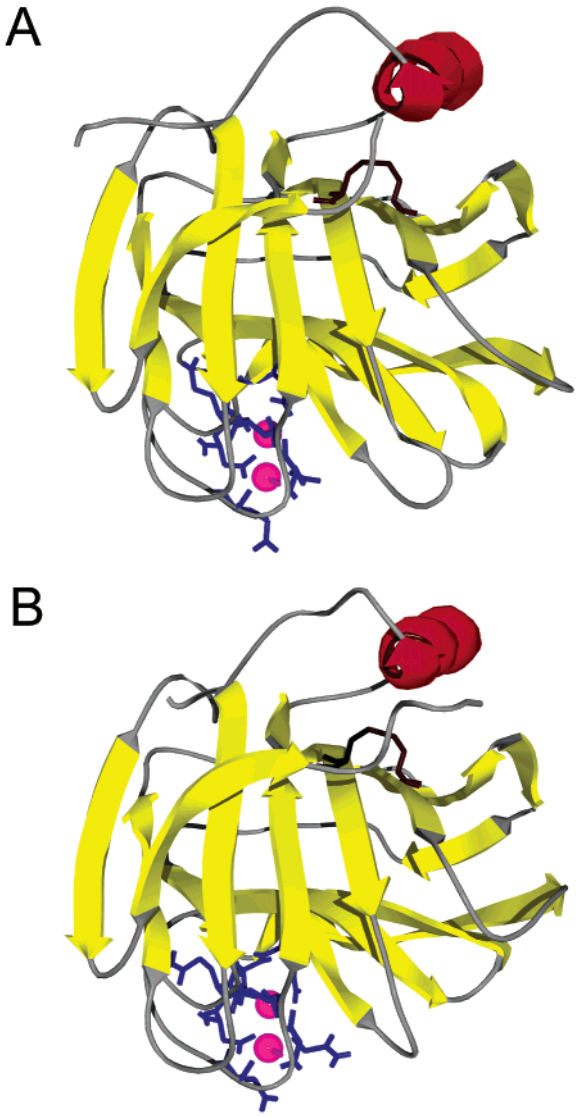

FIGURE 4: Structural model of jeltraxin compared to human CRP. The Web-based comparative protein modeling program Swiss-Model (version 3.5) was used to predict the three-dimensional structure of jeltraxin using the Brookhaven Protein Data Bank (PDB) structures for human CRP (entries 1GNH and 1B09) and SAP (entries 1LGN and 1SAC). The atomic coordinates were optimized using the energy minimization program Grosmos96 and visualized with SwissPDBviewer and POV-ray programs. (A) Crystal structure of human CRP (1B09). The color scheme is as follows: yellow for β-sheet structures with the arrow pointing toward the carboxy terminus, red for an α-helix, gray for random coil stretches, black for the two cysteines, blue for the side chains of residues that coordinate calcium as shown in Figure 1B and pink spheres for calcium ions. (B) Structural model of jeltraxin with calcium ions superimposed from CRP.

Calcium-Dependent Carbohydrate Binding to Egg Jelly Components. We examined the possibility that jeltraxin participates in calcium-dependent lectin−ligand binding interactions with egg jelly macromolecules by Western blotting and a plate binding assay (Figures 5 and 6 and Table

Table 1: Sugar Inhibition of Biotinylated Jeltraxin Binding to Egg Jelly Using the ELISA Plate Binding Assay[a]

	Glu	Gal	Fuc	Man	Glc-NAc	Gal-NAc	Gal-S	Glc-NAc-S	Gal-NAc-S	Mel	Lac
Δ405 nm/min	56 ± 7	15 ± 5	43 ± 9	62 ± 4	57 ± 6	45 ± 4	33 ± 9	59 ± 5	56 ± 7	29 ± 6	9 ± 4
% inhibition	6.5	75	28	0	5	25	45	1.5	6.5	52	85

[a] Biotinylated jeltraxin was incubated in jelly-coated ELISA plate wells in the presence of 10 mM Ca^{2+} and 100 mM monosaccharide or dissacharide solutions. The percent binding inhibition (% inhibition) was calculated by dividing the average change in absorbance (Δ405 nm/min) of the tested sugar by the control Ca^{2+} incubation without sugar (from Figure 6; 60 ± 5). The following sugars were tested, in order from left to right: glucose, galactose, fucose, mannose, glucosamine, galactosamine, galactosamine 6-*O*-sulfate, glucosamine 6-*O*-sulfate, galactosamine 6-*O*-sulfate, melibiose, and lactose.

