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Identification of a Sperm Penetration Factor in the Oviduct of the Golden Hamster[1]

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ABSTRACT

Previously, we found oviductal eggs to be significantly more penetrable and fertilizable in vitro than ovulated eggs collected from the ovarian bursa, while bursal eggs were comparable to mature (unovulated) follicular eggs. Incubation of follicular eggs with a soluble eluate of oviductal egg cumulus complexes (COF) increased sperm penetration: the activity was macromolecular, was destroyed at 56°C, and was produced in the oviduct. We now report purification of this oviductal factor that enhances penetration of follicular eggs and have identified it as oviductin (OVN). Oviducts, 1–1.5 h post-LH from eCG-primed females, were homogenized and the cytosolic fraction was chromatographed on a *Helix pomatia* lectin affinity column; specific proteins were eluted with 0.2 M N-acetyl-D-galactosamine. Fractions were monitored by dot-blot assay using as the primary antibody monoclonal antibody (mAb) 1C4 against OVN. Proteins were resolved by one-dimensional SDS-gel electrophoresis, followed by electrotransfer and immunostaining of Western blots. OVN fractions were indexed to COF by quantitative dot-blot assay, and activity was bioassayed by penetration of follicular eggs within 1 h of coincubation with precapacitated sperm ± factors: COF and BSA (high and low controls, respectively) and fractions from the lectin-isolated peak. The mean penetration rates for three isolations were 17 ± 4.0[a], 51.7 ± 5.0[b], and 49 ± 2.7[b]% for BSA, COF, and column fractions, respectively ($p \leq 0.05$). Purified OVN bound to follicular zonae during culture. Acrosome-intact sperm heads bound OVN during 30 min of incubation both before (t = 0 h) and after capacitation (t = 5.5 h) (visualized by indirect immunofluorescence). We conclude that OVN enhances penetration and fertilization by altering both sperm and eggs.

INTRODUCTION

In vivo, fertilization is highly efficient, since very few of the sperm stored in the caudal isthmus move into the ampulla in the preovulatory to early postovulatory period [1–4]; this results in nearly every ampullary sperm finding and penetrating an egg until all eggs are penetrated [3, 5–7]. Sperm spend very little time traversing the egg investments [8, 9] and are rarely found on the surface of the zona pellucida [6]. This contrasts with the in vitro situation, in which large numbers of sperm per egg are required for efficient penetration and the penetrating sperm may remain bound to the zona pellucida for prolonged periods before undergoing the acrosome reaction [10, 11] or entering into the perivitelline space [12]. There is considerable interest in identifying molecules present at the site of in vivo fertilization that act as cofactors for penetration and fertilization, potentially acting as acrosome reaction-inducing factor(s) (ARIF; [13–16]). Previously, we reported that the ovulated hamster egg cumulus complex contained a soluble, non-albumin ARIF that stimulated hamster sperm acrosome reactions and enhanced penetration through the zona pellucida [13, 16, 17]. The egg penetration-enhancing substance for follicular oocytes appeared to be of oviductal origin and functioned partly to increase the ARIF and sperm-binding activities of the zona pellucida [17]. The objectives of the current study were to identify the oviductal component that enhances sperm penetration into follicular eggs and to determine whether or not its mechanism of action is to stimulate acrosome reactions in capacitated sperm. We now report that this oviductal factor that enhances penetration of follicular eggs has been isolated and has the properties of oviductin (OVN), including tight binding to the zona pellucida [18]. OVN, and oviductal molecules molecularly related to OVN (i.e., oviductal glycoproteins with homology to chitinase [19–22]), have previously been shown to bind tightly to oviductal eggs and early embryos, but evidence for a function mediated by this association was lacking [23–33]. We also present evidence that, in addition to associating with the zona pellucida of unfertilized eggs and increasing sperm penetration into eggs, OVN also binds directly to sperm heads overlying the acrosome. With its dual affinity for eggs and sperm, OVN is an ideal molecule to modulate in vivo fertilization.

MATERIALS AND METHODS

Unless otherwise specified, chemicals (with the exception of inorganic salts used for media preparation) were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Biological Test Fluids

Cumulus oophorus fluid (COF) eluate was prepared as previously described [16, 17]. OVN was isolated from excised oviducts of mature female hamsters (n = 6 hamsters per collection) that were injected with 25 IU of eCG (Diosynth, Chicago, IL) on the morning of the post-estrus discharge [34] and then killed by cervical dislocation at 1500

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h on Day 4. The oviducts were homogenized in PBS (pH 7.3) containing 5 mM benzamidine, 1 mM PMSF, 5 mM EDTA, and 5 mM N-ethylmaleimide. The homogenate was centrifuged at 20 000 × *g* for 30 min; then the supernatant was centrifuged at 100 000 × *g* for 1 h to prepare the cytosolic fraction (final supernatant). OVN was isolated by affinity column chromatography of the cytosolic fraction (7–13 mg total protein) on *Helix pomatia* lectin cross-linked to 4% beaded agarose [18]. Nonspecific (unbound) proteins were eluted with PBS. OVN was eluted with 0.2 M N-acetyl-D-galactosamine in PBS, and 1-ml fractions were collected. The presence of OVN in each of the chromatographic fractions was monitored by dot-blot assay on nitrocellulose membrane using primary antibody (anti-OVN monoclonal antibody [mAb] 1C4 [35]) diluted 1:5000 in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl [pH 7.5], also containing 1% BSA [fraction V, fatty acid-free; Pentex Miles Inc., Kankakee, IL] and 0.05% Tween 20 [Bio-Rad, Richmond, CA]; TTBS-BSA) and peroxidase-conjugated secondary antibody (anti-mouse immunoglobulin; Zymed Labs., San Francisco, CA) diluted 1:2000 in TTBS-BSA, followed by substrate solution; development was carried out until the desired intensity was reached. BSA (3%) in TBS was used as the blocking agent. From each 1-ml fraction containing OVN, the sugar was removed and the OVN was concentrated by ultrafiltration on a Centricon device (Amicon Corp., Danvers, MA; 30-kDa cutoff). Protein concentrations of each fraction were determined by absorbance at 280 and 260 nm and calculated according to the method of Layne [36]. The fractions constituting the "tail" of each OVN peak were pooled and reconcentrated. For bioassay of each OVN-positive column fraction, the amount of OVN used was determined by comparison to the COF standard using a dot-blot dilution assay.

