

FLB1, a Human Protein of Epididymal Origin That Is Involved in the Sperm-Oocyte Recognition Process

FRANCK BOUÉ, CLOTILDE DUQUENNE, BRUNO LASSALLE, ANICK LEFÈVRE, and CATHERINE FINAZ¹

INSERM Unit 355/CNRS Maturation Gamétique et Fécondation, 92140 Clamart, France

ABSTRACT

CA6 antibody was selected out of a monoclonal antibody library raised against human sperm proteins primarily for its ability to recognize an epididymal antigen and to modify sperm adhesion to zona-free hamster oocytes. In the present study, CA6 was shown to decrease sperm binding to zona-free hamster and human oocytes by 40–92% and 38–48%, respectively. The corresponding protein, which was referred to as FLB1, was found to be secreted by the epididymis and to bind specifically to a human, macaque, and rodent subacrosomal sperm region. Western blotting revealed a molecular mass of 94 kDa in human epididymal extracts and of 100 kDa in human, macaque, mouse, rat, and hamster sperm, suggesting further modifications after its binding to sperm. An equivalent protein was not observed in human liver, ovary, testis, plasma, or epidermis. Two-dimensional electrophoresis showed that FLB1 is formed of two subunits with the same 47-kDa molecular mass and slightly different pI (5.8, 5.9). Microsequencing of the protein revealed a partial homology with human cytokeratins 1 and 10. These results suggest that FLB1 is an epididymis-specific cytokeratin-like protein that is involved in the sperm-oocyte recognition process.

INTRODUCTION

Sperm maturation is a complex process during which the spermatozoon surface undergoes a series of modifications essential in the development of its fertilizing capacity. Current evidence indicates that specific proteins that bind to spermatozoa during the course of epididymal transit play a central role in the mammalian sperm maturation process. The synthesis and secretion of epididymal proteins is now well documented in different species from mice to primates [1–4]. Moreover, some of these proteins have been shown to coat spermatozoa [5–7] and to be involved in sperm binding to the zona pellucida (ZP) and oocyte plasma membrane [8,9]. The acquisition of fertilizing ability of spermatozoa has finally been correlated both to the new organization of their membrane and to their progression from the caput to the cauda epididymidis [10, 11].

One may hypothesize that sperm-oocyte recognition requires complementary receptor molecules at the surface of the two gametes. Interestingly, recent data have pointed to the likely involvement in fertilization of adhesive components such as galactosyltransferase [12], integrin receptors [13–16], fibronectin [17], epithelial cadherin [18], and arg-gly-asp peptide [19], which had already been shown to play a role in cell-to-cell interaction.

The mechanisms by which sperm mature in humans have not been extensively studied for ethical reasons. However, several investigators have reported on the development of the capacity of human spermatozoa to bind to and penetrate the oocyte as they progress along the epididymis [20, 21]. In addition, the production of several androgen-dependant proteins has been reported in epididymis, the caput seg-

ment being the most active region [22]. Moreover, the association with spermatozoa of secretory proteins from the epididymis has been evidenced by means of polyclonal antibodies against surface sperm antigens [23, 24]. At the same time, it was reported that pregnancies could result from the insemination of spermatozoa recovered from caput epididymidis and even from vasa efferentia [25, 26].

It was thus of great importance to specify whether or not human sperm maturation requires specific epididymal secretions. The present report is the first tentative identification of a human epididymal protein involved in the gamete interaction process. In a previous study, we selected mAbs raised against human sperm proteins of epididymal origin [27]. One of the mAbs, designated CA6, interacted with a 100-kDa sperm protein and was able, in a preliminary assay, to decrease sperm attachment to zona-free hamster oocytes. In the present study, CA6 mAb was shown to significantly reduce the binding of human spermatozoa to zona-free hamster and human oocytes without affecting their attachment to zonae pellucidae; the corresponding protein, which was referred to as FLB1, appeared to progressively coat spermatozoa along the length of the epididymis. Its synthesis and secretion by the epididymis was assessed. Subsequently, its partial characterization with regard to molecular mass and organ and species specificity was achieved using purified antibody, and data from microsequencing revealed a substantial homology with human cytokeratins 1 and 10.