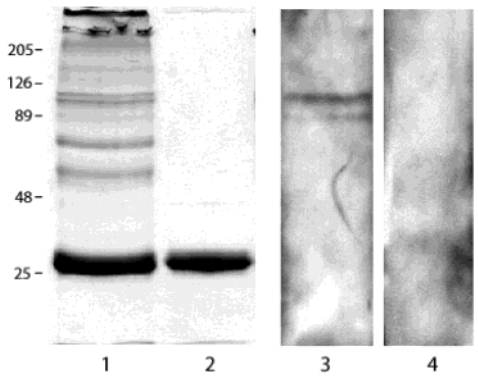

FIGURE 5: Calcium-dependent binding of biotinylated jeltraxin to jelly components as assessed by Western blotting analysis. Purified jeltraxin was biotinylated and used to probe electrophoretically separated jelly components transferred to membranes. Membrane strips were incubated in biotinylated jeltraxin (0.25 mg/mL) solutions containing either calcium (10 mM) or EGTA (10 mM), and then incubated in avidin-bound HRP. Lanes 1 and 2 contained SDS−PAGE-separated and Coomassie-stained jelly and purified jeltraxin preparations, respectively. Lanes 3 and 4 contained the Western blot jelly preparations incubated in biotinylated jeltraxin with calcium and EGTA, respectively.

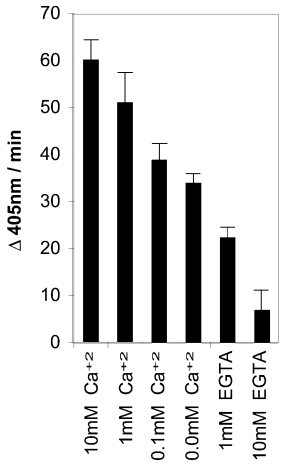

FIGURE 6: Calcium dependence of the binding of biotinylated jeltraxin to jelly using an ELISA plate assay. Biotinylated jeltraxin was added in the presence of calcium or EGTA at varying concentrations to ELISA plate wells coated with egg jelly. After addition of avidin-bound HRP, the enzyme reactivity was monitored kinetically at 405 nm. Error bars represent the standard deviation of three measurements.

1). Purified jeltraxin was biotinylated and then added to Western blots of egg jelly in the presence of either 10 mM calcium or EGTA (Figure 5). Biotinylated jeltraxin bound to jelly macromolecules in the presence of calcium with apparent sizes of approximately 100 and 90 kDa, and also to components of >200 kDa that did not enter the separating gel. In the plate binding assay, binding of biotinylated jeltraxin to egg jelly was clearly dependent on calcium, and the level was reduced dramatically (level of relative inhibition of 92%) when the jeltraxin was incubated with 10 mM EGTA (Figure 6). Without any added calcium or EGTA, jeltraxin could still bind egg jelly which suggests that calcium was retained by jeltraxin. When monosaccharides and disaccharides were added to jelly-coated ELISA plate wells in the presence of 10 mM Ca^{2+} and biotinylated jeltraxin (Table 1), lactose was the most potent inhibitor of binding tested followed by galactose (levels of relative inhibition of 85 and 75%, respectively). These results suggest that jeltraxin−jelly ligand binding is dependent on calcium and specific for terminal β-galactosides.

Oligomerization. Purified jeltraxin was dialyzed extensively against water to remove residual ions and then subjected to mass spectrometry analysis by MALDI-TOF (Figure 7). The size of the monomer was 27.7 kDa, which is 2 kDa smaller than the apparent size of 29.7 kDa estimated by SDS−PAGE (*17*). This is consistent with the amount of carbohydrate estimated previously (6%) and the predicted polypeptide size (23.6 kDa). The signal intensity for the monomer and that for the dimer were the strongest, but definitive signals were detected for all oligomeric sizes ranging up to the decamer. Doubly charged species were also identified probably due to calcium association. Comparison of the residues involved in subunit contacts derived from the human SAP decameric complex (*22*) with jeltraxin revealed that 75% of the aligned amino acids (18 of 24) were either identical or conservative substitutions (Table 2).