Electrophoresis and Western Blots

Fractions (6 μg protein/lane) were resolved by one-dimensional SDS-gel electrophoresis on a 6% gel with a 4% acrylamide stacking gel under reducing conditions using a Mini-Protean II Electrophoresis Cell [37]. Gels were run at constant 200 V for 1 h or until the tracking dye was 3–5 mm from the bottom. Molecular mass standards were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), and ovalbumin (45 kDa). One gel was stained (Coomassie Brilliant Blue R-250); the other was electrotransferred to Immobilon-P polyvinyldifluoride (PVDF) membrane (Millipore, Bradford, MA).

Protein bands were transferred in 25 mM Tris, 192 mM glycine for 90 min with use of a constant voltage of 100 V. Nonspecific binding sites on the Immobilon membranes were blocked overnight at 37°C by means of a solution of 1% nonfat dry milk, 2% fetal calf serum, 10 mM Tris (pH 8.8), and 0.9% NaCl. Blots of transferred proteins were immunostained with primary antibody (anti-OVN mAb IC4 [35]) diluted 1:5000 in antibody dilution buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, and 0.05% Tween 20) followed by secondary antibody (anti-mouse IgG alkaline phosphatase conjugate) diluted 1:1000 in the same buffer. Both incubations were at 22°C for 2 h. The blots were incubated with color development solution (Boehringer-Mannheim, Indianapolis, IN; 50 mM Tris [pH 9.8], 3 mM $MgCl_2$, 200 μl 0.5% NBT [4-nitro blue tetrazolium chloride], and 100 μl of 5 mg/ml BCIP [5-brom-4-chlor-3-indolylphosphate]) for 10 min, washed in water, and air-dried.

Sperm Preparation and Egg Penetration Bioassay

Female golden hamsters were injected i.p. with 25 IU of eCG on the morning of the post-estrus discharge [34]; 64 to 66 h later, 25 IU of hCG was injected i.p [34]. Follicular oocytes were recovered 12 h post-hCG. At this time, all eggs are at metaphase II [38]. Egg handling and cumulus cell removal with hyaluronidase were performed in TALP (Tyrode's albumin lactate pyruvate)-HEPES-polyvinyl alcohol (PVA) at 37°C in air [17, 34, 35].

For egg penetration experiments, cauda epididymal sperm were obtained undiluted under oil and, after staggering inseminations by 2–5 min, incubated under capacitating conditions (2–4 × 10^6/ml) for 5 h in medium TALP-PVA with sperm motility factors as previously described [16, 17, 39, 40]. Then sperm were diluted 1:10 into drops of TALP-PVA containing 14–16 follicular eggs ± biological factors. COF (0.12–0.14 mg protein/ml) was used as a high control, and an equivalent amount of BSA as the low control. OVN fractions, indexed to COF (see *Preparation of Biological Test Fluids*), were used at dilutions of 1:10 to 1:3. Equal volumes of column buffer (PBS, 15 μl) ± OVN and Tyrode's lactate (TL)-HEPES-PVA (COF elution buffer, 10 μl) were added to all drops (final drop vol = 50 μl). Sperm-egg incubation continued for 1 h under 2.5% CO_2 (to compensate for lower HCO_3^- in drops) in air at 37°C; then eggs were fixed and scored for penetration. Eggs were scored as penetrated if one or more sperm heads had entered into the perivitelline space or further into the egg [17, 35]. For each preparation (replicate), eggs from 8 females were proportionately pooled. Within each replicate, there were six duplicates for BSA control, four for COF control, and two for each fraction tested (6–7 fractions per replicate).

Immunofluorescent Localization of OVN Binding to Eggs and Sperm

Follicular eggs were recovered from microscope slides after fixation and scoring for penetration in the presence or absence of COF and/or OVN fractions. Additionally, freshly recovered follicular eggs were incubated with the intact cumulus in OVN fractions for 1 h; then cumulus cells were removed with hyaluronidase as described above. Eggs (plus and minus exposure to oviductal components) were fixed for 30 min in 1% paraformaldehyde and then washed three times in 0.2 M glycine PBS-PVA, pH 7.4. Eggs were incu-

bated with primary antibody, mAb 1C4 (1:2500 dilution in PBS-PVA), for 30 min at 37°C and then with secondary antibody (goat anti-mouse IgG fluorescein isothiocyanate [FITC] conjugate; 1:100 dilution in PBS-PVA) for 30 min at 37°C. Eggs were washed thoroughly in PBS-PVA, mounted on glass slides with either PBS or mounting buffer (see below), and examined under either an epifluorescence or a confocal scanning laser microscope.

