BIOLOGY OF REPRODUCTION **65,** 1000–1005 (2001)

# Evidence That Human Epididymal Protein ARP Plays a Role in Gamete Fusion Through Complementary Sites on the Surface of the Human Egg[1]

**Debora J. Cohen,[3] Diego A. Ellerman,[3] Dolores Busso,[3] Mauro M. Morgenfeld,[3] Alejandra D. Piazza,[4] Masaru Hayashi,[5] Edgardo T. Young,[4] Masanori Kasahara,[5] and Patricia S. Cuasnicu[2,3]**

*Instituto de Biología y Medicina Experimental,[3] (1428) Buenos Aires, Argentina*
*Instituto de Ginecología y Fertilidad,[4] (1122) Buenos Aires, Argentina*
*Hokkaido University School of Medicine,[5] Sapporo 060, Japan*

## ABSTRACT

**Human epididymal sperm protein ARP, a member of the cysteine-rich secretory proteins (CRISP) family, exhibits significant homology with rat epididymal protein DE, a candidate molecule for mediating sperm-egg fusion in rodents. The aim of this study was to investigate the involvement of ARP in human gamete fusion. Sequential extraction of proteins from ejaculated human sperm revealed the existence of a population of ARP that is tightly associated with the sperm surface and thus, potentially capable of participating in gamete interaction. Exposure of capacitated human sperm to a polyclonal antibody against recombinant ARP (anti-ARP) produced a significant and concentration-dependent inhibition in the ability of human sperm to penetrate zona-free hamster eggs. This inhibition was not due to a deleterious effect on the gametes because anti-ARP affected neither sperm viability or motility, nor egg penetrability. The antibody did not inhibit the occurrence of spontaneous or $Ca^{2+}$ ionophore-induced acrosome reaction, nor did it inhibit the ability of sperm to bind to the oolema, supporting a specific inhibition of the antibody at the sperm-egg fusion level. As a relevant evidence for a role of ARP in gamete fusion, the existence of complementary sites for this protein on the surface of human eggs was investigated. Experiments in which zona-free human oocytes discarded from in vitro fertilization programs were exposed to ARP, fixed, and subjected to indirect immunofluorescence revealed the presence of specific ARP-binding sites on the entire surface of the human egg, in agreement with the fusogenic properties of the human oolema. Together, these results strongly support the participation of ARP in the sperm-egg fusion process, suggesting that this protein would be the functional homologue of DE in humans.**

*epididymis, fertilization, gamete biology, ovum, sperm*

## INTRODUCTION

Fertilization in mammals consists of a sequence of specific cell-cell and cell-matrix interactions that culminates in sperm-egg membrane fusion. Evidence suggests that the recognition between sperm and egg plasma membranes occurs through a multistep process involving different cell adhesion molecules on the surfaces of both gametes [1, 2].

In the rat, the epididymal sperm protein DE is a candidate molecule to mediate gamete membrane fusion [3]. DE is synthesized in an androgen-dependent manner by the proximal segments of the epididymis, and associates to the sperm plasma membrane during epididymal transit [4, 5]. Originally localized on the dorsal region of the sperm head, DE migrates to the equatorial segment concomitantly with the occurrence of the acrosome reaction [6]. Sequential extraction of proteins from epididymal sperm has revealed the existence of two populations of DE bound to sperm: a major population removable by ionic strength and loosely associated to the cells; and another one, resistant to this treatment, which behaves as an intrinsic protein [7]. Experiments performed in order to associate these two populations with functional events undergone by sperm, indicated that whereas the loosely bound population is released from sperm during capacitation, the tightly associated protein remains after capacitation and migrates to the equatorial segment [7].

The relocation of DE to the equatorial segment, the region through which the sperm fuses with the egg [8, 9], together with experiments showing that the polyclonal antibody against DE significantly inhibited the percentage of penetrated zona-free rat eggs, supported a role for this protein in sperm-egg fusion [10]. Subsequent studies in which the exposure of zona-free eggs to purified protein DE significantly reduced the percentage of penetrated eggs without affecting sperm-egg binding confirmed the involvement of this protein in gamete fusion through DE-complementary sites on the egg surface. Indirect immunofluorescence (IIF) studies showed that these DE-binding sites are localized over the fusogenic region of the egg surface [3, 11].

Sequence analysis of rat DE revealed that the protein belongs to the CRISP (cysteine-rich secretory proteins) family, and exhibits significant homology with two other epididymal proteins: mouse protein AEG-1/CRISP-1 (70%) [12, 13] and human protein ARP/hCRISP-1 (40%) [14, 15]. Recently, we have described the participation of the mouse homologue of DE in sperm-egg fusion through binding sites on the surface of mouse eggs [16].

In regard to the human protein, several characteristics such as its epididymal origin, secretory nature, molecular weight, and localization on the sperm head [14] suggest that it could correspond to the human molecule that performs a function equivalent to that of DE in rodents. However, the involvement of this protein in gamete fusion has not yet been investigated. In the present study we provide evidence supporting the participation of the human epididymal protein in gamete fusion through complementary sites localized on the surface of the human egg.

