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A Fragment of Prosaposin (SGP-1) from Rooster Sperm Promotes Sperm-Egg Binding and Improves Fertility in Chickens

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ABSTRACT: A protein isolated from the supernatant of cryopreserved rooster sperm was found to increase the capability of cryopreserved rooster sperm to bind in vitro to the perivitelline membrane of a chicken egg and substantially raise fertility after artificial insemination (AI). That activity was partially purified and termed universal primary sperm-egg binding protein (UPSEBP). Insufficient protein remained from 6×10^{11} sperm, despite retention of bioactivity, to allow sequencing. We deduced that the protein may be related to prosaposin (also termed SGP-1, for sulfated glycoprotein-1), and we used published amino acid sequences of prosaposin as a guide for synthesis of peptides. Certain peptides were found to increase in vitro sperm-egg binding and increase fertility of frozen-thawed or fresh rooster sperm, in a manner similar to semipurified UPSEBP. Active epitopes were in a 60 amino acid sequence, reflecting the intervening sequence between saposins A and B, plus short extensions into saposins A and B. Highest activity was found when this synthetic peptide was oxidized to form a disulfide bond between terminal cysteines. Antibody against a synthetic peptide consisting of 58 of these 60 amino acids bound to a 7–9 kilodalton protein in UPSEBP. Collectively, the data support the conclusion that UPSEBP is a fragment of prosaposin. Because prosaposin is in semen in humans and animals, these observations have broad implications for possible cause and therapy of one type of subfertility.

Key words: Profertility peptides, fertilization, seminal plasma, male reproductive tract.

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Initial loose binding of a spermatozoon to the zona pellucida, to prevent swimming away, may require only 10 linkages (Baltz and Cardullo, 1989) and need not be species specific. However, if initial loose binding between a spermatozoon and the zona pellucida fails, subsequent steps cannot occur. Binding of sperm to the zona pellucida, and later the oolema, involves multiple and synergistic ligands found on the surface of sperm (Yanagimachi, 1994; Thaler and Cardullo, 1996; Tanpaichitr et al, 1998, 1999; Cardullo, 1999; Thaler, 1999; Töpfer-Petersen, 1999). Many sperm ligands are similar across species (Kirchoff, 1998; Welch et al, 1998; Töpfer-Petersen, 1999). Some of these originate from the epididymis, the seminal plasma, or both (Yanagimachi, 1994), and after association with the sperm surface, could serve as a ligand to initially bind sperm to the zona. Certain of these molecules may have utility as profertility molecules, and may be appropriate for therapeutic use.

Literature on profertility peptides on sperm, or present in seminal plasma, is burgeoning. Most reports are of an unidentified spot on a gel, but specific sequences with potential profertility action are being identified (eg, Boué and Sullivan, 1996; Cancel et al, 1997; Naz and Zhu, 1997; Gerena et al, 1998; Welch et al, 1998; Töpfer-Petersen, 1999). Most have been studied via use as an antigen to produce antibodies. The antibody has been mixed with sperm and demonstrated to localize to a specific region of a spermatozoon or to suppress success rate during in vitro fertilization (IVF; eg, Naz and Bhargava, 1990; Bellin et al, 1998). Addition of such molecules (eg, Killian et al, 1993; Henault et al, 1995; Jean et al, 1995), gonadotropin-releasing hormone (Funston and Seidel, 1995), or pGlu-Glu-Pro (Fraser et al, 1997) to a sperm suspension has increased success in IVF. We are unaware of increased production of young from exposure of sperm to these molecules.

Prosaposin (~70 kilodalton [kd], also termed SGP-1 for sulfated glyprotein-1) is found in semen, is a major protein in secretions of Sertoli cells, and also is secreted

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by epididymal principal cells (Sylvester et al, 1984, 1989; Collard and Griswold, 1987; Collard et al, 1988; O'Brien and Kishimoto, 1991; Hermo et al, 1992; Hiraiwa et al, 1993b; Igdoura et al, 1993; Rosenthal et al, 1995; Morales et al, 1998). Despite the presence of prosaposin in cauda epididymal plasma at ~0.01 mg/mL (Sylvester et al, 1989), it was undetected on rat epididymal sperm using a fluorescent antibody approach. No reproductive function had been assigned to seminal prosaposin or its products.

Prosaposin is produced in many tissues from a highly conserved gene (O'Brien and Kishimoto, 1991; Kishimota et al, 1992; Mumford et al, 1995; Leonova et al, 1996; Azuma et al, 1998). Typically, prosaposin is targeted to lysosomes and processed to 4 biologically active saposins (each 7–10 kd, termed saposins A–D) plus 3 residual peptide-intervening sequences of 15 to ~50 amino acids. Fate or function of the intervening sequences is uncertain. The prosaposin family has been extensively studied in nervous tissues, where the detergent-like saposins modulate hydrolysis of lipids in lysosomes. Recently, it was shown that prosaposin is involved in development and maintenance of the accessory sex glands in mice (Morales et al, 2000a, 2000b).

Herein we provide detailed information leading to the conclusion that a seminal molecule, universal primary sperm-egg binding protein (UPSEBP), has a structure closely related to a subdomain of prosaposin, functions as species nonspecific ligand to aid initial sperm-egg binding, and can serve as a profertility molecule. We found UPSEBP in supernatant fluids from rooster sperm that had been cryopreserved by conventional methods which, although allowing survival of many cells, also induces high-salt extraction of surface proteins. Because of freeze-thaw alteration, those sperm had low fertilizing potential, which reduced capability to bind to the egg surface (Alexander et al, 1993). Herein, we describe the biologic activity of UPSEBP and the path of deductions and conclusions on its nature. We concluded that the intervening sequence between saposins A and B (Figure 1) and UPSEBP were similar and had similar biological activity; namely, increasing egg binding and fertility of rooster sperm. An abstract (Hammerstedt et al, 1997) presented early data, but 4 reports on bioactivity of the synthetic sequence (Amann et al, 1999a, 1999b, 1999c; Gill et al, 2000) bypassed the biological underpinning and path to the sequence used. This biological information is provided herein.

Prosaposin molecule (~70 kD) showing how it typically is proteolytically processed to provide Saposin A, B, C & D plus 3 intervening sequences

A B C D

UPSEBP

It is postulated that tissue-specific, alternative folding of prosaposin exposes alternative proteolytic cleavage sites and enables formation of UPSEBP in male reproductive tract

60-61 AA rat and human sequences apparently contain epitopes necessary for biological activity of UPSEBP. The few amino acid substitutions are highly synonymous.

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           Saposin A|
         1                   11                    21
Rat     -C-Q-S-L-Q-E-Y-L-A-E-Q-N-/-Q-R-Q-L-E-S-N-K-I-P-E-V-D-L-A-R-V-V
Mouse                                K                       M
Human      E       K-H       L   H   K                   L   M-T-E
Chicken            K-H     A-M-K-L   K     Q             L   F-S-E-L-T

         31                  41                  51         |Saposin B
Rat      A-P-F-M-S-N-I-P-L-L-L-Y-P-Q-D-R-P-R-S-Q-P-Q-P-K-A-N-E-D-V-C-
Mouse                                  H
Human            A                     G       K         D   G
Chicken  S       A   V                 K   K-/   /-K-S     T
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Figure 1. Representation of prosaposin and saposin structures, and postulated UPSEBP. Data summarized from Furst and Sandoff (1992), Kishimoto et al (1992), Mumford et al (1995), Ponting and Russell (1995), and Azuma et al (1998). The chicken sequence had not been reported when these studies were conducted.

Materials and Methods

General Methods

Semen was obtained from chickens with distinct genotypes (Barbato et al, 1998). Only ejaculates with normal sperm concentration and percentage of motile sperm (Buss, 1993) were used. Experiments requiring fresh sperm utilized the 42H subline (selected at 42 days of age for high growth rate) because that population most closely matches birds used in the broiler industry. Fresh semen was extended in Minnesota A extender (MnA; Tajima et al, 1989) and used within 1 hour after collection. When cryopreserved sperm were needed, the 42L subline (selected at 42 days of age for low growth rate) was used because sperm from these males display good post-thaw fertility (those of the 42H subline do not). Sperm were cryopreserved from individual males or from large pools of semen (from >12 roosters of line 42L) exactly as described in Alexander et al (1993). Cryopreserved semen was accumulated over time and allocated for use so that any experiment included containers of several individual or pooled ejaculates.

Quality of processed or treated sperm was evaluated as 1) percentage of motile sperm (Buss, 1993), 2) exclusion of propidium iodide as assessed by flow cytometry (Watson et al, 1992), and 3) in vitro sperm-egg binding (detailed below).