To test the ability of OVN to induce the acrosome reaction and bind to sperm, a total of four males were bioassayed. One male was used for each of the three OVN preparations employed for egg bioassay, and one male was used for an additional OVN preparation not tested by egg penetration. Sperm were incubated for 0 or 5.5 h under capacitating conditions; then ± COF or OVN was added for 30 min (final vol = 200 μl; sperm concentration/ml = 1.6 × 10^6). Suspensions were layered onto two-step TL-HEPES plus Ficoll gradients (0.5%, 500 μl; 1.5%, 100 μl also containing 1% paraformaldehyde) in 1-ml microcentrifuge tubes in order to separate sperm from medium containing unbound oviductal molecules, wash the sperm, and fix them. Gradients were centrifuged at 300 × *g* for 20 min. After 15-min fixation, sperm were washed two times with 0.2 M glycine-PBS-PVA and held on ice. Sperm suspensions (10–20 μl) were centrifuged onto a ≅ 7-mm circular area on poly-L-lysine-coated slides by means of a Cytospin centrifuge (Shandon Southern Inst., Sewickley, PA); then sperm lawns were washed three times (5 min each) with 0.3% BSA in PBS. Slides were incubated with primary antibody (mAb 1C4, 1:1200 dilution in wash buffer) for 1 h at 22°C or overnight at 4°C, washed three times with washing buffer, incubated with secondary antibody (goat anti-mouse IgG FITC conjugate, 1:75 dilution) for 1 h 22°C, and then washed three to five times with washing buffer. Controls included 1) replacement of mAb 1C4 with a nonspecific IgG primary antibody ($IgG_{2\alpha}$ kappa, 5 μg/ml) or 2) omission of the primary antibody. Sperm preparations were covered with 15 μl of mounting medium (0.1 M n-propyl gallate in glycerol-PBS [9:1], pH 8.0), mounted under 18-mm coverslips, and then sealed with nail varnish [39]. Sperm were viewed immediately on an epifluorescence microscope equipped with FITC filters and a 40× neofluor objective.

Acrosome Reaction

Sperm were incubated plus or minus OVN and prepared as described above (in *Immunofluorescent Localization of OVN Binding to Eggs and Sperm*). Acrosomal status was determined by phase-contrast and brightfield microscopy at 400× [41]. A minimum of 150 sperm were counted per slide.

Data Analysis

For egg penetration experiments, there were two main effects, preparation number (= replicate) and sperm:egg coincubation treatments (BSA, COF, and OVN). For this analysis, initially, all OVN fractions within each preparation were treated as a single group. Then the accuracy of the dot-blot dilution assay for indexing the bioassay fractions to COF (high control standard) was also tested. Within each of the three egg bioassay runs, all of the penetration responses were divided by the mean COF penetration for that run and multiplied by 100. Then all of the OVN test fractions were segregated into three groups based on the dilution factor used to index them to the COF standards. One-way ANOVA was used to compare groups. After verification that each group containing bioactive factors (e.g., COF and OVN fractions) was significantly greater than the BSA low control, data were reanalyzed to compare OVN fractions to the COF control alone. For effects of OVN on acrosome reaction induction in sperm, main effects were preparation plus male (n = 4), capacitation time (0 and 5.5 h), and

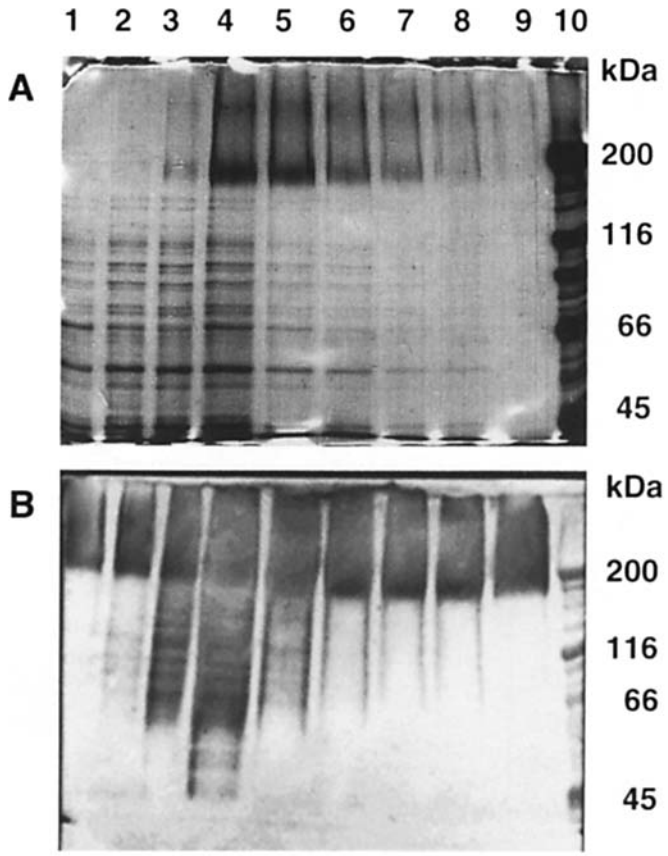


FIG. 1. Lectin affinity isolation and characterization of hamster OVN used for bioassays. A) OVN (200–240 kDa) was purified from oviduct extract by *Helix pomatia*-agarose affinity chromatography. Proteins (6 μg/lane) were electrophoresed on 6% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250 as described in *Materials and Methods*. B) Electrophoresed samples were transferred onto PVDF membrane, incubated with mAb 1C4 and then with alkaline phosphatase-conjugated goat anti-mouse IgG, and developed as described in *Materials and Methods*. Lanes A, 1–8, and B, 1–8: first eight 1-ml fractions eluted with 0.2 M N-acetyl-D-galactosamine that were dot blot-positive with mAb 1C4. Lanes A,9 and B,9: pooled fractions 11–14. Lanes A,10 and B,10: molecular mass standards, myosin (200 kDa), β-galactosidase (116 kDa), BSA (66 kDa), ovalbumin (45 kDa).