[1]Supported by World Health Organization grant H9/181/R429, CONRAD grant MFG-97-29, and National Ministry of Health grant to P.S.C., and PLACIRH Re-entry grant PRE-014/97 to D.A.E. D.J.C., D.A.E., and D.B. are Research Fellowship recipients from the National Research Council of Argentina (CONICET). P.S.C. is a Research Career Award recipient from CONICET.

[2]Correspondence: Patricia S. Cuasnicu, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, (1428) Buenos Aires, Argentina. FAX: 54 11 4786 2564; e-mail: cuasnicu@dna.uba.ar

Received: 2 February 2001.
First decision: 27 February 2001.
Accepted: 8 May 2001.

ISSN: 0006-3363. http://www.biolreprod.org

## MATERIALS AND METHODS

### Preparation of Sperm Extracts and Tissue Cytosols

Ejaculates were obtained from healthy donors whose initial semen characteristics fell within the World Health Organization (WHO) criteria for normality [17]. Semen samples were washed three times in PBS containing 0.2 mM PMSF (PBS-PMSF) (Sigma Chemical Co., St. Louis, MO), and then incubated for 30 min in 2 M NaCl PBS. The suspension was centrifuged 10 min at 700 × *g* and the supernatant was centrifuged at 13 000 × *g*, dialyzed against deionized water at 4°C, and lyophilized. The sperm pellet was incubated once again with 2 M NaCl as described above and after centrifugation at 700 × *g*, sperm were incubated for 30 min with 1% Triton X-100 (Sigma) in PBS. Following this incubation, the suspension was centrifuged for 10 min at 13 000 × *g*, and the supernatant was precipitated at −20°C with 10 volumes of acetone.

Human testes and epididymides were obtained from patients (aged 50–80 yr) undergoing orchidectomy as treatment for prostatic carcinoma and who had not received radiation, hormonal, or chemotherapy treatments. Testicular and epididymal cytosols were prepared as previously described [16].

### Western Blots

Samples were separated in nonreducing 12% SDS-polyacrylamide gels, according to the method of Laemmli [18], and proteins were electrotransferred to nitrocellulose [19]. The membranes were blocked for 1 h with powdered skim milk (2% in PBS) and incubated for 3 h with a 1:1000 dilution of rabbit polyclonal antibodies raised against either the human recombinant ARP molecule coupled to maltose binding protein (MBP; anti-ARP) [14], MBP (anti-MBP; New England BioLabs, Inc., Beverly, MA), or human recombinant testicular TPX-1 protein also coupled to MBP (anti-TPX-1) [20]. Anti-TPX-1 antibody was prepared as previously described for anti-ARP [14]. Membranes were washed thoroughly before incubation for 1 h with biotin-conjugated anti-rabbit immunoglobulin G (IgG; 1:500 dilution, Sigma). After extensive washing, the membranes were incubated for 1 h with ExtrAvidin-horseradish-peroxidase (1:1000 dilution, Sigma), and reactive bands were visualized with 3,3′-diaminobenzidine (40 μg/ml [Sigma] in Tris 0.1 M [Sigma] pH 7.5, and 0.01% v/v $H_2O_2$). All incubations were carried out at room temperature.

### Human Sperm Capacitation

After complete liquefaction, the semen was diluted with six volumes of Biggers, Whitten, Whittingham (BWW) solution [21], centrifuged (10 min at 300 × *g*), and washed again with 1 ml of BWW containing 3.5% HSA (Sigma; BWW-HSA [human serum albumin]). Sperm samples were then allowed to swim up for 1 h at 37°C in 1 ml of BWW-HSA, and motile selected spermatozoa were diluted to a final concentration of 5–10 × $10^6$ cells/ml, and incubated 18 h at 37°C in BWW-HSA in an atmosphere of 5% $CO_2$.

### Sperm Viability and Computer-Assisted Semen Analysis

Semen samples from normal donors were capacitated as previously described. After 18 h of capacitation, 100 μl of the sperm suspension were incubated for 30 min in BWW-HSA alone or containing anti-ARP or anti-MBP (1:50 dilution), and sperm viability and motility were evaluated. For viability assessment, 40 μl of sperm suspension was stained with prewarmed 0.5% eosin (yellowish; Sigma) in saline solution, and the incorporation of the dye was evaluated by light microscopy. The percentage of viability was calculated as the number of sperm that did not incorporate the dye over the total number of sperm counted.

For motility assessment, aliquots of 5 μl of sperm were placed in a Mackler chamber (Sefi-Medical Instruments, Rehovot, Israel) and motility parameters were measured in a computerized sperm analyzer (Hamilton Thorn IVOS/09279/V10.8s; Hamilton Thorn, Danvers, MA). For each sample the mean value of more than 200 cells (from at least eight different fields) was calculated. The following parameters were measured: motility (percentage), progressive motility (percentage), path velocity (VAP, μm/sec), track speed (VCL, μm/sec), lateral head displacement (LHD, μm), and linearity (LIN, percentage).