DISCUSSION

We have identified a member of the pentraxin protein family from the egg jelly of an anuran amphibian and have shown that it has calcium binding and oligomeric properties such as other serum pentraxins. Pentraxins have been identified in the matrices from mammalian gametes such as apexin in the guinea pig acrosomal matrix (*32*, *33*) and PTX3 in the mouse cumulus cell matrix surrounding eggs (*34*). Unlike apexin and PTX3, jeltraxin was not a fusion pentraxin. Jeltraxin was clearly related to serum pentraxins CRP and SAP by the criteria of size, level of sequence identity, and phylogenetic relationship. Interestingly, all other serum-related pentraxins are synthesized in the liver, whereas the site of synthesis for jeltraxin was the oviduct. Jeltraxin was previously shown to be glycosylated which is a common feature of serum pentraxins (*42*).

Jeltraxin was most closely related to *X. laevis* CRP which could indicate that it originated after the separation of amphibians from other tetrapods. If jeltraxin arose before the split, the cDNA sequence should be equally as divergent from all CRP and SAP members, assuming a constant rate

Table 2: Comparison of the Human SAP Residues Involved in Oligomeric Subunit Contacts with the Aligned Jeltraxin Sequence[a]

residue no.[b]	15	17	46	48	93	95	98	102	112	115	117	126	130	131	132	133	169	170	183	217	219	221	224	225
hSAP	V	**P**	**Y**	D	**K**	*I*	F	*P*	E	S	I	*P*	*V*	K	K	G	**G**	**E**	**P**	*Y*	I	K	*V*	W
jeltraxin	L	**P**	**Y**	E	**K**	*D*	V	*L*	D	T	L	*L*	*P*	R	R	I	**G**	**E**	**P**	*L*	V	R	*Q*	F

[a] The human SAP amino acids that contact neighboring subunits in the crystallographic structure of the decamer (*22*) were compared to the aligned jeltraxin residues from Figure 1B. The shading of residues refers to the following: bold type for conserved residues, regular type for conservative substitutions, and italic type for nonconservative substitutions. [b] Jeltraxin's amino acid position in Figure 1B.

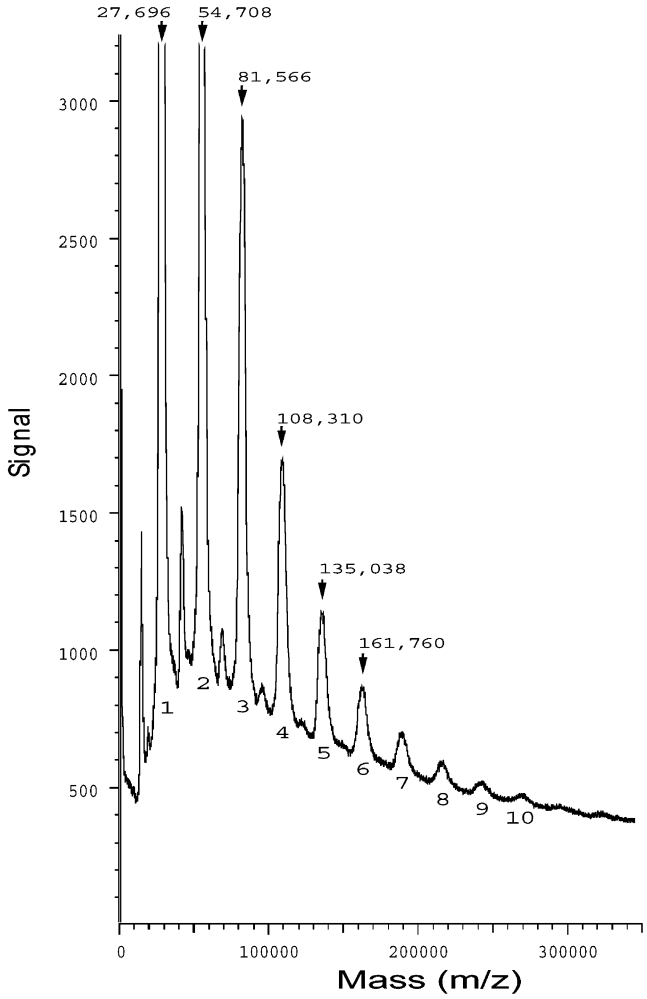

FIGURE 7: MALDI-TOF analysis of jeltraxin. Jeltraxin (2 mg/mL) was mixed with sinapinic acid in a 1:1 ratio and deposited on the MALDI target. This spectrum was recorded in the positive ion mode and is an average of 200 nitrogen laser shots (337 nm). Oligomeric configurations are numbered below respective peaks with mass estimates presented above the peaks for the monomer up to the hexameric complex (singly charged). Doubly charged complexes were also observed but are not labeled.