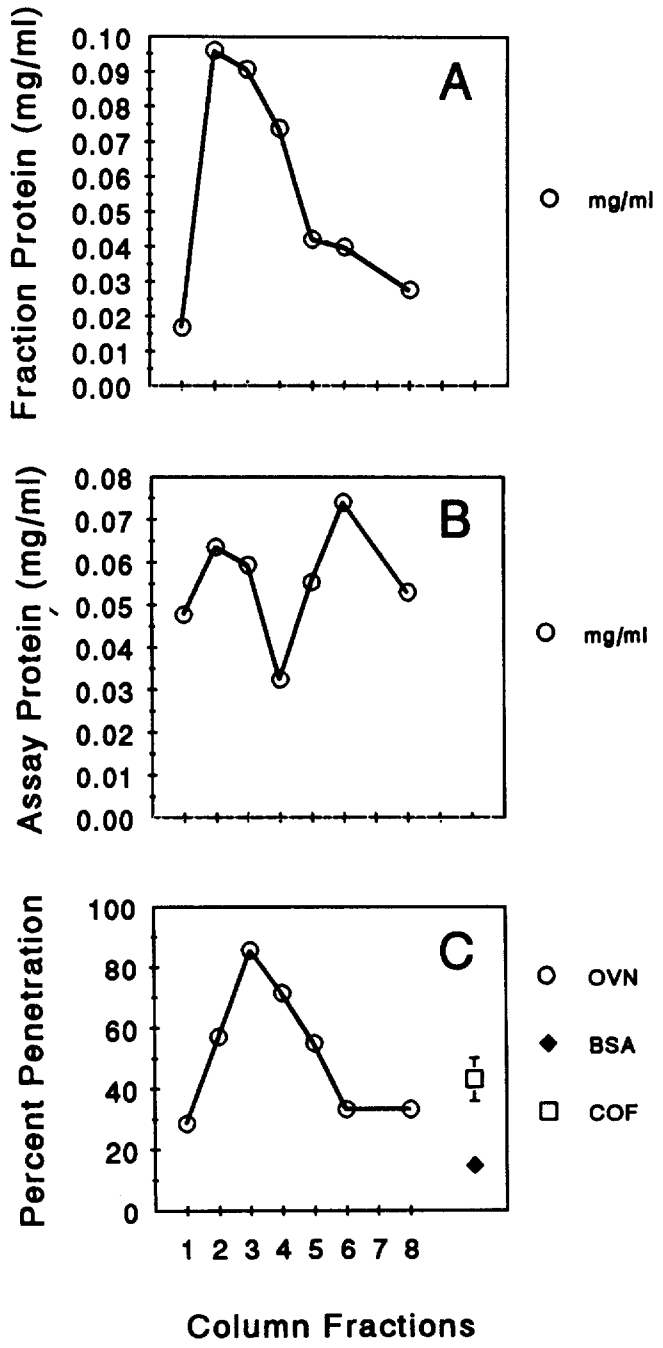


FIG. 2. Relationship between lectin affinity-isolated OVN fractions and bioactivity as assayed by sperm penetration into eggs. Panels A-C are from a representative run. A) Protein concentrations in each 1-ml fraction eluted from affinity column with 0.2 M N-acetyl-D-galactosamine. B) Final OVN protein concentrations (after microconcentration and buffer exchange) used in the egg bioassay. C) Percentage egg penetration with the protein concentrations in (B). Column fractions 1–6: first six 1-ml fractions that were dot blot-positive with mAb 1C4. Fraction 8: pooled fractions 10–16.

treatment (± OVN). Where appropriate, multiple comparisons were made using Fisher's least significant difference at $p \leq 0.05$. The NCSS statistical package (Kaysville, UT) was used for analyses.

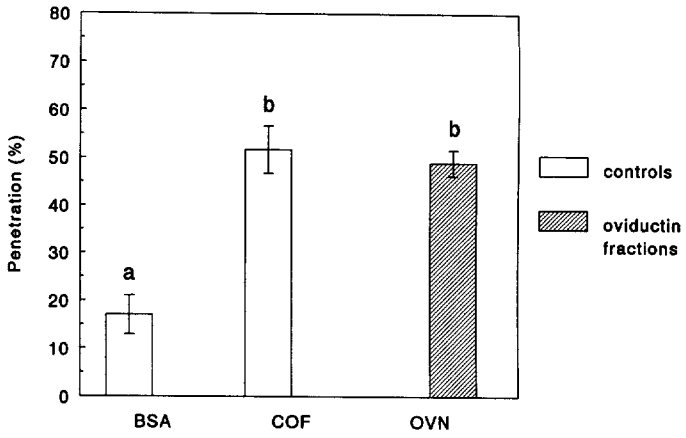


FIG. 3. Comparison of pooled egg penetration responses (all OVN fractions combined) to those of high and low controls. The OVN fractions were prepared and used as described in *Materials and Methods* (see also Fig. 2). COF eluate is the high control, and BSA alone is the low control. Treatments with different superscripts are significantly different, $p \leq 0.01$.

RESULTS

Fractionation and Electrophoresis

Approximately 10% of the oviductal cytosolic protein that was loaded onto the lectin affinity column was eluted by 0.2 M N-acetyl-D-galactosamine (i.e., bound to the affinity matrix); the remainder eluted with PBS (i.e., was nonspecifically bound). On average, fractions 3–18 after addition of competing sugar were OVN-positive on dot blots. On the electrophoretograms, the sugar-eluted fractions were en-