MATERIALS AND METHODS

Monoclonal Antibody Production

A BALB/c mouse was immunized with 2 i.p. injections of 5×10^6 washed human spermatozoa administered 3 wk apart. After 3 more weeks, the mouse was given a booster

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¹Correspondence: C. Finaz, INSERM Unit 355, 32 rue des Carnets, 92140 Clamart, France. FAX: 1-46-32-48-90.

injection of 1×10^7 spermatozoa and killed 4 days later. The spleen cells were fused with SP2/O myeloma [28], the hybridomas were selected with 20 mM azaserine, and the supernatants of confluent cultures were screened for anti-sperm antibody production with ELISA as described below. The selected hybridoma was subcloned by limiting dilution. Antibody was grown on a larger scale in ascites form in BALB/c mice primed with pristane (Aldrich Chemical Co., Milwaukee, WI) or by large expansion of cell culture. CA6 antibody was of the IgG1 subclass, and an anti-myelin basic protein mouse IgG1 (Boehringer, Mannheim, Germany) was utilized as a control in fertilization tests and immunoblotting.

ELISA

Culture supernatants were screened for antibody synthesis with 0.45- μ m Multiscreen-HV filtration plates (Pharmacia LKB, Upsala, Sweden) through the use of live washed human spermatozoa as target cells in order to select antibodies directed against presumed surface proteins; 96-well plates were coated with 1×10^6 spermatozoa per well for 1 h and washed three times with PBS. At each stage, great care was taken not to allow the spermatozoa to dry in order to preserve their integrity. Nonspecific binding sites were saturated with 1% BSA in PBS for 1 h. Assays were performed with either 100 μ l of immune serum diluted 1:100 for positive control or 100 μ l of hybridoma supernatant; preimmune serum and SP2/0 culture medium were used as negative controls. After incubation for 1 h at 37°C, the plates were washed three times with 0.05% Tween 20 in PBS (PBS-Tween). Second antibody was a 1:1000 dilution of a goat anti-mouse immunoglobulin IgG (H+L) conjugated to horseradish peroxidase (Zymed Labs., S. San Francisco, CA). After a 1-h incubation at 37°C, the plates were washed four times with PBS-Tween. The bound peroxidase was revealed by the addition of 100 μ l of 1.8 mM o-phenylenediamine in 100 mM phosphate citrate buffer (pH 5) and 0.05% H₂O₂. The reaction was stopped with 2N HCl. After transferral to standard 96-well plates, the absorbance of each well was measured at 492 nm on an automatic plate reader.

Immunohistochemical Tissue Staining

Human epididymides were surgically removed from three subjects in a transplant program; none of them had received any hormonal treatment. Epididymides were dissected in three zones (caput, corpus, and cauda) and immediately frozen in liquid nitrogen. Fragments were then placed in Tissue Tek II embedding medium (Miles; Elkhart, IN) at -30°C. Sections 5 μ m thick were cut with a cryotome, placed on slides, air dried, fixed with methanol at -20°C for 10 min, and kept at -20°C. Antigen localization was performed by avidin-biotin peroxidase staining (Vectastain ABC kit; Vector Labs., Burlingame, CA). Sections were

incubated for 20 min with 1% horse serum to eliminate nonspecific binding and then with either CA6 cell supernatant or a nonsecreting supernatant for 1 h at room temperature. After three washings with PBS, the sections were treated for 30 min with horse biotinylated anti-mouse IgG (Vector Labs.), washed three times with PBS, and then incubated for 30 min with Vectastain ABC reagent. After three washings in PBS, the reaction was revealed with 118 mM amino ethyl carbazol AEC (Sigma Chemical Co., St. Louis, MO) as peroxidase substrate plus 0.02% H₂O₂. Sections were rinsed, stained with Harris hematoxylin (Ortho Diagnostic Systems, Loudwater, UK), and mounted in glycerol solution (Dako, Carpinteria, CA).