Initial experiments were semiquantitative because the sperm-binding assay (see later discussion) was being developed in conjunction with characterization of UPSEBP. As is traditional in isolation of biological factors, these semiquantitative data with partially purified extracts are presented graphically as 1 replicate typical of a specified number of replicates, rather than as means with standard deviations. Follow-up studies provided quantitative, refined estimates and were analyzed statistically, as described in the discussions of individual experiments.

Assay of Sperm-Egg Binding

A sperm binding assay (SBA) was essential to monitor bioactivity of protein extracts or synthetic peptides, and one was evolving (Barbato et al, 1998) concurrent with initial studies in this project. Assay 1 used native perivitelline membrane from laid

hen's eggs placed on a glass slide, with a template overlaid, and enumeration of number of sperm bound using a microscope. Assay 2 used a heat-solubilized extract of hen's egg perivitelline membrane deposited in wells of a 96-well plate, and enumeration of number of sperm bound using a microscope. Assay 3 used a heat-solubilized extract of hen's egg perivitelline membrane deposited in wells of a 96-well plate, and automated enumeration of number of sperm bound. DNA was stained, quantified using a microwell-plate reader, and converted to number of sperm bound. Each assay was used as detailed in Barbato et al (1998).

To test effects of substances on sperm binding, cells were prepared at 5×10^8 sperm/mL in MnA. Components (eg, crude UPSEBP, peptides, antibodies) were added to the stipulated concentration and sperm suspensions were incubated at room temperature for 10 minutes. Samples were diluted 10-fold and dispensed into microwells for analysis of binding capability during a 1-hour incubation.

To test the ability of crude UPSEBP to block binding of sperm to the egg-membrane substrate, selected microwells received 100 μL of MnA containing UPSEBP (0, 5, to 15 μL of ~0.25 mg/mL solution). After 90 minutes at room temperature, wells were washed 3 times with MnA. Then cryopreserved sperm were added and binding was measured by SBA.

The definitive evaluation of peptides via SBA used cryopreserved sperm, treated as described above, and evaluated using assay 3. Each of 3 to 5 replicates pools of semen was split, allocated to treatments, and each treatment was dispensed into 3 wells of an SBA plate. Resultant data were analyzed via nested analysis of variance (ANOVA) and the model $Y_{ij} = m + P_i + R_{ij} + e_{ijk}$, where i = the peptide tested (fixed effect), j = the random variable of plate used, and k = the random variable of wells per plate. Differences between mean of control and test peptide were tested by both the least significant range and Duncan's procedures (PC-SAS, version 6.11).

Isolation of Crude UPSEBP and Denaturation Studies

Seminal plasma recovered by centrifugation of fresh semen had <5% of the UPSEBP activity found in supernatants from cryopreserved sperm. Hence, semen from 10 to 50 males was pooled, extended with MnA containing 9% (w/v) glycerol, and frozen to −196°C. In early trials, after thawing, sperm were sedimented by centrifugation and the supernatant was concentrated with a Centriprep 10 cartridge (10 kd cutoff filter, Amicon YM10 13632; Fisher Scientific, Pittsburgh, Pa). The retentate (UPSEBP-1) was stored at −20°C.

We found that all bioactivity was retained by a 100-kd cutoff filter (Amicon YM100 14432; Fisher Scientific). Extraction of the 100-kd retentate with $CHCl_3$:CH_3OH (Mills et al, 1984) gave a preparation with greater than twice the bioactivity. This was termed UPSEBP-2.

To prepare purer material, supernatants from thawed sperm (from 30–300 ejaculates) were pooled and concentrated on a 300-kd cutoff filter (Amicon XM300 14332; Fisher Scientific). All biological activity (based on assay 2) was retained, and >98% of materials (protein) absorbing at 280 nm was removed in the filtrate. After removal of lipids from the retentate with butanol and isopropyl ether (Mills et al, 1984), the aqueous fraction contained all the original activity. This was concentrated by lyophilization. The powder was dissolved in and dialyzed (3.5 kd cutoff membrane) against Tris buffer (150 mM NaCl in 10 mM Tris, pH 7.4) to change the solution to one that was appropriate for chromatography. Size exclusion chromatography (Supelco G3000 SWXL column; size 0.78 × 30 cm; Supelco Inc, Bellefonte, Pa; Tris buffer) yielded a 5- to 10-kd fraction containing >75% of initial biological activity. This active fraction was subjected to high-performance liquid chromatography (HPLC; Rivier, 1978) using a C-4 column (Supelco LC-304; 5 micron, 0.46 × 25 cm) and a gradient elution of water: acetonitrile (ACN) + 0.05% trifluoroacetic acid (TFA; VWR Inc, Pittsburgh, Pa). Material eluting at ~30% ACN contained ~90% of the bioactivity placed on the column and was lyophilized (UPSEBP-3).

Sensitivity of UPSEBP to proteolytic treatment was assessed by adding 1 mg chymotrypsin (Sigma C7762; Sigma Chemical Company, St Louis, Mo) to 0.25 mL of UPSEBP-2 (0.25 mg/mL protein), incubation at 37°C for 18 hours, addition of 20 μmol of phenylmethylsulfonyl fluoride (Sigma P7626) in isopropanol to inactivate the enzyme, lyophilization to remove solvents, and resuspension in 250 μL water. A control treatment without UPSEBP was conducted to assure that chymotrypsin was inactivated. Sensitivity to heat was evaluated by placing 0.25 mL of UPSEBP-2 in a boiling water bath for 5 minutes followed by cooling. Alkali treatment was evaluated by adding NaOH to 0.25 mL of UPSEBP-2 to bring the solution to a pH of ~9.0, boiling for 5 minutes, cooling, and readjustment of pH to ~7.0 with HCl. Resultant materials, with and without supplementation with untreated UPSEBP-2, were evaluated for bioactivity using cryopreserved sperm and assay 1.

Prosaposin Preparations and Antibodies

Rat prosaposin (SGP-1), purified from medium around cultured Sertoli cells (Sylvester et al, 1989), was provided by M.D. Griswold. Human saposins A, B, C, and D (Morimoto et al, 1988; Hiraiwa et al, 1993a; Fu et al, 1994) were provided by J.F. O'Brien. Protein content of each was measured with the bicinchoninic acid (BCA assay; Sigma B-9643 using bovine serum albumin, Fraction V, ICN 160069 as standard). Polyclonal antibody to rat prosaposin (Sylvester et al, 1989) was provided by M.D. Griswold. Antibody to human saposins A, B, C, and D and to a synthetic sequence representing the carboxyl 15 amino acids in the intervening A–B sequence (Morimoto et al, 1988, 1989; Fu et al, 1994) were provided by J.F. O'Brien. They were used undiluted or after 1:4, 1:5, and 1:10 dilution. Normal rabbit serum was used as a control.

Synthetic Peptides

The peptide sequence for rat prosaposin (Figure 1), and early binding data using preparations of prosaposin, saposins, and antibodies, guided decisions on what peptide sequences to evaluate. Initial emphasis involved short sequences at the interface of the A–B intervening sequence and saposin B. Several peptides (5 to 15 amino acids) were prepared and tested in SBAs. Subsequently, larger peptides reflecting the complete sequence from near the carboxy terminal of saposin A into the amino terminal of saposin B were studied (Figure 2; up to 61 amino acids).

Peptides were prepared using an ABI Model 431A synthesizer

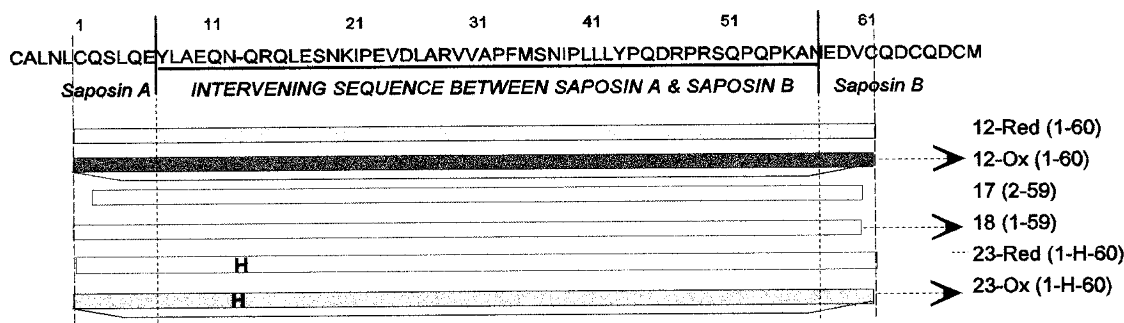

Figure 2. Representation of peptides synthesized and tested for bioactivity. Numbering sequence of amino acids (1–60) starts with the cysteine toward the amino terminus (see also Figure 1). Saposin A ends at residue 6 (E; vertical solid line) and saposin B begins at residue 57 (E; vertical solid line). All peptides shown, except peptide S23, were of the rat sequence. Peptide S23 was of the human sequence, which has minor differences throughout plus an additional histidine (H; see Figure 1). For peptides S12 and S23 the reduced form is indicated by the box only, whereas the disulfide oxidized form has a line connecting the amino and carboxyl terminal cysteines. Dotted horizontal lines orient schematic boxes with peptide identification numbers. Shaded boxes designate peptides with activity, either by assay 3 or fertility assays, and the darkest shading represents most active peptide.