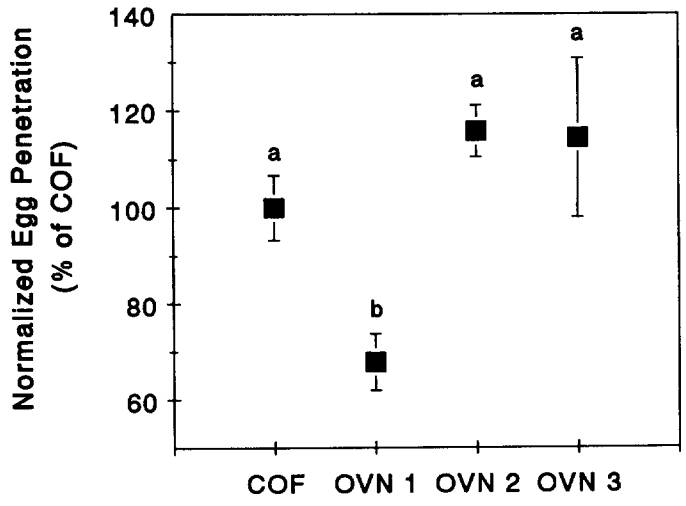


FIG. 4. Accuracy of using immunoreactive equivalency of OVN concentration in bioassay for predicting sperm penetration response. Pooled fractions in OVN 1, graded initially as 2.5–5 times more *dilute* than the COF standard by the dot-blot procedure, were bioassayed at an OVN concentration 0.33–0.67 times that of COF (the high control). Pooled fractions in OVN 2 and OVN 3 were graded initially as equal to COF and 2 times more concentrated, respectively, and tested at OVN concentrations equivalent to that in COF. Penetration responses were normalized to those in the COF controls as described in *Materials and Methods*. Treatments with different superscript letters are significantly different, $p \leq 0.05$.

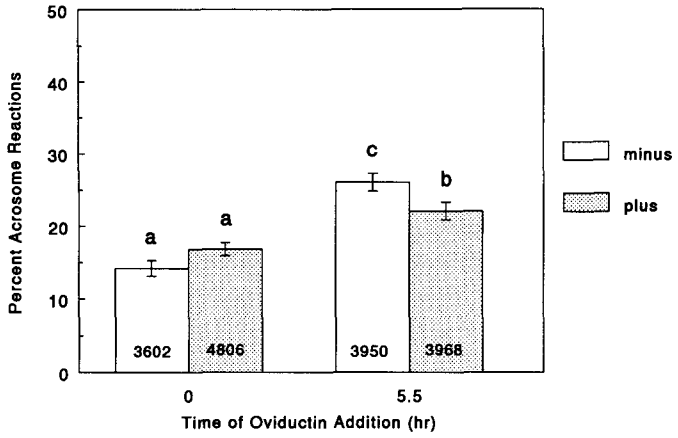


FIG. 5. Effects of OVN on sperm acrosome reactions of uncapacitated and capacitated sperm. OVN was added (i.e., "minus" or "plus") to sperm in TALP-PVA for 30 min at either 0 h (uncapacitated) or 5.5 h (capacitated) of incubation. The median OVN protein concentration used was 0.08 (0.097–0.059, 75–25 percentile) mg/ml. Sperm were then washed, fixed, and scored as described in *Materials and Methods*. The total number of sperm counted in each treatment is labeled on each bar. Treatments with different superscript letters are significantly different, $p \leq 0.05$.

riched in two diffuse bands at 200–240 kDa (Fig. 1A) that were identified by immunolabeling with mAb 1C4 as OVN (Fig. 1B).

Egg Penetration Bioassay

The relationship between the original protein concentration of the OVN-positive fractions from the affinity columns, the protein used in the egg bioassay, and the egg penetration activity is illustrated in Figure 2. The median protein concentration used for the OVN-positive test fractions in the egg penetration assays was 0.08 (0.097–0.059, 75–25 percentile) mg/ml. The penetration activity was positively correlated with the protein concentration in the bioassay; $r = 0.4756$, $p = 0.002$, error $df = 38$ (data not shown). The penetration data for all OVN-positive fractions compared to low (BSA) and high (COF) controls are illustrated in Figure 3. The OVN-positive fractions (pooled data; 48.96 ± 4.96%) elicited an average 2.9-fold increase in penetration compared to the BSA control (17.02 ± 4.05%) and were comparable to the COF standard (51.74 ± 4.96%). The accuracy of the qualitative dot-blot dilution indexing procedure, used in an attempt to test fractions at OVN levels comparable to those in COF, was analyzed. One group of fractions (OVN 1), initially judged to be 2.5–5 times more *dilute* than the COF standard, was bioassayed at a concentration only 0.33–0.67 times the strength of COF as estimated by the dot-blot procedure. The other two groups, one judged initially to be equal to COF (OVN 2), the other 2 times more concentrated (OVN 3), were tested at equivalency to COF. Egg penetration in all of the test groups with bioactive fractions (COF and OVN groups) was significantly greater than in the normalized BSA low control, 30 ± 2.8%