of evolution. Thus, a jeltraxin homologue might not exist in mammals, although it is still possible. Even though mammalian eggs do not accumulate jelly coat layers like those of amphibians, oviduct-specific glycoproteins are known to associate with the extracellular matrices of mammalian eggs (*43*). Nevertheless, it is probable that the egg jelly from other amphibians has a jeltraxin homologue. In *X. laevis*, a predominant component of diffusible egg jelly was approximately 28 kDa in size but not further characterized (*4*). It will be of interest to determine whether a jeltraxin homologue exists in other species, and also if other pentraxins exist in amphibian gamete matrices such as apexin and PTX3.

Jeltraxin was predicted by homology modeling to be a globular protein with one intrachain disulfide bond and a jellyroll topology equivalent to that of human CRP and SAP. Calcium binding by jeltraxin most likely occurs at the same residues and structural location that coordinate calcium in human CRP and SAP. The coordinating residues were strictly conserved between jeltraxin and human SAP, whereas human CRP had one conservative amino acid change from an aspartic acid to a glutamic acid (jeltraxin residue 162, Figure 1B). It is thought that the longer side chain for glutamic acid enables CRP to bind a second calcium ion with higher affinity than SAP. As evidence in support, human SAP's affinity for calcium has been estimated to be 10-fold lower than that of CRP by proteolytic protection assays (*44*). Also, crystallographic analyses of SAP show that the second calcium was coordinated by an acidic functional group (i.e., acetate ion) provided in the medium rather than by its own side chains (*22*). Attempts to quantify the affinity of the SAP calcium binding sites by equilibrium dialysis have been unsuccessful due to precipitation of SAP in the presence of calcium, but the binding of both calcium ions by human CRP has been determined to have equivalent dissociation constants of 6×10^{-5} at neutral pH (*44*, *45*). The conformation and residues responsible for binding the first calcium ion are nearly identical for human SAP and CRP so the dissociation constant for this site should be equivalent, whereas the second calcium likely has an affinity reduced by 1 order of magnitude. Thus, we predict that jeltraxin will bind two calcium ions in a manner similar to that of human SAP. This could allow jeltraxin to lose or donate one of the two calcium ions more readily. It is possible that acidic jelly ligands may also participate in coordinating the second calcium ion.

Another property similar to that of SAP was that jeltraxin subunits form a decameric complex. Jeltraxin purified from egg jelly had a molecular mass of 245 kDa as determined by sedimentation equilibrium and 263 kDa by gel permeation chromatography (*17*), which is comparable in size to the 250 kDa human SAP decamer (*46*, *47*). Mass spectrometry of jeltraxin using MALDI-TOF provided evidence that it formed a decameric complex comprised of monomers 27.7 kDa in size. We suspect that our higher estimate for the jeltraxin subunit of 29.7 kDa by SDS−PAGE was caused by anomalous migration in gels due to its glycoproteinaceous nature. Decamers of human SAP have been shown to consist of two pentameric complexes associated with relatively few contacts between them (*20*). Jeltraxin decamers may be configured like SAP. Of the 24 amino acid contacts between SAP subunits, 18 of these were either identical or conservative substitutions in the jeltraxin sequence (Table 1). SAP decamers have a spherical diameter of 10 nm, and it should be noted that globular structures of this size were observed in electron micrographs of *X. laevis* egg jelly (*12*).