(PE Biosystems, Foster City, Calif), 4-hydroxymethyl phenoxylmethyl (HMP) resin, and F-moc chemistry. For peptides S12, S17, S18, and S23, synthesis was interrupted approximately halfway through the sequence, a portion of the resin was removed from the reaction vessel, and synthesis continued to complete a peptide containing the desired 58 to 61 amino acids. Peptide was cleaved from the resin, precipitated with ether, washed, and lyophilized. Peptides were purified by reverse-phase HPLC (Ranin 300A C-18 column [0.46 × 10 cm]; Ranin Inc, Woburn, Mass; 0.3% trialkyl ammonium phosphate as mobile phase at 1 mL/min; Rivier, 1978) and characterized. For example, peptide S12-Red eluted at 31 to 33 minutes, and based on mass spectral analysis had a purity of >95% and a molecular weight of 6911 (theoretical 6909; within the error of the determination).

To establish molar content, aliquots of peptide solutions were hydrolyzed and subjected to quantitative amino acid analysis (Barbato and Wideman, 1990). The molar content of amino acid residues was consistent with the expected sequence and allowed calculation of molar concentration of peptide. For peptides S12, S17, S18, and S23, solutions also were analyzed by the BCA protein assay. It was found that 0.2 μmol of peptide were equivalent to 1.0 mg of bovine serum albumin.

Peptides S12 and S23 were oxidized (Weissman and Kim, 1991) to form an internal disulfide linkage. The reaction mix was fractionated on an open C-18 column (Supelco 57012) conditioned with 20 column volumes of 80% ACN plus 0.1% (v/v) of TFA in water. After loading the sample, unwanted material was eluted with 3 volumes of 0.1% TFA in water followed by 3 volumes of 10% (v/v) ACN in 0.1% TFA in water. Unreacted linear peptide was eluted with 5 volumes of 30% ACN in water, after which the desired cyclic form (internal disulfide bond) was eluted with 5 volumes of 55% ACN in water. Solvent was removed by lyophilization. The sample was dissolved in 55% ACN in water and stored at −20°C. Protein (see above) and sulfhydryl (SH; Sigma D-8130; Ellman, 1959) contents were determined and the molar ratios of free SH to peptide for the linear and cyclic forms were calculated. Ratio for the linear form was 1.8 (theoretical value, 2.0) and that for the oxidized form was 0.04 (theoretical value, 0.0).

Preparation of Antibodies to Peptide S17

To assure that their preimmune serum did not react with bull or rooster sperm, candidate female New Zealand White rabbits were evaluated. Sperm were washed twice, fixed in 1% (v/v) glutaraldehyde, exposed for 30 minutes to 1:100 diluted preimmune serum (in phosphate buffered saline; PBS), washed twice in PBS, exposed to anti-rabbit immunoglobulin G (IgG) labeled with fluorescein isothiocyanate, and counterstained with propidium iodide. Flow cytometry was used to identify sperm as 1) fluoroscein negative, propidium iodide positive; or 2) fluoroscein positive, propidium iodide positive. Rabbits were rejected if >3% of the stained sperm were in the second category when treated with preimmune serum.

Three rabbits passing the prescreen were selected and immunized (Hurn and Chandler, 1980) with peptide S17 dissolved in PBS and combined (1:1) with Freunds complete adjuvant to 1 mg/mL. Each rabbit was injected with 100 μg peptide distributed in 20 sites. Booster injections (50 μg peptide S17 in Freunds incomplete adjuvant) were administered on days 28, 63, and 110, with exsanguination on day 125. Antibody was purified (NH_4SO_4 precipitation, diethylaminoethyl [DEAE] column chromatography) and stored at 4°C in the presence of 0.02% NaN_3. Antisera from all 3 rabbits were tested against the antigen, and each provided similar qualitative results. The reagent with the highest titer (antibody code 5438B, DEAE purified) was used.

Comparisons Between Peptides of Known Structure and Partially Purified UPSEBP

Two indirect approaches were used to assess the relationship of known compounds to UPSEBP based on their biological activity. First, an antibody to peptide S17 was used to determine if that preparation affected sperm-egg binding similarly to saposin antibodies. Fresh rooster sperm (5×10^8 sperm/mL in MnA) were treated for 15 minutes at ambient temperature with 1) no addition, 2) 1:5 dilution of preimmune serum, or 3) 1:5 to 1:500 dilution of antibody against peptide S17. Samples were diluted 1:10 with MnA and evaluated using assay 3.

Second, chromatographic characteristics of UPSEBP were studied using the purification scheme found to separate peptides

S12-Red and S12-Ox. UPSEBP-1 (~10 mg) was applied to open C-18 columns, and processed as described to yield materials 1) not binding to the column, 2) eluted with 10% ACN (where polymerized oxidized forms of peptide S12-Ox eluted), 3) eluted with 30% ACN (where monomeric peptide S12-Red eluted), or 4) eluted with 55% ACN (where monomeric peptide S12-Ox eluted). Fractions were evaluated for effects on sperm binding using assay 3.

Assay of Fertility

In chickens, a single ovulation usually occurs daily, followed by a ~15-minute interval when fertilization can occur, and the egg is laid the next day (Bakst et al, 1994; Etches, 1996). Fertilization after ~15 minutes is precluded by deposition of a layer of lipid and proteinaceous material over the ovum. However, fertilization of successive ova can occur for a number of days after a single artificial insemination (AI). The eggs have a decreasing probability of being fertilized over days 1 to 21 after a single AI (day 0). The interval over which eggs are fertilized after a single AI, and percentage fertilized, reflect quality and number of sperm inseminated, plus an effect of hen (Wishart, 1995; Wishart and Staines, 1999). With multiple AIs, practiced commercially to maximize overall fertility, outcome is likely dominated by the features of the latest sperm inseminated. We measured percentages of laid eggs providing a live chick after 21 days of incubation, an outcome termed "hatch."

Because large male-to-male differences in response to treatment are common, and were anticipated, whenever possible we compared effects of treatment or treatments on hatch on a within-male basis, using split ejaculates and inseminations into ≥6 hens per treatment. We used commercial (Dekalb, DeKalb, Ill) White Leghorn hens, because they lay an egg almost every day, are highly fertile, and were unrelated to the roosters. This minimized confounding of treatment effects due to sperm by male-female interactions.

Generally, we tested hatch for individual males or pooled semen via 3 successive AIs into hens, at 3-day intervals, using a limited number of sperm. This allowed pooling of outcome data across hens and interval after the first, or each subsequent, AI. Percentage data for hatch were transformed (arc sin$\sqrt{\%}$) to improve normality before one-way ANOVA (PC-SAS, version 6.11).

In fertility trials, subtle treatment affects can be masked by insemination of an excessive number of sperm (Amann and Hammerstedt, 1993; Hammerstedt, 1996). With poultry, subtle differences among populations of sperm, or treatments, can be detected via the slope of the decline from maximum hatch after a single AI or the last of a series of AIs (Kirby and Froman, 1990; 1991). We analyzed certain data sets with this approach, using the equation:

$$f(x) = \frac{\gamma}{1 + e^{\beta(\tau - x)}},$$

where γ = initial specific hatch, β = rate of decay from initial specific hatch, and τ = inflection point of the decline of hatch (ie, days until hatch becomes ½ of the initial specific hatch). The program described by Kirby and Froman (1990) was modified by Barbato (1991) to improve concordance between estimated and observed values. We used a constant for initial specific hatch, calculated as the mean for the first 3 days after the final AI. Statistical comparisons were performed using PROC GLM in PC-SAS (version 6.11).