### Acrosome Reaction Induction

Capacitated sperm incubated in medium alone or in medium containing either anti-ARP or anti-MBP (1:50) were incubated with $Ca^{2+}$ ionophore A23187 (final concentration 5 μM; Sigma) at 37°C. After 1 h, sperm suspensions were washed, fixed for 10 min with 2% paraformaldehyde in PBS, washed again, placed on polylysine-coated slides, and air-dried. Slides were immersed for 5 min in methanol at 4°C for cell permeabilization [22]. Acrosome reaction was evaluated by sperm staining with 50 μg/ml of fluorescein isothiocyanate (FITC)-labeled *Pisum sativum* aglutinin (PSA, Sigma) [23]. Sperm were scored as acrosome intact when a bright staining was observed on the acrosome, or as acrosome reacted when either fluorescent staining was restricted to the equatorial segment or no labeling was observed.

### Zona-Free Hamster Oocyte Penetration Test

This test was performed as described in the WHO *Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction* [17]. Immature (4- to 6-wk-old) hamster (*Mesocricetus auratus*) females were superovulated by an i.p. injection of 25 IU of eCG (Syntex, Buenos Aires, Argentina), followed by the i.p. administration of 25 IU of hCG (Sigma) 48 h later. Eggs were recovered from the oviducts of superovulated animals 14–16 h after hCG administration. Cumulus cells were dispersed with 0.1% hyaluronidase (Sigma, type IV) prepared in 4 mg/ml BSA (Sigma) in PBS. After washing in BWW, the zona pellucidae were removed by treatment with 0.1% trypsin (Sigma) in BWW. Finally, zona-free oocytes were thoroughly washed in capacitation medium and distributed among treatment groups.

Only those human sperm samples presenting more than 70% motile sperm after capacitation were used for insemination. Aliquots containing 3.5 × $10^5$ motile cells were diluted to 100 μl with BWW-HSA, and incubated with anti-ARP (1:5 to 1:500), anti-MBP (1:50), or medium alone at 37°C under paraffin oil (saybolt viscosity 125/135; Fisher Scientific Co., Pittsburgh, PA). After 30 min, 15–20 zona-free hamster eggs were added to each drop, and gametes were coincubated for 2–3 h at 37°C in an atmosphere of 5% $CO_2$. The oocytes were then freed from unbound and loosely bound spermatozoa by serial aspiration through a finely drawn pipette, fixed in 2.5% glutaraldehyde, mounted on slides, and observed under the microscope (400×) after staining with 1% aceto-carmine solution. The number of eggs presenting decondensing sperm heads or pronuclei and sperm tails in the egg cytoplasm, as well as the number of spermatozoa bound per egg were recorded.

### Indirect Immunofluorescence of Human Oocytes

Human unfertilized oocytes, identified by the absence of two pronuclei and failure to divide 48 h after conventional in vitro fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) attempts, as well as noninseminated surplus oocytes discarded from an IVF program with patients' informed consent were used for these experiments. Cumulus cells were dispersed by treatment with 0.1% hyaluronidase in BWW, and the zona pellucidae were removed with acid Tyrode solution (pH 2.5) [24]. Zona-free human oocytes incubated for 30 min at 37°C in medium alone, or medium containing 6 μM of either recombinant ARP, MBP (expressed in the same system as ARP), or rat epididymal protein DE, were fixed for 1 h in 1 ml of 2% paraformaldehyde at room temperature. After washing with PBS containing 10 mg/ml BSA (PBS-BSA10; Sigma), oocytes were incubated for 30 min at 37°C in 100 μl of 5% normal goat serum (NGS) in PBS-BSA10. Eggs were then exposed to anti-ARP, anti-MBP, or anti-DE (1:50) for 2 h at 37°C. After washing in PBS-BSA10 containing 0.02% v/v Tween 20, eggs were incubated for 30 min at 37°C in FITC-conjugated goat-anti-rabbit IgG (1:50 in PBS-BSA10; Sigma), washed, mounted in 90% glycerol in PBS, and finally examined with a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics.

### Statistical Analysis

Results are expressed as mean values ± SEM for each series of experiments. Statistical significance of the data were analyzed using a one-way ANOVA for the number of bound sperm per egg and sperm motility values, and a chi-square test for the percentages of both oocytes with bound or fused sperm, and acrosome reacted sperm, with significant differences being defined by a $P$ value of less than 0.05.

## RESULTS

### Interaction of Human Epididymal Protein ARP with the Sperm Surface

The fact that in the rat, the population of DE protein tightly bound to the sperm surface would be the one finally

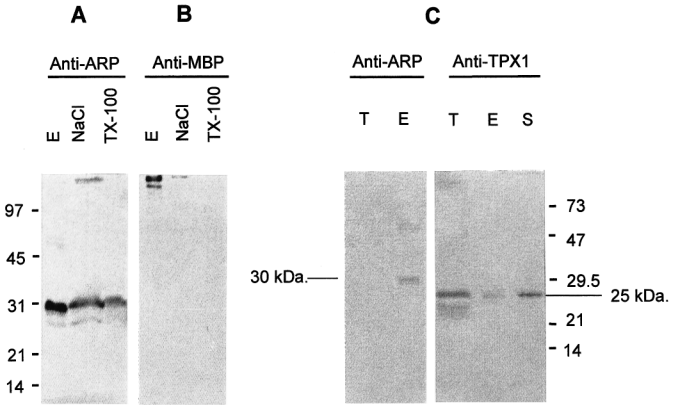


FIG. 1. Sequential extraction of ARP from human ejaculated sperm. Human testicular (T) and epididymal (E) cytosols as well as protein extracts obtained by either sequential exposure of ejaculated human sperm to 2 M NaCl (NaCl) and 1% Triton X-100 (TX-100), or direct extraction with 1% Triton X-100 (S) were subjected to electrophoresis on nonreducing 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by Western blot using anti-ARP (1:1000) (**A**), anti-MBP (1:1000) (**B**), or anti-TPX-1 (1:1000) (**C**) as primary antibodies.