A property conserved for nearly all pentraxins that have been examined has been calcium-dependent ligand binding. Both jeltraxin and apexin have been found to associate with other components located in its extracellular matrix in a calcium-dependent manner. It has been suggested that apexin coordinates the assembly and/or release of acrosomal contents through calcium-dependent interactions with other acrosomal contents and possibly the acrosomal membrane (*32*). Jeltraxin has calcium-dependent β-galactose-specific lectin associations with egg jelly. Jeltraxin's binding specificity is consistent with that of human SAP which is known to bind the 4,6-cyclic pyruvate acetal of β-galactose (*48*). The significance of jeltraxin's lectin activity is at present unclear. The role of jeltraxin in egg jelly could be strictly structural, but it is likely that the calcium binding property of jeltraxin facilitates fertilization in some manner. The structural integrity of jelly at the time of fertilization is important. Egg jelly coats swell when eggs are spawned into hypotonic media such as pond water and eventually become impenetrable to sperm due to the loss of diffusible factors (*4*, *49*, *50*). Binding of jeltraxin to jelly components might maintain the structure in a state conducive for sperm penetration. Alternatively, jeltraxin itself might be important for interactions with sperm and remain in the egg jelly through these matrix associations. Jeltraxin could serve as an essential reservoir of calcium ions for the induction of the acrosome reaction since this event requires calcium (*8*).

Further studies will be necessary to determine the role of jeltraxin in the egg jelly, and whether homologues are to be found in the egg jelly from other anuran species. We anticipate that further studies on jeltraxin and the identification of additional jelly components will help clarify the role of egg jelly during fertilization.

ACKNOWLEDGMENT

We are grateful to Youngming Xie in Dr. Carlito Lebrilla's laboratory for performing the MALDI-TOF analyses. We also thank Dr. LeAnn Lindsay for critical review of the manuscript.