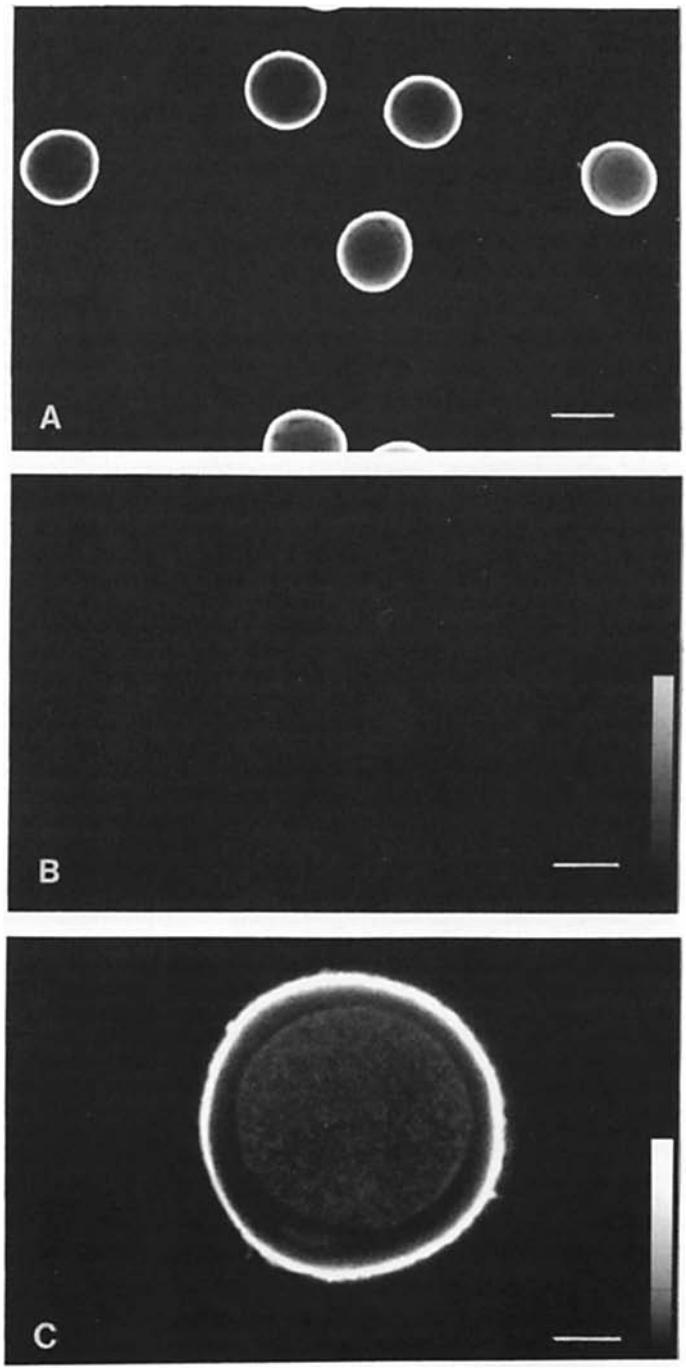
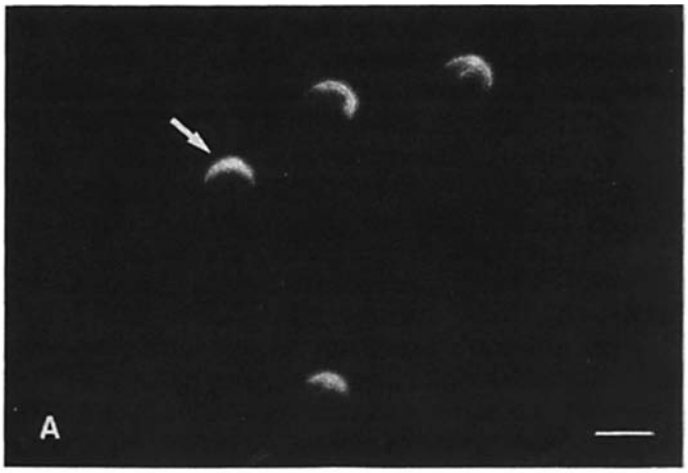


FIG. 6. Confocal localization of lectin affinity-isolated OVN binding to follicular oocytes. Freshly recovered follicular eggs were incubated with cumulus intact in lectin affinity-isolated OVN fractions for 1 h; cumulus cells were removed with hyaluronidase and eggs were then fixed and processed for indirect immunofluorescent visualization using the anti-OVN mAb, 1C4, as described in *Materials and Methods*. A) Follicular eggs incubated with OVN for 1 h; magnification bar = 96 μm. B) Follicular eggs incubated without OVN for 1 h; magnification bar = 96 μm. C) Single follicular egg from (A) enlarged to show relationship between vitellus, which is negative for OVN binding, and zona pellucida, which is intensely positive; magnification bar = 23 μm.



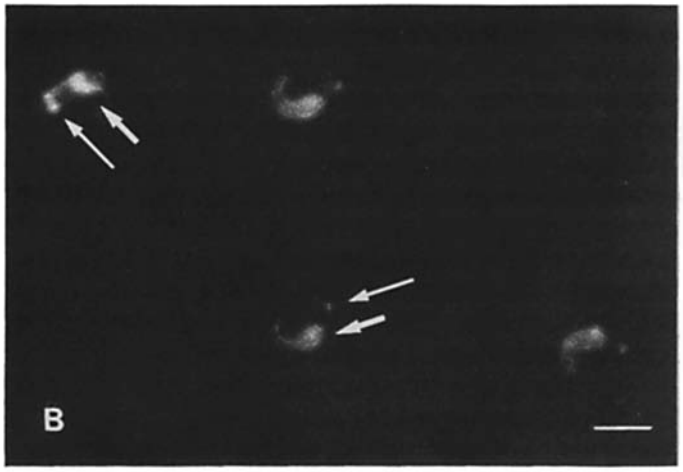


FIG. 7. Immunofluorescent patterns of OVN binding to acrosome-intact hamster sperm at two times during capacitation incubation. A) Patterns of OVN binding to the acrosomal crescent (arrow) of sperm when added for 30 min at 0 h of capacitation. B) Patterns of OVN binding to acrosome-intact sperm when added for 30 min at 5.5 h of capacitation. Note patterns of no fluorescence overlying the acrosomal crescent; dull fluorescence on the ventral surface of the acrosomal "hook"; either a bright diffuse area or a discrete spot over the equatorial segment (large arrows) as well as a bright spot or band posterior to it (small arrows). Magnification bars = 8.3 μm.

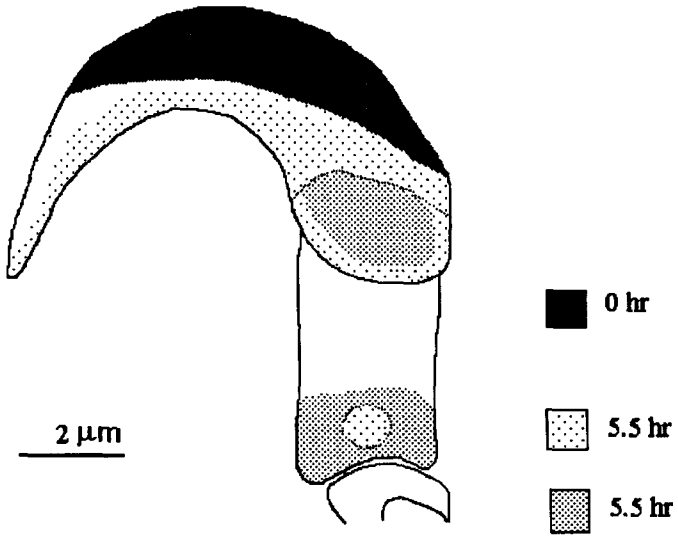


FIG. 8. Diagram of changes in OVN binding patterns to acrosome-intact sperm between 0 and 5.5 h of capacitation. The dark pattern over the acrosomal crescent predominated at 0 h (see Fig. 7A); the other two patterns, marked by two densities of stippling, were seen at 5.5 h (see Fig. 7B).