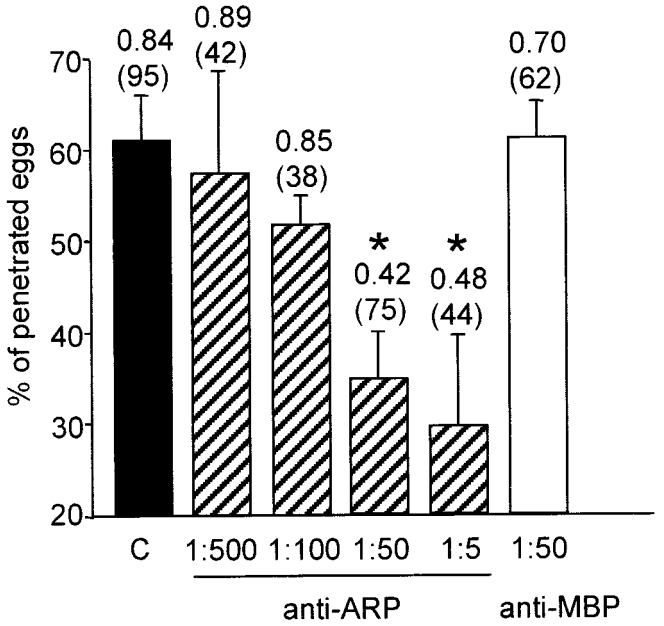


FIG. 2. Effect of anti-ARP on the ability of human sperm to penetrate zona-free hamster eggs. Capacitated human sperm were incubated for 30 min in medium alone (C), or medium containing either different concentrations of anti-ARP (1:5 to 1:500) or anti-MBP (1:50). Zona-free hamster eggs were then added to sperm and, after 3 h of gamete coincubation, eggs were recovered, fixed, stained with 1% aceto-carmine, and the percentage of penetrated eggs evaluated. The average number of sperm penetrated per egg as well as the total number of eggs observed (in parenthesis) are shown on the top of each bar. Results represent the mean value ± SEM of five independent experiments. $*P < 0.05$ vs. controls.

involved in gamete fusion [7], led us to first explore the existence of a firmly bound population of the human epididymal protein on sperm. For this purpose, ejaculated human sperm were subjected to sequential extraction of proteins with 2 M NaCl and 1% Triton X-100, and the presence of the protein in the sperm extracts was evaluated by Western blot using a polyclonal antibody against recombinant protein ARP (anti-ARP) as primary antibody. Because this antibody was raised against ARP expressed as a fusion protein with MBP, an antibody against MBP (anti-MBP) was used as a control. Human epididymal cytosol was included among the samples as a positive control. Results indicated that whereas treatment of sperm with 2 M NaCl was able to remove a substantial amount of ARP (detected as a strong band of ~30 kDa and a faint band of ~26 kDa, corresponding to the deglycosylated form of the protein [14]; Fig. 1A, lane 2), a population of ARP remained on sperm after this treatment as judged by the presence of the protein in a Triton X-100 extract obtained after two consecutive extractions with 2 M NaCl (Fig. 1A, lane 3). No bands were detected when the membranes were incubated with anti-MBP as primary antibody (Fig. 1B).

Because the existence of TPX-1, a testicular homologue of ARP, has also been described in humans [20], the possibility that the population released by Triton X-100 could correspond to this intra-acrosomal testicular protein was evaluated. For this purpose, human testicular and epididymal cytosols as well as a sperm protein extract were analyzed by Western blot using either anti-ARP or anti-human TPX-1 as primary antibodies. Results shown in Figure 1C indicated that whereas anti-ARP did not cross-react with testicular cytosol, anti-TPX-1 was able to detect a band of a different molecular weight (25 kDa) in both testicular and epididymal cytosols as well as in the sperm protein extract.

### *Participation of ARP in Sperm-Egg Fusion*

The involvement of ARP in sperm-egg fusion was studied by the use of the hamster oocyte penetration test, which evaluates the ability of human sperm to fuse with the plasma membrane of hamster eggs [25]. Capacitated human sperm were exposed to zona-free hamster eggs in the presence of different dilutions of the specific antibody anti-ARP. Sperm incubated in medium alone or medium containing anti-MBP were used as controls. Results indicated that exposure of sperm to a dilution ≤1:50 of anti-ARP produced a significant inhibition in both the percentage of penetrated eggs and the average number of sperm penetrated per egg compared with controls (Fig. 2). In view of these results, subsequent experiments were performed using a 1:50 dilution of anti-ARP.