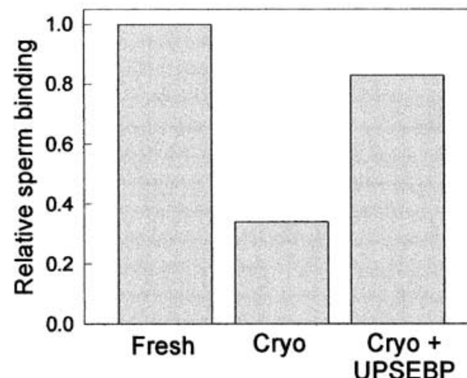

Figure 3. Restoration by UPSEBP-1 of binding capacity of cryopreserved rooster sperm to an egg membrane, as evaluated by assay 1. Cryopreserved sperm were exposed to crude UPSEBP-1 (0 or 25 μg in 0.1 mL; cryo or cryo + UPSEBP, respectively), incubated 10 minutes, diluted 1:10, and assayed concurrently with fresh sperm. Relative sperm binding is ratio of percentage sperm bound for a treated sample divided by percentage sperm bound for control sample. In this case, treated is cryopreserved sperm or cryopreserved sperm exposed to UPSEBP and the control is fresh sperm. Data are representative of 3 replicates, each using different pools of fresh and cryopreserved semen.

Results

Effects of UPSEBP on Sperm Binding and Fertility

Cryopreservation reduced binding of rooster sperm in a SBA (assay 1) by ~65% (Figure 3). However, binding capability was substantially restored by 10-minute treatment of the sperm suspension with UPSEBP-1 (25 μg per 0.1 mL) before 10-fold dilution and evaluation by SBA. One interpretation was that UPSEBP-1 acted as a "bridging ligand" between a spermatozoon and the perivitelline membrane. If so, preincubation of perivitelline membrane with UPSEBP-1 before addition of sperm should substantially reduce binding. Increasing doses of crude UPSEBP-1 reduced binding to <20% of the control (Figure 4). The r^2 value for the semi-log plot for pooled data from 3 replicates was 0.999, which is consistent with 1 class of ligand with a single binding. The SBA provided a bioassay useful during isolation and characterization of putative UPSEBP.

Efficacy of UPSEBP-2 in restoring binding (assay 1) and fertilizing potential to cryopreserved sperm was studied, along with other attributes. Data were normalized against concurrently run fresh sperm. Cryopreservation reduced ($P < .05$) the percentage of motile sperm and the percentage of sperm with an intact plasma membrane (Figure 5), and exposure of sperm to UPSEBP-2 had no effect on either attribute. Although sperm binding was

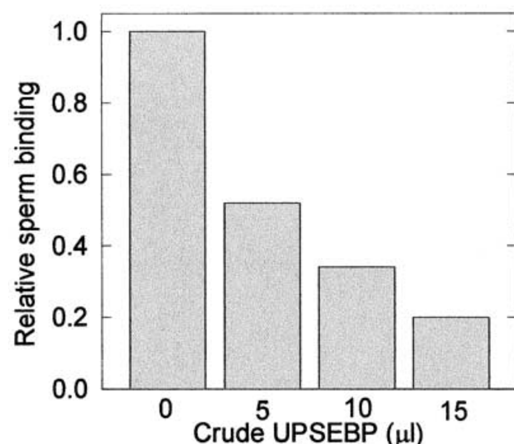

Figure 4. Ability of UPSEBP-1 to block binding of fresh sperm to egg membrane. Egg membranes were exposed to 0 to 15 µL of crude UPSEBP-1 (250 µ/mL) for 90 minutes before washing once and adding sperm suspensions for determination of binding capacity by assay 1. Data are representative of 3 replicates, each using a different pool of semen.

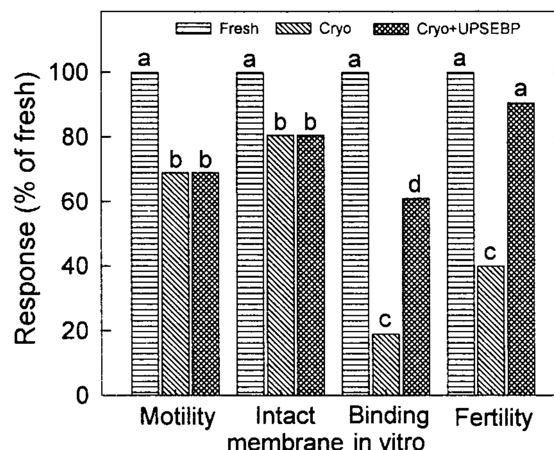

Figure 5. Ability of UPSEBP-2 (250 µg/mL) to restore attributes of cryopreserved rooster sperm. On each of 3 days, at 3-day intervals, pooled semen was thawed and cells were treated and compared with a contemporary pool of fresh semen, as described in text. Data for each pool of thawed sperm were expressed relative to the contemporary value for fresh semen and the resultant values were averaged across the replicates. Data for hatch were based on >300 eggs laid by the 17 to 19 hens in each treatment group. For a given attribute, means with different letters differ (a, b, d, $P < .05$; c, $P < .01$; statistical analyses based on absolute means).

greatly reduced by cryopreservation ($P < .01$), exposure of thawed sperm to UPSEBP-2 increased ($P < .01$) binding to >60% of the value for fresh sperm. When binding was expressed on the basis of motile sperm or sperm with an intact plasma membrane, binding for thawed sperm exposed to UPSEBP-2 approached 85% that of fresh cells. Fertility of cryopreserved sperm exposed to UPSEBP-2 was similar to that of fresh sperm ($P < .05$) and better ($P < .01$) than with untreated sperm (Figure 5). Exposure to UPSEBP-2 repaired a form of cryodamage by a mechanism not involving induction of sperm motility.

Individual roosters have a broad range in fertility when the AI dose is relatively low, and in part this is due to defective sperm-egg binding (Barbato et al, 1998). Hence, we used split ejaculates from individual roosters to compare fertility of sperm exposed to UPSEBP-1 vs untreated control sperm. AI used 50×10^6 fresh sperm and was repeated 3 times every third day. Of the 9 males with fertility below the 95% confidence interval for the population (Figure 6), 6 had their fertility improved ($P < .05$) by exposure of sperm to UPSEBP-1. One of the 9 most fertile males had increased fertility. Exposure of sperm to UPSEBP-1 reduced fertility in none of the males.

Initial Characterization and Partial Purification of UPSEBP from Rooster Sperm

Initial evaluations suggested that UPSEBP might be a macromolecular complex with an apparent molecular weight >500 kd, because all bioactivity in the original isolate was excluded from the molecular sizing column (Figure 7, upper). UPSEBP-2 is a protein because bioactivity in an SBA was lost by treatments that typically denature proteins. Compared with untreated UPSEBP-2, 100% of bioactivity was lost by chymotrypsin treatment and >80% of bioactivity was lost by treatment at 100°C, or adjustment to pH 9.0 followed by 100°C treatment. We suspected that the apparent large size of UPSEBP, evidenced in the upper panel in Figure 7, reflected a protein-lipid complex rather than a very large protein. Hence, lipids were extracted from a similar aliquot of pooled extract, and the lipid-free material was subjected to size exclusion chromatography (Figure 7, lower). Because all

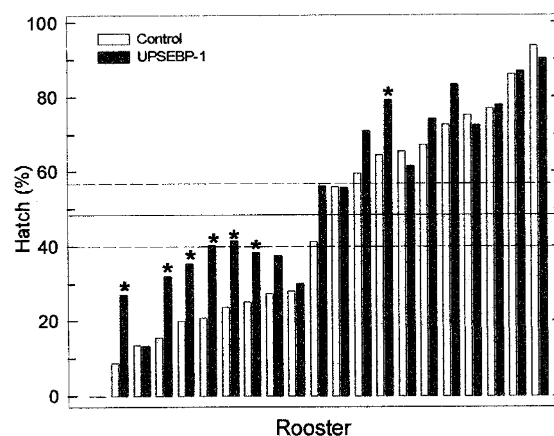

Figure 6. Effect of UPSEBP-1 on fertility of fresh semen from 21 roosters. Each ejaculate was divided into 2 parts (no supplement control vs 100 µg UPSEBP-1 per 0.1-mL AI dose; 50×10^6 sperm/AI). Means for 3 sequential AIs at 3-day intervals. Each bar represents ~200 eggs per male. Asterisk indicates positive effect ($P > .05$) on hatch for indicated male. Mean hatch (solid line) and 95% confidence limits (dashed lines) for the control treatment are shown.