REFERENCES

1. Hedrick, J. L., and Hardy, D. M. (1991) *Methods Cell Biol. 36*, 231−247.
2. Iwao, Y. (2000) in *Fertilization in Protozoans and Metazoan Animals* (Tarin, J. J., and Cano, A., Eds.) pp 147−191, Springer-Verlag, New York.
3. Olson, J. H., Xiang, X., Ziegert, T., Kittelson, A., Rawls, A., Bieber, A. L., and Chandler, D. E. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 11205−11210.
4. Olson, J. H., and Chandler, D. E. (1999) *Dev. Biol. 210*, 401−410.
5. Campanella, C., Carotenuto, R., Infante, V., Maturi, G., and Atripaldi, U. (1997) *Mol. Reprod. Dev. 47*, 323−333.
6. Picheral, B. (1977) *J. Ultrastruct. Res. 60*, 181−202.
7. Onitake, K., Takai, H., Ukita, M., Mizuno, J. I., Sasaki, T., and Watanabe, A. (2000) *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 126*, 121−128.
8. Ueda, Y., Yoshizaki, N., and Iwao, Y. (2002) *Dev. Biol. 243*, 55−64.
9. Wyrick, R. E., Nishihara, T., and Hedrick, J. L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2067−2071.
10. Ishihara, K., Hosono, J., Kanatani, H., and Katagiri, C. (1984) *Dev. Biol. 105*, 435−442.
11. Bonnell, B. S., and Chandler, D. E. (1996) *Mol. Reprod. Dev. 44*, 212−220.
12. Bonnell, B. S., Reinhart, D., and Chandler, D. E. (1996) *Dev. Biol. 174*, 32−42.
13. Shimoda, Y., Kitajima, K., Inoue, S., and Inoue, Y. (1994) *Eur. J. Biochem. 223*, 223−231.
14. Arranz, S. E., and Cabada, M. O. (2000) *Mol. Reprod. Dev. 56*, 392−400.
15. Quill, T. A., and Hedrick, J. L. (1994) *Dev. Growth Differ. 36*, 615−620.
16. Quill, T. A., and Hedrick, J. L. (1996) *Arch. Biochem. Biophys. 333*, 326−332.
17. Carroll, E. J., Jr., Wei, S. H., and Nagel, G. M. (1991) *Arch. Biochem. Biophys. 284*, 346−351.
18. Perchez, M. A., and Carroll, E. J., Jr. (1996) *Dev. Growth Differ. 38*, 15−22.
19. Gewurz, H., Zhang, X. H., and Lint, T. F. (1995) *Curr. Opin. Immunol. 7*, 54−64.
20. Hohenester, E., Hutchinson, W. L., Pepys, M. B., and Wood, S. P. (1997) *J. Mol. Biol. 269*, 570−578.
21. Thompson, D., Pepys, M. B., and Wood, S. P. (1999) *Struct. Folding Des. 7*, 169−177.
22. Emsley, J., White, H. E., O'Hara, B. P., Oliva, G., Srinivasan, N., Tickle, I. J., Blundell, T. L., Pepys, M. B., and Wood, S. P. (1994) *Nature 367*, 338−345.
23. Steel, D. M., and Whitehead, A. S. (1994) *Immunol. Today 15*, 81−88.
24. Lin, L., and Liu, T. Y. (1993) *J. Biol. Chem. 268*, 6809−6815.
25. Jensen, L. E., Hiney, M. P., Shields, D. C., Uhlar, C. M., Lindsay, A. J., and Whitehead, A. S. (1997) *J. Immunol. 158*, 384−392.
26. Lund, V., and Olafsen, J. A. (1998) *Dev. Comp. Immunol. 22*, 185−194.
27. Robey, F. A., and Liu, T. Y. (1981) *J. Biol. Chem. 256*, 969−975.
28. Seery, L. T., Schoenberg, D. R., Barbaux, S., Sharp, P. M., and Whitehead, A. S. (1993) *Proc. R. Soc. London, Ser. B. 253*, 263−270.
29. Tsui, C. C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Barnes, C., and Worley, P. F. (1996) *J. Neurosci. 16*, 2463−2478.
30. Polentarutti, N., Bottazzi, B., Di Santo, E., Blasi, E., Agnello, D., Ghezzi, P., Introna, M., Bartfai, T., Richards, G., and Mantovani, A. (2000) *J. Neuroimmunol. 106*, 87−94.
31. Breviario, F., d'Aniello, E. M., Golay, J., Peri, G., Bottazzi, B., Bairoch, A., Saccone, S., Marzella, R., Predazzi, V., and Rocchi, M. (1992) *J. Biol. Chem. 267*, 22190−22197.
32. Noland, T. D., Friday, B. B., Maulit, M. T., and Gerton, G. L. (1994) *J. Biol. Chem. 269*, 32607−32614.
33. Reid, M. S., and Blobel, C. P. (1994) *J. Biol. Chem. 269*, 32615−32620.
34. Varani, S., Elvin, J. A., Yan, C. N., DeMayo, J., DeMayo, F. J., Horton, H. F., Byrne, M. C., and Matzuk, M. M. (2002) *Mol. Endocrinol. 16*, 1154−1167.
35. Ruibal, R., and Thomas, E. (1988) *Copeia 1988*, 591−604.
36. 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., and Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
37. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem. 162*, 156−159.
38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Moleular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
39. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng. 10*, 1−6.
40. Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., and Hochstrasser, D. F. (1999) *Methods Mol. Biol. 112*, 531−552.
41. Shrive, A. K., Cheetham, G. M., Holden, D., Myles, D. A., Turnell, W. G., Volanakis, J. E., Pepys, M. B., Bloomer, A. C., and Greenhough, T. J. (1996) *Nat. Struct. Biol. 3*, 346−354.
42. Tennent, G. A., and Pepys, M. B. (1994) *Biochem. Soc. Trans. 22*, 74−79.
43. Buhi, W. C. (2002) *Reproduction 123*, 355−362.
44. Kinoshita, C. M., Gewurz, A. T., Siegel, J. N., Ying, S. C., Hugli, T. E., Coe, J. E., Gupta, R. K., Huckman, R., and Gewurz, H. (1992) *Protein Sci. 1*, 700−709.
45. Kinoshita, C. M., Ying, S. C., Hugli, T. E., Siegel, J. N., Potempa, L. A., Jiang, H., Houghten, R. A., and Gewurz, H. (1989) *Biochemistry 28*, 9840−9848.
46. Pinteric, L., Assimeh, S. N., Kells, D. I., and Painter, R. H. (1976) *J. Immunol. 117*, 79−83.

47. Osmand, A. P., Friedenson, B., Gewurz, H., Painter, R. H., Hofmann, T., and Shelton, E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 739−743.
48. Hind, C. R. K., Collins, P. M., Renn, D., Cook, R. B., Caspi, D., Baltz, M. L., and Pepys, M. B. (1984) *J. Exp. Med. 159*, 1058−1069.
49. Del Pino, E. M. (1973) *J. Exp. Zool. 185*, 121−132.
50. Wolf, D. P., and Hedrick, J. L. (1971) *Dev. Biol. 25*, 348−359.

BI035314O