To investigate whether the inhibition observed was due to a deleterious effect of anti-ARP on sperm cells, the occurrence of agglutination, or a decrease in the viability or motility of antibody-treated capacitated sperm were examined. Motility was objectively analyzed using a computer-assisted semen analysis (CASA) system. Determinations were performed immediately before addition of the eggs. Results indicated that anti-ARP neither induced sperm agglutination nor affected sperm viability or any of the motility parameters analyzed compared with controls incubated in either medium alone or medium containing anti-MBP antibody (Table 1).

TABLE 1. Effect of anti-ARP on human sperm viability and motility. Results represent the mean value ± SEM of four independent experiments.

| Parameters analyzed | Medium alone | Anti-MBP | Anti-ARP |
|---|---|---|---|
| Viability (%) | 91 ± 4 | 89 ± 5 | 88 ± 7[a] |
| Motility (%) | 76 ± 3 | 74 ± 5 | 79 ± 4[a] |
| Progressive motility (%) | 55 ± 7 | 49 ± 7 | 56 ± 10[a] |
| VAP (μm/sec) | 54 ± 3 | 51 ± 3 | 56 ± 2[a] |
| VCL (μm/sec) | 85 ± 7 | 82 ± 6 | 89 ± 3[a] |
| LHD (μm) | 5.1 ± 0.3 | 4.9 ± 0.4 | 5.2 ± 0.4[a] |
| LIN (%) | 55 ± 2 | 53 ± 3 | 55 ± 4[a] |

[a] No significant differences were observed among groups (ANOVA test).

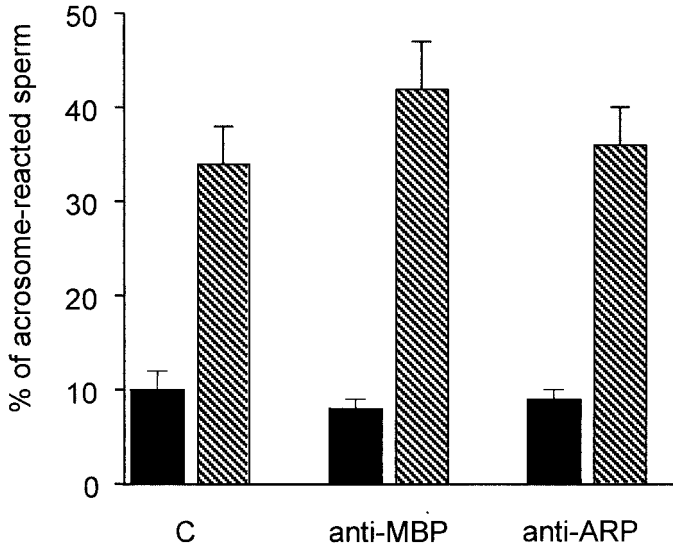


FIG. 3. Effect of anti-ARP on the occurrence of spontaneous and induced acrosome reaction. Capacitated sperm were incubated in medium alone (C) or medium containing anti-MBP (1:50) or anti-ARP (1:50), in either the absence (solid bars) or presence (hatched bars) of 5 μM calcium ionophore A23187. After 1 h, sperm were washed and the occurrence of the acrosome reaction was evaluated by staining sperm with FITC-PSA. Results represent the mean value ± SEM of three independent experiments. No significant differences were observed among groups (chi-square test).

To exclude the possibility that the observed inhibition were due to an effect of anti-ARP on egg penetrability, zona-free hamster eggs were incubated 30 min with anti-ARP or anti-MBP (1:50), washed, and then inseminated with capacitated human sperm. Results indicated that none of these treatments produced a significant effect in the percentage of penetrated eggs compared with controls (anti-ARP, 78% ± 1%; anti-MBP, 86% ± 19%; medium alone, 91% ± 12%).

Because the occurrence of the acrosome reaction is a requisite for fusion of human sperm with hamster oolema [26], the inhibition in egg penetration observed in the presence of anti-ARP could be due to an effect of the antibody on this exocytotic event. To explore this possibility, human sperm were incubated under capacitating conditions, and both the spontaneous and $Ca^{2+}$ ionophore (A23187)-induced acrosome reaction were evaluated by the PSA technique. As shown in Figure 3, spermatozoa exposed to anti-ARP exhibited percentages of spontaneous or induced acrosome reaction not significantly different from those corresponding to sperm incubated under control conditions.

Considering that binding of sperm to the oolema is a necessary step for sperm-egg fusion, the effect of anti-ARP on the sperm ability to bind to zona-free hamster eggs was also evaluated. Results shown in Table 2 indicated that neither the percentage of zona-free eggs with bound sperm, nor the number of sperm bound per egg were significantly different from controls.