Sperm Immunostaining

Immunofluorescence staining was carried out on live sperm. Ejaculated sperm from men and from the macaque, as well as spermatozoa aspirated from cauda epididymidis of the mouse, rat, hamster, and rabbit, were washed with PBS, diluted to 1×10^{-5} /ml with PBS containing 1% BSA, and incubated for 1 h at room temperature. After centrifugation, pellets were diluted with either CA6 supernatant or CA6 IgG1 and incubated for 1 h at room temperature. The cells were washed twice with PBS-Tween, and a 1:40 dilution of goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody was added for 1 h. After three washings, a drop of the sperm suspension was smeared on a glass slide, air dried, mounted with PBS-glycerol (1:1, v/v), and observed by fluorescence microscopy.

Indirect avidin-biotin peroxidase staining was performed with washed human spermatozoa that were first smeared on slides and fixed for 10 min with methanol at -20°C. Staining was then performed as described above for epididymal sections.

Purification of CA6 Antibody

CA6 mAb isotype was determined on culture supernatants through the use of an immunoassay isotyping kit (Boehringer-Mannheim, Indianapolis, IN) and was shown to be of the IgG1 subclass. After unfruitful tests with protein A, the antibody was purified by means of protein G Sepharose 4 Fast Flow (Pharmacia). The protein G Sepharose column was equilibrated with 20 mM phosphate buffer (pH 7.0) and charged with ascites fluid diluted 1:2 with phosphate buffer or culture supernatant adjusted at pH 7.0 at a flow rate of 0.5 ml/min. Protein G Sepharose was washed with phosphate buffer, and the immunoglobulin was eluted with citrate buffer (pH 6), neutralized with Tris-HCl (pH 9), and exhaustively dialyzed against PBS overnight. The purity of the preparation was checked by SDS-PAGE.

Evaluation of Sperm Fertilizing Ability

Effect on acrosomal reaction. The effect of CA6 mAb on the acrosome reaction was tested through the use of

fluorescinated *Pisum sativum* according to the method described by Cross et al. [29] after a 30-min incubation with either CA6 supernatant or a negative hybridoma supernatant. The acrosomal status of treated spermatozoa was evaluated 3 or 18 h later. For each experiment, spontaneous and induced acrosome reaction was evaluated. The acrosome reaction was induced with 1 μ M ionophore A23187 (Sigma) added to the sperm suspension during the last 30 min of incubation. Supravital staining was performed with Hoechst 33258 (Sigma) in parallel, and 200 viable spermatozoa were counted each time.

ZP binding assay. ZP binding tests were done according to established protocols [30, 31]. Briefly, salt-stored human oocytes that failed to fertilize in an in vitro fertilization (IVF) program were washed twice with PBS-BSA solution and stored in B2 medium. Human ejaculated sperm were washed with PBS-BSA and incubated at 37°C for 1 h with either CA6 medium or a negative supernatant. Control and treated washed sperm were stained with either FITC or tetramethyl rhodamine isothiocyanate (TRITC), and a mixture of equal numbers of each sperm sample (0.2×10^5 in B2 medium) was added to about 20 oocytes. Sperm and oocytes were incubated at 37°C for 1 h in 5% CO₂. The oocytes were washed twice with B2 medium to eliminate non-adherent sperm; FITC- and TRITC-labeled sperm were counted by using a light fluorescence microscope with excitation at 450 to 490 and 546 nm, respectively.

Fertilization tests. Sperm fertilizing ability was evaluated by using both a homo- and a hetero-specific IVF procedure. Virgin golden hamsters were induced to superovulate by an i.p. injection of 40 IU eCG (Intervet, Angers, France) followed by 40 IU hCG (Intervet) 72 h later. Animals were killed 15–17 h after hCG injection. Cumulus cells were dispersed with 0.1% hyaluronidase (bovine testis, type I; Sigma), and ZP were removed by treatment with 0.05% trypsin (type I; Sigma) solution in PBS plus 0.4% (w/v) BSA (Sigma) (PBS-BSA) according to Lassalle and Testart [32]. Zona-free oocytes were thoroughly rinsed in fresh PBS-BSA solution after each enzymatic treatment and finally placed in B2 medium (Api System, La Balme les Grottes, France) at 37°C under 5% CO₂.

Human oocytes that did not fertilize in a human IVF program because of sperm failure were utilized. The experiments were approved by the National Ethical Committee. After selection of mature unfertilized oocytes, digestion of ZP was performed with 0.1% pronase (Sigma) according to Lassalle and Testart [33].