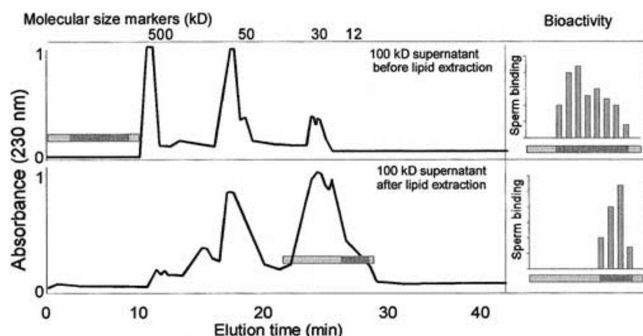

Figure 7. HPLC fractionation of UPSEBP. Supernatant protein recovered after cryopreservation was concentrated on a 100-kd filter, as for preparation of UPSEBP-2. Samples were subjected to size exclusion chromatography on a Supelco G-3000 column before (upper) and after (lower) lipid extraction. In each panel, absorbance shows the elution of protein over time, with high molecular weight proteins eluting first as designated by the size markers. The fractions later found to have bioactivity (assay 2) are designated by the dark stipple, and the increase in relative sperm binding over control values is detailed to the right insert. Upper panel: 0.2 mg protein before lipid extraction, with all bioactivity eluting before the protein peaks. Lower panel: 0.9 mg after lipid removal by treatment with $CHCl_3$:CH_3OH, with all bioactivity eluting in the region of 8–12 kd proteins, after most of the protein peaks.

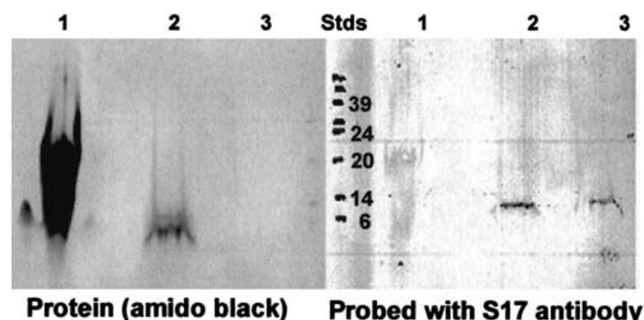

Figure 8. Western blot of proteins in UPSEBP-2. Lanes 1, 2, and 3 represent material eluting at 30%, 55%, or 80% ACN from a LC-18 column. The majority of the protein eluted at 30% ACN (ie, lane 1, left). Although protein was detected in lane 2 (left), the antibody bound to protein of greater molecular weight (right), approximately 8–9 kd. The S17 antibody binds to peptide S12-Ox (data not shown). Because peptide S12-Ox elutes from a similar LC-18 column at 55% ACN, whereas peptide S12-Red elutes at 30% ACN, we concluded that the UPSEBP was present as the oxidized hairpin form. Approximately 150 mg of UPSEBP-2 (held several years at −20°C) was acidified to pH 2.0 with trifluoroacetic acid, and centrifuged. The supernatant was loaded onto a preconditioned Supelclean LC-18 column (Supelco #57055) and fractions eluting at 30%, 55%, and 80% ACN were dried down and dissolved in 60 μL of gel buffer. Approximately 25 μL (reflecting ~60 mg of UPSEBP) of each preparation was brought to ~6% 2-mercaptoethanol and loaded onto an SDS gel (18% polyacrylamide) and run at constant current. Proteins were electroblotted onto nitrocellulose, stained with amido black, a digitized image prepared, destained, exposed to antibody against peptide S17, and again a digitized image prepared.

bioactivity now eluted with a retention time slightly longer than that of a 12-kd molecular weight standard, we concluded that UPSEBP-2 actually is a ~10 kd protein.

UPSEBP is very potent. A 6-step protocol ending with hydrophobic-interaction chromatography was replicated 4 times, each using a different pool of material from 30 to 50 cryopreserved ejaculates. Fractions were assessed for protein content and for biological activity (assay 2). In the final step with each replicate, all detectable bioactivity was eluted with ~30% ACN. No protein was detected in those fractions with the BCA assay.

We attempted to obtain material to sequence in a purification starting with 42 mg UPSEBP-2 (from 300 ejaculates; $\sim 6 \times 10^{11}$ sperm). The material was fractionated in 35 separate size-exclusion chromatographic runs, with peak bioactivity at ~10 kd (>75% in 1 tube) Fractions were pooled, lyophilized, dissolved, dialyzed as described earlier, and analyzed. There was ~0.19 mg protein. An aliquot (0.14 mg protein) was made 25% in ACN and fractionated on a micro C-4 column. No protein was detectable by absorbance at 230 nm for any fraction (ie, <0.01 OD), but all bioactivity was eluted at ~30% ACN. It appeared that UPSEBP was a proteinaceous material found in trace amounts on or in sperm. Because isolation by this approach of sufficient protein for analysis likely would have required >100 times more initial material, we abandoned attempts at purification and sequencing.

What Next?

At this time, it was evident that UPSEBP had an affinity for lipids, as evidenced by the change in chromatographic characteristics after removal from lipids (Figure 7), and that it was unlikely that we could purify microgram amounts and sequence the molecule. One of the authors (R.H.H.) was reviewing literature on SGP-1 (prosaposin) and mentally linked 1) our observations on seminal UPSEBP; 2) contents of a review by O'Brien and Kishimoto (1991), including discussion of the cleavage of prosaposin to form saposins interacting with lipids; 3) published statements (eg, Sylvester et al, 1984, 1989; Hiraiwa et al, 1993b) that prosaposin was present in cauda epididymal plasma and semen and that prosaposin was very sticky and lipophilic; and 4) the absence of a known role for the abundant prosaposin to which sperm are exposed. This led to the hypothesis that UPSEBP might be a fragment of prosaposin and initiation of studies to test the hypothesis by indirect approaches, as sequencing was not an option.

Since the initial submission of this manuscript, we had an opportunity to use a new polyclonal antibody against peptide S17 provided by BioPore Inc (ID #5438). to probe an aliquot of UPSEBP-2 that had been delipidated as described above, but not chromatographed before storage at −20°C. Drawing on our recent experiences on chromatography of synthetic peptides, this material (~150 mg) was processed and fractionated on a C-18 column. Resulting materials were reduced with 2-mercaptoethanol and used to prepare Western blots (Figure 8; details in legend). Most protein eluted with 30% ACN. The 50% ACN fraction contained protein of lower mo-

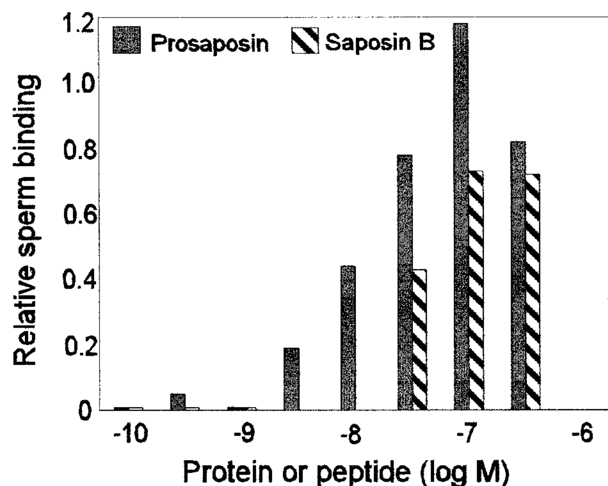

Figure 9. Restoration by highly purified prosaposin and saposin B of binding capability of cryopreserved rooster sperm to egg membrane, as evaluated by assay 1. Pooled cryopreserved sperm (line 42L) were thawed, processed to remove glycerol, exposed to proteins at the stipulated doses, incubated 10 minutes, diluted 1:10, and assayed concurrently with aliquots of the suspension exposed to UPSEP-1 at the most effective dose. Data are representative of 3 replicates, each using different pools of fresh and cryopreserved semen. Saposins A, C, and D had no effect at 10^{-10} to 10^{-5} M (data not shown).

lecular weights (see lanes 2 and 3, left) than that binding antibody against peptide S17 (see lanes 2 and 3, right). The only material binding the antibody eluted with 50% or 80% ACN; possibly, use of more 50% ACN would have eluted all material. With synthetic peptide, S12-Red elutes at 30% ACN and S12-Ox elutes at 50% ACN. We consider this as strong evidence that UPSEBP-2, prepared from supernatants from cryopreserved rooster semen, contains a protein or proteins with similarity to synthetic peptides S17 and S12, and likely has a disulfide linkage as does peptide S12-Ox.

Effects of Prosaposin and Saposins on Sperm Binding

Ability of purified prosaposin or individual saposins at 10^{-14} to 10^{-9} M to restore binding of cryopreserved sperm, after 1:10 dilution, was evaluated with assay 1. Relative to the maximum response with UPSEBP-1, maximum response with rat prosaposin was at $10^{-7.5}$ to $10^{-6.5}$ M, at which concentrations it was 0.8 to 1.1 times as effective as UPSEBP (Figure 9). Human saposin B was 0.8 times as effective at $10^{-7.0}$ to $10^{-6.5}$ M, and human saposins A, C, and D had no effect at up to 10^{-5} M (data not shown). It appeared that prosaposin was equally as good as UPSEBP-1 in restoring sperm binding capability to thawed sperm, and saposin B was partially effective.