### *Complementary Sites for ARP on the Human Egg Surface*

As another approach toward the study of the participation of ARP in human sperm-egg fusion, the existence of ARP-binding sites on the surface of the human egg was examined. For this purpose, zona-free human oocytes discarded from IVF programs were incubated with 6 μM recombinant ARP (recARP), fixed, and finally subjected to IIF using anti-ARP or anti-MBP as primary antibodies. Zona-free oocytes incubated under the following different conditions were used as controls: 1) eggs incubated with MBP and anti-MBP, 2) eggs incubated in medium alone and then exposed to anti-ARP or anti-MBP, and 3) eggs incubated with epididymal protein DE and anti-DE. From a total of 95 oocytes analyzed, 34 corresponded to eggs that failed to form two pronuclei by conventional IVF or ICSI procedures, and 61 corresponded to oocytes that had not been exposed to sperm. Results showed that all oocytes incubated with recARP, followed by either anti-ARP or anti-MBP (n = 60), presented a patchy fluorescence over the entire egg surface (Fig. 4a). The same fluorescent pattern was observed in eggs exposed or unexposed to sperm. In contrast, eggs incubated with MBP and anti-MBP exhibited occasional small patches of fluorescence with the majority of the plasma membrane being very sparsely labeled, whereas those exposed only to anti-ARP or anti-MBP, or to DE/anti-DE were completely negative (Fig. 4b).

TABLE 2. Effect of anti-ARP on the ability of human sperm to bind to zona-free hamster eggs. Values represent the mean value ± SEM of four independent experiments. No significant differences were observed among groups (chi-square test and ANOVA).

| Treatment | No. of eggs | Eggs with bound sperm (%) | No. of sperm bound/egg |
|---|---|---|---|
| Control | 86 | 100 | 13 ± 2 |
| Anti-MBP | 70 | 88 ± 9 | 14 ± 2 |
| Anti-ARP | 61 | 100 | 11 ± 3 |

## DISCUSSION

Previous results from our laboratory reported the participation of sperm epididymal protein DE in both rat and mouse sperm-egg fusion [3, 16]. In the present study we provide evidence supporting the involvement of the human epididymal homologue ARP in gamete fusion.

The analysis of successive extractions of proteins from human sperm revealed the existence of two populations of ARP associated to sperm; one population removable by ionic strength, and another one that remains on sperm even after exposure to 2 M NaCl, and which was released by detergent treatment. The possibility that this tightly bound protein could correspond to TPX-1, an intra-acrosomal, testicular member of the CRISP family, was considered. However, the lack of cross-reaction of anti-ARP with testicular

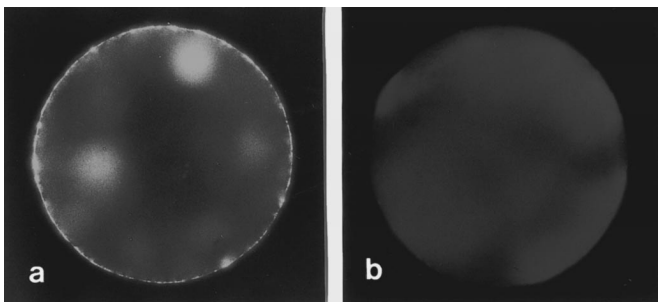


FIG. 4. Immunofluorescent localization of ARP-complementary sites on the surface of the human egg. **a**) Zona-free human eggs were incubated with recombinant ARP (6 μM) for 30 min, fixed, and exposed to anti-MBP/FITC-anti-rabbit IgG. A patchy distribution of fluorescence over the entire egg surface is observed. **b**) Zona-free human eggs were fixed and then exposed to anti-MBP/FITC-anti-rabbit IgG. Note the absence of labeling on the egg surface. Identical results were obtained for eggs incubated with DE/anti-DE (magnification ×250).

cytosol, and the finding that anti-TPX-1 recognized a band in testicular tissue and sperm protein extract with a molecular weight different from that corresponding to ARP, supported the epididymal origin of both populations of ARP. Our results differ from those of Kratzschmar and colleagues [15] who reported the complete release of the human protein by detergent-free solutions. Considering that these investigators used approximately two to three times less protein in their SDS-PAGE studies, and that the tightly bound protein represents a minor population according to densitometry analysis (data not shown), their results might be attributed to a lack of detection of the tightly bound population. In view of our observations in the rat indicating that the tightly associated protein would correspond to the one finally involved in gamete fusion [7], the existence of such a population of ARP supports a role for this protein in sperm-egg interaction.

The participation of ARP in gamete fusion was then evaluated by studying the effect of an antibody against recARP [14] on the ability of capacitated human sperm to penetrate zona free-hamster eggs. Results showed that exposure of capacitated human sperm to anti-ARP significantly decreases their ability to penetrate hamster eggs under standardized conditions. Several lines of evidence support the specificity of this inhibition: 1) the effect was concentration-dependent and was not observed in either the absence of antibody or the presence of a control antibody (anti-MBP); 2) anti-ARP did not produce sperm agglutination, and did not affect sperm viability or a series of sperm motility parameters analyzed objectively; 3) anti-ARP did not affect egg penetrability, as judged by the fact that those oocytes exposed to anti-ARP and washed, could then be normally penetrated.

The possibility that the inhibition observed resulted from an effect of the antibody on the occurrence of functional events required for gamete fusion was then investigated. Results indicated however, that anti-ARP affected neither the spontaneous or ionophore-induced acrosome reaction nor the binding of sperm to the egg surface. Although a possible inhibition of sperm penetration due to steric hindrance by the antibody cannot be completely excluded, the observation that the first stage of sperm-egg binding was not affected supports a specific effect of anti-ARP in an event subsequent to sperm-egg binding and leading to fusion.