(mean ± SEM, not shown). As expected, the normalized penetration response mean of OVN 1, 68 ± 5.9%, was significantly less than the normalized COF mean, 100 ± 6.7% ($p \leq 0.05$; Fig. 4). The normalized means of OVN 2 and 3, 116 ± 5.3% and 114 ± 16.4%, respectively, were not different from the normalized COF mean (Fig. 4). However, Bartlett's test for homogeneity of variance showed that variances were not equal ($p \leq 0.01$). A Dunnett-type test, comparing the variances of OVN 2 and 3 to that of the COF control, showed that the penetration response for OVN 3 (fractions initially judged as more concentrated than the COF standard), but not OVN 2 (fractions initially judged as equal to the COF standard), was more variable than for the COF standard group ($p \leq 0.01$; [42]). This indicated difficulty in interpolating values for samples that did not closely match the color of the controls.

Immunofluorescent Localization

Some of the eggs were recovered after scoring for sperm penetration and were assayed for the presence of OVN on their zonae pellucidae. All of these sampled eggs were immunopositive for the presence of OVN. Additional follicular eggs not exposed to sperm were incubated in OVN containing medium for 1 h. OVN binding was localized on their zona pellucidae using confocal microscopy (Fig. 6, A and C).

Intense immunofluorescence of OVN was observed bound to the acrosomal crescent of intact sperm (Figs. 7A and 8), particularly at the 0-h capacitation time, when 82.5 ± 3.5% were labeled, compared to 13.7 ± 3.4% at 5.5 h (Fig. 9). Areas of the postacrosomal region of some acrosome-intact sperm were also labeled (Fig. 7B), but the frequency and fluorescence intensity were greater at the 5.5-h capacitation period (10.24 ± 10.26% vs. 60.35 ± 9.1%, 0 and 5.5 h, respectively; Fig. 9). The postacrosomal regions of acrosome-reacted sperm labeled with equal frequency at both time periods (31.8 ± 15.5% and 46.4 ± 13.8%, 0 and 5.5 h, respectively; not illustrated), although the intensity appeared greater at 5.5 h.

Acrosome Reactions

OVN was added to sperm suspensions for 30 min at two time points during capacitation incubation, at 0 and 5.5 h. Acrosome reactions increased significantly between the two times ($p \leq 0.0001$), averaging over all treatments 15.6% at

0.5 h and 24.5% at 6 h (not shown). Since the interaction term for time and OVN treatment was significant ($p = 0.0078$; not shown), data were reanalyzed by time. Addition of OVN at 5.5 h, but not at 0 h, had a small but statistically significant effect ($p = 0.027$) on inhibiting the acrosome reaction (Fig. 5).

DISCUSSION

In the golden hamster, we have previously shown that changes occur in both eggs and sperm during their residence in the oviduct—changes that increase the efficiency of fertilization [17, 35, 43]. Mature eggs that have not been exposed to the oviduct, whether ovulated (bursal) or unovulated (follicular), are penetrated less efficiently than are oviductal eggs [17, 35, 43]. Changes clearly occur in the structure of zonae pellucidae after oviductal exposure: the diameter of the zona pellucida increases without any apparent thinning of the zona wall, the perivitelline space enlarges, and OVN binds to the zona pellucida [18, 23, 35, 44]. Sperm are also exposed to oviductal molecules during capacitation in the isthmus prior to their encounter with the zona pellucida of the egg in the ampulla, and there is evidence that sperm interactions with isthmic cells and molecules underlie the mechanism of in vivo capacitation [2, 4, 45]. However, for already capacitated sperm, it has been unclear whether there are any additional changes, mediated by ampullary molecules, that influence sperm interactions with the zona pellucida.

Our initial hypotheses had been threefold: oviductal exposure of mature eggs and already capacitated sperm increases the fertilization rate; this change occurs because of an increased speed of acrosome reactions of sperm bound to the zona [17, 43, 44]; and the agent responsible for these changes is OVN. In support of the "acrosome reaction" hypothesis, we had previously observed that crude preparations of postovulatory oviduct fluid (i.e., COF) stimulated acrosome reactions in unattached sperm and also enhanced penetration of follicular eggs ([16, 17, 35]; present report [Figs. 2 and 3]). However, experiments we performed on sperm zona-binding and acrosome reactions of zona-bound sperm were equivocal in supporting the hypothesis that the speed of acrosome reaction was more rapid on zonae that had been exposed to the oviduct (and by inference, to OVN; [17]). Our results suggested that while the number and type of sperm (i.e., acrosome intact) that bound initially to oviductal and follicular eggs were similar, as were the rates (speeds) of acrosome reaction during the first 25 min after sperm binding, more sperm remained adherent to the oviductal eggs for longer periods, allowing acrosome-reacted sperm to accumulate more rapidly [17].