Effects of Antibodies Against Prosaposin and Fragments on Sperm Binding

We then used antibodies against purified prosaposin or individual saposins to neutralize binding capacity of UPSEBP-1. Cryopreserved rooster sperm were preincubated with sufficient crude UPSEBP-1 to provide maximum sperm binding and parallel aliquots were coincubated with antibody, before evaluation with assay 1. Antibody against rat prosaposin, part of the human intervening A–B sequence, and human saposin A were similarly effective (data not shown). They suppressed binding by ~50% at 1:4 dilution and ≥95% when used undiluted. Antibody against human saposin B was less effective, giving ≥80% suppression when used undiluted. Antibodies against human saposin C, human saposin D, and unrelated antigens or preimmune serum had no effect. Note that the antibody against prosaposin is known to bind to each of the saposins as well as prosaposin, and that concentrations of IgG in the antisera were unknown.

In the same study, polyclonal antibody against synthetic peptide S17 also suppressed binding (assay 1), but required use of a 1:2 dilution to suppress binding by ~50%, as compared with the 1:4 dilution, which was equally effective with antibodies against saposins A or the intervening A–B sequence (data not shown). However, this might be misleading because concentrations of IgG in the antisera were unknown. Later, the same antibody against peptide S17 was compared with a control antibody for its effect on sperm binding, using cryopreserved sperm and assay 3. Antibody against peptide S17 progressively reduced binding to a maximum of 0.2× the control, as its relative concentration was increased (data not shown), whereas the control antibody had no effect on binding. Thus, UPSEBP bioactivity was substantially decreased by both antibody against a portion of the human intervening A–B sequence or the entire rat intervening A–B sequence. Apparently, bioactivity involved in sperm-egg binding is in or near the A–B intervening sequence of prosaposin.

Effects of Synthetic Peptides on Sperm Binding

Given the impact of prosaposin and saposin B on sperm binding (Figure 9), we used synthetic peptides to localize the sperm-egg binding bioactivity in prosaposin. Twenty-five different peptides were synthesized, most based on the rat sequence for prosaposin (Figure 1). Initial evaluations tested a 10^4 range in concentrations of each peptide in this assembly (as 2 overlapping sets, each with 6 concentrations) to identify concentrations to probe in detail. This was to ensure that there was ''enough'' to interact with sperm to stimulate binding (potential positive effect), but not ''too much'' so that carryover of free peptide competed with sperm for sites on the egg membrane (inhibit binding of competent sperm). For example, with peptide S-12-Ox, the optimum dose was near 1 μM, but concentrations <0.5 μM had no effect and those >4 μM suppressed sperm binding.

Preliminary screening revealed that short peptides had

no effect or required very high concentrations (ie, 30 μM) to restore binding to cryopreserved sperm (see Table 1 in Hammerstedt et al, 1999). These included 1) 7 peptides, including the intersection of the carboxy terminal of the intervening A–B sequence with saposin B (positions 42 to 66 in Figure 2; including the 15 amino acid rat sequence similar to the human sequence used to prepare antibody against the intervening A–B sequence); and 2) 2 peptides, including the intersection of the amino terminal of the intervening A–B segment with saposin A (positions 1 to 21 of Figure 2). It is important that observations directed attention to peptides spanning the entire region between saposins A and B (ie, residues 1 and 61 in Figure 2).

Six peptides (Figure 2) were extensively tested for capacity to restore binding capacity of cryopreserved sperm. These included 1) large portions of the intervening A–B segment plus short stretches of saposins A and B (peptides S12, S17, and S18), 2) synthesis based on the human rather than rat sequence (peptide S23), and 3) reduced (linear) or oxidized (folded) versions of the same sequences (peptides S12-Red vs S12-Ox; S23-Red vs S23-Ox). Peptides S17 and S18, which lack one or both cysteines, did not stimulate binding, although peptide S18 inhibited ($P < .05$) binding at 50 μM (the highest concentration tested; data not shown). Rat-based peptides, including 2 cysteines increased binding, with both S12-Red and S12-Ox effective ($P < .01$) at 0.8 μM. This concentration is similar to that found effective for natural materials. Peptides S23-Red and S23-Ox had no effect with rooster sperm under conditions tested (S23-Ox is effective with human sperm; Amann et al, 1999c).

Chromatographic Characteristics of UPSEBP and Peptides S12-R or S12-Ox

To compare similarity in chromatographic characteristics of UPSEBP and synthetic peptides, 4 different UPSEB-1 preparations were loaded onto open C-18 columns, and processed with the procedure used to separate unbound, reduced, and oxidized forms of peptide S12. Bioactivity for each fraction was determined (assay 3; data not displayed). About half of the bioactivity in UPSEBP-1 applied to the column was not retained and washed through the column. No activity was found in the 30% ACN fraction (where peptide S12-Red elutes). All retained activity eluted in the 55% ACN fraction where peptide S12-Ox elutes. The substantial wash-through was not due to overloading the column, but likely was a ≥300 kd protein-lipid complex (see above). In follow-up experiments, application of one-half the amount of UPSEBP-1 to the column gave the same distribution across fractions.

Effects of Synthetic Peptides on Sperm Fertility

Evaluation of UPSEBP-1 for effect on fertility (Figure 6) used a 1 mg/mL solution. If one assumed 100% purity (unlikely) and a mass of 7000 kd, that solution would have been 140 μM in concentration. That value was chosen as the ‘‘highest likely dose’’ for testing a synthetic peptide in fertility trials. Peptide S12-Red, which had a strong effect on sperm binding, was evaluated sequentially at 140 μM, followed by 0.05 μM, and finally 5.0 μM. Logistical constraints precluded simultaneous testing, but each test used semen pooled from several lots of cryopreserved sperm (subline 42L). Hens were randomized again for assignment to either control or peptide treatment. Confounding factors included 1) age of hen, 2) season, and 3) number of previous inseminations (virgin hens used for 140 μM dose vs recycled hens for other doses). There was no difference in control fertility ($P < .05$) across the 3 successive tests. The 0.05 μM dose of peptide S12-Red had no effect, 5.0 μM raised fertility ($P < .05$), and 140 μM depressed fertility ($P < .05$; data not shown). Values relative to the contemporary control were 98%, 115%, and 65%.

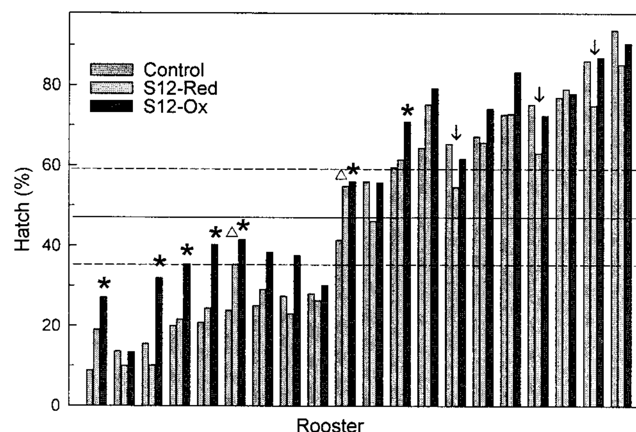

Figure 10. Evaluation of 5 μM peptide S12-Red or S12-Ox for capacity to improve fertility of fresh sperm from individual roosters. Each ejaculate was divided into 3 parts (no supplement vs addition of 5 μM peptide S12-Red or S12-Ox; 50×10^6 sperm/AI). Each bar is mean percentage of hatch (~60 eggs per male) for eggs laid after three sequential AIs at 3-day intervals. A triangle or asterisk designates positive ($P < .05$) effect for S12-Red or S12-Ox, respectively, and an arrow a negative ($P < .05$) effect on hatch for designated male.

To further test peptide 12-Red and evaluate the importance of the oxidized vs the reduced form of the peptide, fertility of sperm exposed to 5 μM peptides S12-Red or S12-Ox were compared with that of control sperm. Fresh sperm from individual males of subline 42H was split, mixed with peptide, and immediately used at 50×10^6 sperm/AI. With control semen, differences among males were evident (Figure 10). Peptide S12-Red raised fertility of 1 low-fertility and 1 average-fertility male, but depressed fertility for 3 high-fertility males. Peptide 12-Ox raised the fertilities of 5 of the 9 low-fertility males and 2 of the 3 males with average fertility; it did not depress fertility for any male. We concluded that peptide S12-Ox 1) had greater biopotency than peptide S12-Red, 2) was

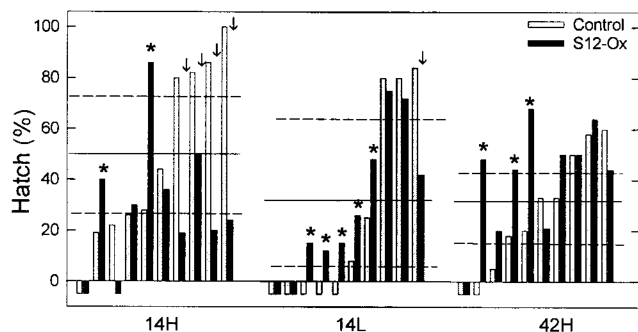

Figure 11. Evaluation of 5 μM peptide S12-Ox for capacity to improve fertility of fresh sperm from individual roosters of 3 genotypes. Each ejaculate was divided into 2 parts (no supplement vs addition of 5 μM S12-Ox peptide; 50 × 10^6 sperm/AI). Mean percentage hatch (~60 eggs per male) for 3 sequential AIs at 3-day intervals. An asterisk designates a positive and an arrow designates a negative effect ($P < .05$) on hatch of designated male, relative to its control value.

more likely to increase fertility of low-fertility males, and 3) might be less likely than S12-Red to depress fertility of high-fertility males.