Considering that these studies involved the use of a heterologous system (human sperm and hamster eggs), the existence of ARP binding sites on the surface of human eggs represented a strong indication in favor of the participation of ARP in human gamete fusion. IIF experiments showed that all eggs incubated with ARP and then with either anti-ARP or anti-MBP presented fluorescent labeling on the egg surface. This labeling was specific for ARP as judged by the absence of fluorescence on those eggs exposed to antibodies only, or to other proteins and their corresponding antibodies (MBP/anti-MBP; DE/anti-DE). The same fluorescent pattern was observed in eggs that had not been exposed to sperm, or those that failed to be fertilized after IVF or ICSI, indicating that perturbation of the egg plasma membrane by these techniques does not affect the distribution of ARP-binding sites on the egg surface. The physiological significance of these observations remains to be established.

ARP-incubated human eggs exhibited a patchy fluorescent labeling over the entire egg surface, which was different from our observations in DE-incubated rodent eggs, which presented a nonlabeled area corresponding to the plasma membrane overlying the meiotic spindle [3, 16]. This result is consistent with previous observations indicating that, in contrast to eggs from other mammals, human oocytes do not exhibit a microvillus-free area or other indications of polarization, and appear to be functionally homogeneous with respect to sperm entry sites [27, 28]. Thus, the distribution of ARP-binding sites on the egg surface is in agreement with the fusogenic properties of the human oolema. To our knowledge, this is the first report on the existence and localization of specific binding sites for a sperm protein on the surface of the human egg.

In the last few years, significant progress has been made toward the identification of molecules that mediate sperm-egg interaction. Two of the best characterized candidates are fertilin, a testicular heterodimer of $\alpha$ and $\beta$ subunits [29], which mediates sperm-oolema binding through its interaction with an integrin on the egg surface [30, 31], and protein DE [3, 16], which participates in a subsequent event leading to fusion through a mechanism not involving the disintegrin-integrin interaction [3, 16]. In humans, recent evidence indicating that fertilin $\beta$ [32, 33] would mediate sperm-egg binding through an integrin receptor on the human egg [34], together with our findings supporting a role for ARP in gamete fusion, suggest that the molecular mechanisms involved in human sperm-egg fusion might be analogous to those operating in other mammalian species.

Although many epididymal proteins have been identified and characterized, the function of most of them remains unknown, particularly in humans. In fact, the controversy of the role of the epididymis in men [35, 36] is still unresolved partly due to the paucity of this information. Thus, the present results may contribute to a better understanding of both the molecular mechanisms involved in human sperm maturation and the role of the epididymis in human physiology.

The strict tissue specificity of ARP makes our findings also of considerable interest to the study of immunocontraception. This possibility is strongly supported by previous results from our laboratory indicating that immunization of rats with protein DE produces a significant and reversible reduction in male and female fertility by a specific inhibition of sperm fertilizing ability [10, 37, 38].

The existence of infertile patients, whose sperm are able to penetrate the zona pellucida in vitro but are unable to fuse with the oolema, has been reported [39]. Therefore, the identification of ARP as a molecule involved in human gamete fusion might contribute to the development of new methods of diagnosis and treatment of male infertility. The relevance of ARP for human fertility is currently under investigation.

## ACKNOWLEDGMENT

The authors thank Dr. Juan Calamera from Laboratorio de Estudios Reproductivos (LER) for his collaboration in the CASA experiments.

## REFERENCES

1. Myles DG. Molecular mechanisms of sperm-egg membrane binding and fusion in mammals. Dev Biol 1993; 158:35–45.
2. Evans JP. Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions. Front Biosci 1999; 4:114–131.
3. Rochwerger L, Cohen DJ, Cuasnicú PS. Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion. Dev Biol 1992; 153:83–90.
4. Kohane AC, Gonzalez Echeverria F, Piñeiro L, Blaquier JA. Interac-