We now present evidence that the class of oviductal molecules that have homology with chitinase, termed "oviductins" or estrogen-dependent glycoproteins [18–22, 32], is responsible for some of the oviduct-mediated effects on

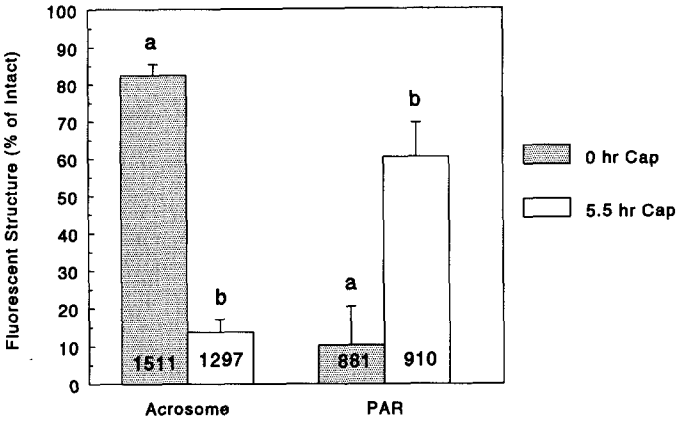


FIG. 9. Quantitation of changes in fluorescence patterns of OVN binding to acrosome-intact hamster sperm at two time periods of capacitation incubation. Acrosome-intact sperm were scored as either having bright fluorescence over the acrosomal crescent (acrosome; see Fig. 7A) or not. Those intact sperm not having bright acrosomal crescent fluorescence were then scored for presence of fluorescence on the postacrosomal region (PAR; see Fig. 7B). Postacrosomal fluorescence patterns included those with dull fluorescence on the ventral surface of the acrosomal "hook" and either a bright diffuse patch or a discrete spot over the equatorial segment as well as a bright spot posterior to it. At 0 h of capacitation, acrosome-intact sperm had predominantly the acrosomal fluorescence (Acrosome; see also Fig. 7A), whereas at 5.5 h the postacrosomal fluorescence patterns were most frequent (PAR; see also Fig. 7B). The total number of sperm counted for each condition is given on each bar. Within each group, Acrosome or PAR, treatments with different superscripts are significantly different, $p \leq 0.01$.

fertilization. Lectin affinity-isolated hamster OVN (Fig. 1, A and B) had the same activity as a complex mixture of postovulatory oviductal and cumulus oophorus fluid (i.e., COF) in stimulating enhanced penetration of follicular eggs (Figs. 2 and 3). There were multiple molecular mass components in the affinity fractions recognized by the anti-OVN mAb (Fig. 1). For the chitinase class of oviductal molecules, multiple proteins that are related molecularly occur quite commonly; but the precise interrelationship(s) between the forms is unknown and the topic is currently under investigation [46–48]. In our study, the amount of immunoreactive OVN in the test fractions was a good predictor of the penetration response (Fig. 4). Although the penetration activity was positively correlated with the protein concentration present in the bioassay ($r = 0.4756$, $p = 0.002$), the correlation was not as high as might have been expected (see also Fig. 2, B and C). However, unexpectedly and contrary to our starting hypothesis, addition of OVN to sperm either before or after capacitation, at concentrations effective for increased egg penetration, did not increase the incidence of acrosome reactions (as detected by phase-contrast and bright-field microscopy; Fig. 5). As expected, OVN bound firmly to zonae pellucidae of eggs (Fig. 6, A and C; [18, 23, 25, 26, 29]), but a new finding was that OVN bound to the acrosomal region of sperm (Figs. 7A, 8, and 9). Binding of OVN to acrosome-intact sperm showed regional and temporal responsiveness to changes in capacitation state. La-

beling moved from a location on the acrosomal rim on the head prior to capacitation, posteriorly, to the equatorial segment and parts of the postacrosomal area at the end of capacitation (Figs. 7, A and B, 8, and 9). The anterior rim of the acrosome is the site used by uncapacitated sperm for attaching to the oviductal epithelium [45], whereas the equatorial segment has been implicated in sustaining the binding of penetrating sperm to the zona pellucida [49]. Thus, redistribution of OVN binding sites on the sperm's head during capacitation may be important for gamete recognition and binding.

Oviductal zona-binding glycoproteins have been observed in a variety of animal species [18, 23, 25, 26, 28–30, 33, 50]. In the pig, one of these oviductal glycoproteins was postulated to be a substrate for acrosin [50]. Acrosin-proacrosin is a serine protease, located in the acrosomal secretory vesicle, that has binding affinity for sulfated glycoconjugates such as those present in the zona pellucida [51, 52] and possibly those present in OVN, a sulfated mucin-like protein [48]. Acrosin assists in maintaining binding of sperm to the zona pellucida [51, 52]. Perhaps OVN, like certain other sulfated polysaccharides, binds to acrosin, inhibiting its enzymatic activity [53] and prolonging its "bindin-like" function [51, 52]. If OVN binding inhibits autocatalysis of acrosin, then dissolution of the acrosomal cap should be retarded once the membrane fusion events of the acrosome reaction (detectable only by electron microscopy) have been initiated. This could explain the slight but statistically significant inhibition of the acrosome reaction (dissolution of the cap) that we observed when OVN was added to sperm for 30 min at 5.5-h capacitation (Fig. 5).

The oviduct has an important role at several stages of gamete preparation for fertilization. It is the major site for sperm storage and capacitation in its distal portion, the isthmus (reviewed in [41]). Fertilization occurs in its proximal region, the ampulla, where the efficiency and speed of sperm penetration through the egg investments appear to be facilitated by specific macromolecules either produced by the oviduct and/or associated with the ovulated egg [17, 41]. Our data suggest that molecules like OVN help the efficiency of sperm-egg interaction in vivo, perhaps by binding sperm to the zona pellucida more tenaciously.

In summary, we have shown that affinity-purified OVN binds both to the acrosomal region of the sperm head and to the zona pellucida of follicular eggs, and substantially enhances the penetration of already capacitated sperm into mature follicular eggs. The amount of immunoreactive OVN in the bioassayed fractions was a good predictor of the penetration response. Data reported in our present and previous studies [17] indicate that OVN enhances binding affinity of sperm for the zona pellucida, perhaps by affecting the time course of acrosomal enzyme secretion and/or degradation. Further study will be required to confirm the mechanism of the enhancement of penetration and fertilization.

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