It was known that roosters of the 14H, 14L, and 42H lines differ in fertility (Barbato, 1999). Hence, fresh sperm from individual males of each line were inseminated after exposure to 0 or 5 μM peptide S12-Ox. For the highest fertility subline (Figure 11, left), 5 μM peptide had a positive ($P < .05$) effect on sperm from 2 of 10 males, but depressed ($P < .05$) hatch for all 4 males with above average fertility. With sperm from males in low-fertility sublines (Figure 11, center and right), hatch was increased ($P < .05$) for 8 of 20 males and decreased ($P < .05$) for only 1 above-average fertility male (Figure 10, center and right). Overall, exposure of sperm to peptide S12-Ox increased ($P < .05$) hatch for 10 of 30 roosters. Apparently, 5 μM was near the optimal dose for use with fresh sperm from 14L or 42H males, but excessive for use with sperm from 14H males.

The final fertility trial directly compared fertility of sperm exposed to 5 μM S12-Red, S12-Ox, or S23-Ox with that for control sperm. Peptides were purified and frozen to provide uniform preparations replicate evaluations at the same season in 2 successive years. Each used pooled, croypreserved sperm roosters of line 42L (2 different generations) and virgin hens from the same supplier. The change in hatch after the last of 3 insemination was similar for both years (Figure 12, panels A and B). Thus, data were pooled (Figure 12, panel C) for analysis. Peptide S12-Ox had a positive effect on hatch, which was especially obvious ($P < .05$) for days 4, 5, and 6. This shifted the decline in hatch to a later period after the last AI. There was no difference between data for peptide S12-Red and the control. In contrast, peptide S23-Ox suppressed hatch for 7 days after the last AI; this peptide

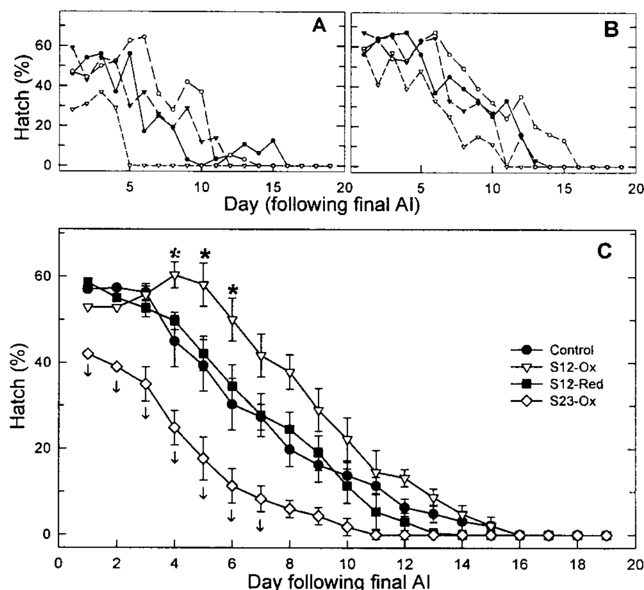

Figure 12. Evaluation of 5 μM peptides S12-Red, S12-Ox, or S23-Ox for capacity to improve fertility of cryopreserved rooster sperm. Pools of cryopreserved sperm (line 42L) were thawed, processed to remove glycerol, exposed to synthetic peptides at 0 μM (control) or 5 μM, and used for AI commencing 5 minutes later. Three sequential AIs at 3-day intervals each used 50 × 10^6 sperm. Hatch was monitored as sperm were depleted from storage glands within the female tract. Data for males of generation P_9 **(Panel A)** and generation P_{10} **(Panel B)** were pooled **(Panel C)**. Asterisk designates a positive and an arrow designates a negative effect ($P < .05$) on hatch.

might have been at an excessive dose or has contraceptive action with chicken sperm.

Discussion

A Unifying Postulate

Based on data presented herein and the literature, we propose that a fragment of prosaposin (SGP-1) becomes bound on the surface of sperm and serves as a bridging ligand during sperm-egg binding. Prosaposin is known to be in seminal plasma and reproductive tissues.

Prosaposin has been intensively studied with regard to across-species conservation, tissue localization, proteolytic processing, and potency of its fragments (see Figure 1 and references previously cited). Careful review of structures for saposins (Mumford et al, 1995; Ponting and Russell, 1995) revealed the importance of internal disulfide bonds within the molecule or molecules, leading to "signature patterns" of repeating amino acid sequences. Building from those data, derived primarily from animal systems, a further extension was made to incorporate the structures of analogous sequences found in plants. They introduced a "swaposin" concept of alternate folding schemes in the parental molecule to provide even more

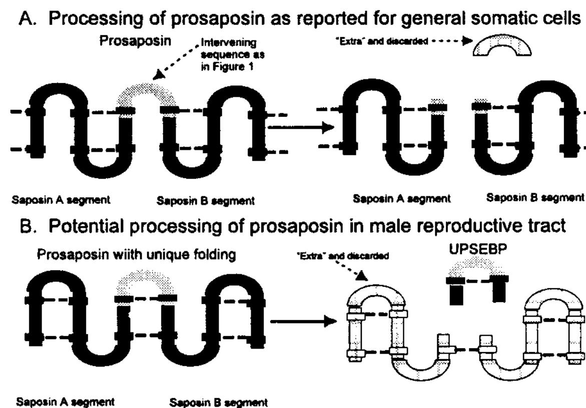

Figure 13. Postulated mechanism for unique processing of prosaposin to generate the fragment termed UPSEBP. The overall linear peptide form of prosaposin (see Figure 1) is simplified here to illustrate only the region of interest. The dark segments represent the saposin protein sequences; the dashed lines, disulfide bonds; and the light colored lines, the intervening peptide sequence between saposins A and B. A detailed description of the saposin/swaposin concept is provided in Mumford et al (1995) and Ponting and Russell (1995). The important element is direction of the disulfide bonds (dashed lines). **(A)** Prosaposin, as found in general somatic cells, is known to have unique interchain disulfide bonds that serve to organize the unique domains destined to be the individual saposin molecules after proteolytic processing. **(B)** Prosaposin as provided in the male reproductive tract might have a unique folding pattern and interchain disulfide bond display that serves to organize the swaposin pattern, which after proteolytic processing provides UPSEBP.

potential molecules from the same gene and protein structures.

To illustrate (Figure 13) how those concepts might explain the observations herein, a schematic diagram (panel A) is provided to illustrate the general path believed (O'Brien and Kishimoto, 1991; Kishimoto et al, 1992) to yield the individual saposin molecules from their precursor, prosaposin. If Sertoli cells in the testis or principal cells of the epididymis had a unique mode of folding the prosaposin (panel B) to the ''swaposin'' disulfide pattern, the resultant product still might be recognized by antibodies raised to saposins or ''conventional'' prosaposin. Tissue-specific proteolysis would yield a peptide with the disulfide-bonding pattern proposed for UPSEBP. Tissues of the male reproductive tract have unique chaperone-like activities such as clusterin (Sylvester et al, 1989; Bailey and Griswold, 1999; Humphreys et al, 1999) and HSP70-2 (Eddy, 1999). Clearly, prosaposin is pluripotent, whereby highly similar DNA sequences, followed by tissue-specific expression, unique folding processes, and endoprotease action could generate related molecules 5%–15% the size of the parent molecule. Controlled degradation of ''all but the part that is needed'' leads to accumulation of highly potent molecules in cells and tissues.

Given a mechanism that might provide UPSEBP for secretion by Sertoli cells or certain epithelial cell lining the excurrent ducts or accessory sex glands, what does UPSEBP do? A fundamental, obligatory step in fertilization is sperm-egg binding. The topic has been extensively studied and many factors have been identified. However, most reviews focus on rodent studies and even broad recent reviews (cited in the first few paragraphs of this article) do not provide an unambiguous description of initial sperm-egg binding. Data herein support the role of yet another molecule, UPSEBP, in this complex process.