tion of proteins of epididymal origin with spermatozoa. Biol Reprod 1980; 23:737–742.
5. Kohane AC, Cameo MS, Piñeiro L, Garberi JC, Blaquier JA. Distribution and site of production of specific proteins in the rat epididymis. Biol Reprod 1980; 23:181–187.
6. Rochwerger L, Cuasnicu PS. Redistribution of a rat sperm epididymal glycoprotein after in vivo and in vitro capacitation. Mol Reprod Dev 1992; 31:34–41.
7. Cohen DJ, Rochwerger L, Ellerman DA, Morgenfeld M, Busso D, Cuasnicu PS. Relationship between the association of rat epididymal protein DE with spermatozoa and the behavior and function of the protein. Mol Reprod Dev 2000; 56:180–188.
8. Bedford JM, Moore HDM, Franklin LE. Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. Exp Cell Res 1979; 119:119–126.
9. Yanagimachi R. Sperm-egg fusion. In: Duzgunes N, Bronner F (eds.), Current Topics in Membranes and Transport. Orlando, FL: Academic Press; 1988: 3–43.
10. Cuasnicú PS, Conesa D, Rochwerger L. Potential contraceptive use of an epididymal protein that participates in fertilization. In: Alexander NJ, Griffin D, Spieler JM, Waites GMH (eds.), Gamete Interaction. Prospects for Immunocontraception. New York: Wiley-Liss; 1990: 143–153.
11. Cohen DJ, Munuce MJ, Cuasnicú PS. Mammalian sperm-egg fusion: the development of rat oolemma fusibility during oogenesis involves the appearance of binding sites for sperm protein ''DE''. Biol Reprod 1996; 55:200–206.
12. Mizuki N, Kasahara M. Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. Mol Cell Endocrinol 1992; 89:25–32.
13. Haendler B, Kratzschmar J, Theuring F, Schleuning WD. Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. Endocrinology 1993; 133:192–198.
14. Hayashi M, Fujimoto S, Takano H, Ushiki T, Abe K, Ishikura H, Yoshida M, Kirchhoff C, Ishibashi T, Kasahara M. Characterization of a human glycoprotein with potential role in sperm-egg fusion: cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEGL1). Genomics 1996; 32:367–374.
15. Kratzschmar J, Haendler B, Eberspaecher U, Roosterman D, Donner P, Schleuning WD. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. Eur J Biochem 1996; 236:827–836.
16. Cohen DJ, Ellerman DA, Cuasnicú PS. Mammalian sperm-egg fusion: evidence that epididymal protein DE plays a role in mouse gamete fusion. Biol Reprod 2000; 63:462–468.
17. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction. 4th ed. Cambridge, UK: Cambridge University Press; 1999.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
19. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350–4360.
20. Kasahara M, Gutknecht J, Brew K, Spurr N, Goodfellow PN. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. Genomics 1989; 5:527–534.
21. Biggers JD, Whitten WK, Whittingham DG. In: Daniel JC (ed.), Methods in Mammalian Embryology. San Francisco: Freeman; 1971: 86–116.
22. Mendoza C, Carreras A, Moos J, Tesarik J. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. J Reprod Fertil 1992; 95: 755–763.
23. Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 1986; 15:213–226.
24. Nicolson G, Yanagimachi H, Yanagimachi R. Ultrastructural localization of lectin-binding sites on the zonae pellucidae and plasma membrane of mammalian egg. J Cell Biol 1975; 66:263–273.
25. Yanagimachi R, Yanagimachi H, Rogers BJ. The use of zona-free animal ova as a test system for the assessment of the fertilizing capacity of human spermatozoa. Biol Reprod 1976; 15:471–476.
26. Yanagimachi R. Zona-free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. Gamete Res 1984; 10:187–232.
27. Santella L, Alikani M, Talansky BE, Cohen J, Dale B. Is the human oocyte plasma membrane polarized? Hum Reprod 1992; 7:999–1003.
28. Dale B, Tosti E, Iaccarino M. Is the plasma membrane of the human oocyte reorganised following fertilisation and early cleavage? Zygote 1995; 3:31–36.
29. Blobel CP, Wolfsberg TG, Turck CW, Myles DG, Primakoff P, White JM. A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. Nature 1992; 356:248–252.
30. Almeida EAC, Huovila A-PJ, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. Mouse egg integrin $\alpha 6\beta 1$ functions as a sperm receptor. Cell 1995; 81:1095–1104.
31. Evans JP, Kopf GS, Schultz RM. Characterization of the binding of recombinant mouse sperm fertilin β subunit to mouse eggs: evidence for adhesive activity via an egg $\beta_1$ integrin-mediated interaction. Dev Biol 1997; 187:79–93.
32. Gupta SK, Alves K, Palladino LO, Mark GE, Hollis GF. Molecular cloning of the human fertilin β subunit. Biochem Biophys Res Commun 1996; 224:318–326.
33. Videaus CM, Von Kap-Herr C, Golden WL, Eddy RL, Shows TB, Herr JC. Human fertilin beta: identification, characterization, and chromosomal mapping of an ADAM gene family member. Mol Reprod Dev 1997; 46:363–369.
34. Bronson RA, Fusi FM, Calzi F, Doldi N, Ferrari A. Evidence that a functional fertilin-like ADAM plays a role in human sperm-oolemmal interactions. Mol Hum Reprod 1999; 5:433–440.
35. Bedford JM. The status and the state of the human epididymis. Hum Reprod 1994; 9:2187–2199.
36. Legare C, Thabet M, Picard S, Sullivan R. Effect of vasectomy on P34H messenger ribonucleic acid expression along the human excurrent duct: a reflection on the function of the human epididymis. Biol Reprod 2001; 64:720–727.
37. Perez Martinez S, Conesa D, Cuasnicú PS. Potential contraceptive use of epididymal proteins: evidence for the participation of specific antibodies against rat epididymal protein DE in male and female fertility inhibition. J Reprod Immunol 1995; 29:31–45.
38. Ellerman DA, Brantua VS, Martinez SP, Cohen DJ, Conesa D, Cuasnicu PS. Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. Biol Reprod 1998; 59:1029–1036.
39. Tesarik J, Thébault A. Fertilization failure after subzonal sperm insertion associated with defective fusional capacity of acrosome-reacted spermatozoa. Fertil Steril 1993; 60:369–371.