Support for the Postulate

The extensive data in support of the concept that a fragment of prosaposin normally is (or can be) bound on the surface of sperm and serve as a bridging ligand during sperm-egg binding can be summarized as follows: Before starting these studies, it was known that cryopreservation of rooster sperm destroyed capacity of thawed sperm to bind to a hen's egg (Alexander et al, 1993). We found that a factor was lost from the sperm surface during cryopreservation, and both in vitro binding capacity and fertility could be restored when that factor (UPSEBP) was added to, or was bound up in, suspensions of thawed or fresh sperm before AI (Figures 3 to 6). Fractionation studies showed that the biological activity of UPSEBP could be highly enriched and had a molecular weight of ~10 kd (Figure 7, lower). The molecule apparently had very high potency, as bioactivity was retained in the absence of measurable protein. For reasons detailed earlier, we postulated that UPSEBP was related to the ''intervening peptide sequence between saposins A and B'' (Figure 1). Binding studies (Figure 9) supported this conclusion.

We then evaluated the bioactivity of each of 25 peptides, chemically synthesized, most based on the sequence of rat prosaposin. Based on the sperm-binding assay, the most active peptides included the A–B intervening sequence of prosaposin plus a few amino acids from saposins A and B at each end, and terminating with a cysteine. Micromolar concentrations of the 60 amino acid peptide, S12, affected sperm. The folded oxidized form (S12-Ox), with a disulfide bond, was more effective than its linear counterpart (S12-Red), based on sperm binding and fertility (Figure 10). For many individual roosters of 3 genetic lines, exposure to 5 μM peptide S12-Ox increased fertility (Figure 11). In the highest fertility line, treated sperm from some of the more fertile males produced fewer chicks. The benefit of 5 μM of the peptide S12-Ox also was shown using a different paradigm; namely, ''loading the system'' with successive AIs and monitoring fertility as the hen's sperm-storage tubules are emptied. Under these circumstances, peptide S12-Ox (rat sequence) elevated hatch, peptide S12-Red was no different from the control, and peptide S23-Ox (human sequence) depressed hatch (Figure 12).

It also was found that antibody against a 58 amino acid peptide S17 (ie, peptide S12 without terminal cysteines) removed UPSEBP bioactivity from solution, as evidenced

by reduced sperm-egg binding in vitro. Finally, we performed Western blot analysis of UPSEBP using a new antibody against peptide S17. For this analysis we drew on the earlier observation that bioactive synthetic peptide S12-Ox eluted from a C-18 column at ≥55% ACN. Native protein binding the antibody against synthetic peptide S17 was found to elute in fractions similar to those in which peptide S17-Ox eluted. This is strong, although indirect, evidence that bioactive epitopes in UPSEBP are represented in synthetic peptide S17-Ox, and that UPSEBP involves the A–B intervening sequence of prosaposin.

Effective Doses of Synthetic Peptides

Repeatedly, low doses of a peptide were ineffective and high doses were inhibitory to sperm binding in vitro or to fertility. Such dose maxima are common in physiology and pharmacology. For the in vitro case, the peptide might facilitate sperm binding by filling available sites on the sperm membrane, making the sperm more "sticky," but then inhibit binding of sperm by competition of excess "free peptide" with sperm for binding sites to an unknown receptor on the zona pellucida or perivitelline membrane.

The in vivo depression of fertility is not as easily treated. Whereas a local excess might be present around the sperm at the instant of AI, sperm migration up the female tract, storage for days, and controlled release into the oviduct daily for fertilization should remove all "free peptide." One explanation (without supporting data) is that after exposure to a high dose of peptide, too many sperm bind to the egg during the available ~15 minutes in a hen's infundibulium. Polyspermy appears to be common and perhaps necessary in birds (Etches, 1996) as 4 to 20 sperm normally enter the germinal disc, but a highly activated sperm preparation could be "too much of a good thing"; a feature of fertilization discussed by Amann and Hammerstedt (1993).

Is the UPSEBP System Important?

In commodity agriculture, intense genetic selection for traits of high economic value (eg, feed conversion, growth rate, milk production) is common, providing ever lower cost protein for human consumption. Reproductive effectiveness declines. With chickens, Barbato (1999) found that capability of rooster sperm to bind to the perivitelline membrane (assay 3) and subfertility had relatively high heritabilities (h^2 ~0.32) and differed among lines. At least 1 cause of such subfertility can be overcome by exposure of sperm to peptide 12-Ox (Figures 9–11). Current status of genetic studies (Barbato et al, unpublished) leads to the conclusion that "few" genes are involved. Future studies need to probe for altered alleles or gene expression affecting 1) putative sperm membrane receptor for UPSEBP, 2) alterations in post-translational processing of prosaposin, 3) availability of UPSEBP to sperm in sufficient amounts, 4) presentation of the UPSEBP to the developing sperm, and 5) defective or insufficient binding sites for UPSEBP on the perivitelline membrane. Defects in synthesis and expression of prosaposin per se are unlikely, because the parent molecule is critical in a host of other processes that are apparently normal in these chickens.

Studies building on observations herein have established that peptide S12-Ox is effective in increasing fertility when used in appropriate concentrations in studies designed to detect differences in fertility if they occurred. In vitro exposure of sperm to peptide S12-Ox increased cleavage rate in IVF with mouse (Magargee et al, 2000) and cattle gametes (Seidel et al, 2001). Exposure of frozen thawed bull (Amann et al, 1999b) or rooster (Gill et al, 1999) sperm increased pregnancy rate or number of chicks hatched. Exposure of fresh turkey sperm to peptide S12-Ox increased poult production (Gill et al, 2000).

Many important genes have been conserved as species evolved. This is true of the UPSEBP sequence (see Figure 1) within prosaposin. Amino acids for rat vs mouse, rat vs human, human vs chicken, and rat vs chicken are 95%, 77%, 69%, and 64% identical. We used alignment techniques (Higgins et al, 1992) to examine conservation of the UPSEBP sequence in comparison to other portions of prosaposin, in terms of synonymous and nonsynonymous amino acid substitutions. Considering data for human, rat, and mouse, no region had a significantly greater value for synonymous amino acid substitutions than UPSEBP. Furthermore, there were fewer ($P < .05$) nonsynonymous amino acid substitutions in UPSEBP than in saposin C, or the B–C and C–D intervening segments. Thus, conservation of the UPSEBP sequence is similar to that for the life-sustaining saposins A, B, and D, and greater than that for saposin C.

The concept of a universal sperm egg binding protein is supported by 1) the high conservation of amino acids in the putative UPSEBP sequence (eg, peptide S12) across species separated by >200 million years, and 2) the observations that "rat" peptide S12-Ox can increase production of progeny in species as diverse as cattle, chickens, mice, and turkeys. The mechanisms of action remain unexplored, but could be addressed with modern molecular techniques and lines of animal models known to differ in function of the system. The narrow range of beneficial doses and inhibitory effects of the "human" peptide S23-Ox on chicken sperm suggest structural features of the UPSEBP system might lead to both profertility and antifertility peptides.

Is Peptide S-12 the Correct Sequence for UPSEBP?

When these studies were performed, the amino acid sequence of chicken prosaposin was unknown, so we used

the rat sequence as the template for peptide synthesis. Conduct of similar studies using rat gametes and rat UPSEBP would be difficult. Repetition of certain experiments using peptides and antibodies based on the chicken intervening A–B sequence should be undertaken. This would minimize the uncertainty associated with our inability to isolate an sufficient mass of bioactive UPSEBP from rooster sperm for sequencing.

Substantial indirect evidence supports the conclusion that UPSEBP is a fragment of prosaposin, essentially the intervening A–B sequence. Central to this conclusion is that exposure of sperm to UPSEBP, synthetic peptides based on the intervening A–B sequence for rat prosaposin, native rat prosaposin, or human saposin B increased in vitro sperm-egg binding, fertility, or both after AI. The beneficial effect of UPSEBP in increasing sperm-egg binding was reduced by antibodies against rat prosaposin, human saposin B, or synthetic peptide S17. Finally, antibody against the synthetic rat peptide sequence S17 localized to a 7–9 kd protein, present in trace amounts, in extracts of cryopreserved rooster semen (ie, UPSEBP). We anticipate that a similar response would have been obtained had the antibody been prepared against the corresponding chicken A–B intervening sequence, given that the amino acid sequences are 64% identical. Although the amount of protein associated with the 7–10 kd band in Western blots is negligible, chromatographic isolation on a C-18 column might provide sufficient material for microsequencing and final confirmation that UPSEBP is primarily the A–B intervening sequence of prosaposin.

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