Frozen ejaculates from six different donors were used to limit misinterpretations due to individual sperm parameters. Sperm thawing was done at room temperature, and content of straws was diluted with 5 ml PBS-BSA. Sperm samples were centrifuged at $600 \times g$ for 7 min; the pellets were resuspended in 1 ml of either CA6 supernatant or a negative hybridoma supernatant, of either the diluted CA6 IgG1 (1 mg/ml) or an irrelevant IgG1 (1 mg/ml). Sperm

suspensions were incubated for 60 min at 37°C under 5% CO₂; they were then washed with PBS-BSA and resuspended in B2 medium. Finally, 500 μ l of sperm suspension containing $2-3 \times 10^4$ motile spermatozoa/ml was placed in petri dishes before the addition of zona-free oocytes. Culture was performed in a CO₂ incubator (37°C, 5% CO₂ in air). For each experiment, the same numbers of treated and untreated spermatozoa exhibiting forward progressive motility were incubated with oocytes.

Sperm binding and oocyte penetration were examined 2–4 h after insemination. Zona-free oocytes were placed between the slide and coverslip for observation under a phase-contrast microscope (1000 \times). The number of spermatozoa bound per egg was estimated, and ova were recorded as penetrated when at least one swollen sperm nucleus or male pronucleus was discernible in the egg ooplasm. Adherent sperm counts were obtained from two independent observations, and variations were always below 5%.

Electrophoresis and Immunoblotting

SDS-PAGE was performed under reducing conditions according to Laemmli [34]. Human, macaque, and rabbit ejaculated sperm and mouse, rat, and hamster sperm (removed from cauda epididymidis by retrograde perfusion of the vas deferens with PBS) were washed in PBS and solubilized into the sample buffer (0.25% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.0625 M Tris-HCl, pH 6.8). Proteins from human organs were extracted by pulverizing thawed tissues in 50 mM Hepes buffer, 500 mM NaCl, 1% Nonidet P-40 (NP-40), and 1 mM PMSF (Sigma). After centrifugation, supernatants were evaluated for protein concentration and the selected amounts were mixed with 2 vol sample buffer. Samples and molecular mass markers (Sigma) previously heated at 100°C for 5 min were run on 8–10% acrylamide gels.

Two-dimensional gel electrophoresis (2D electrophoresis) was based on the procedure of O'Farrell et al. [35]. Samples were solubilized in either a saturating concentration of urea or 9.5 M urea, 2% NP-40, 2% pH 3.5–9.5 ampholines (Pharmacia), and 5% 2-mercaptoethanol in H₂O. Isoelectric focusing in the first dimension was performed with either a saturating concentration of urea or 9.5 M urea in tube gels containing pH 3.5–9.5 ampholines in a 30% acrylamide solution. The second dimension electrophoresis was run in 10% acrylamide gels at 12.5°C.

After migration, the protein samples were transferred onto nitrocellulose membranes according to the method of Towbin et al. [36]. The membranes were saturated with 3% BSA or nonfat milk in PBS for 2 h at room temperature and incubated overnight at 4°C with either CA6 supernatant, purified IgG1 diluted in PBS, or negative hybridoma supernatant. After washing with PBS-Tween 0.05%, the membranes were incubated for 1 h at room temperature with a 1:1000 dilution of peroxidase-conjugate goat anti-mouse IgG (Sigma). Blots were washed three times with PBS-Tween,

and bound peroxidase was detected with 1.5 mM 3,3'-diaminobenzidine in 0.05 M phosphate buffer (pH 7.4) in the presence of 0.03% H₂O₂. If necessary, 1% goat serum was added in the second antibody solution in order to reduce the background.

Immunoprecipitation

Human sperm proteins were extracted by incubation of 1×10^8 washed spermatozoa in 2 ml of lysis buffer (0.14 M NaCl, 0.01 M Tris [pH 8.0] containing 5 µg/10 ml aprotinin [Sigma], 1% NP-40, and 20% glycerol) at 4°C on a rotator for 1 h. The lysate was centrifuged at $100\,000 \times g$ at 4°C for 45 min, and the supernatant was submitted to immunoprecipitation overnight with 5 µl of CA6 ascites fluid at 4°C on a rotator. Immune complexes were collected by absorption to protein G Sepharose beads previously washed with lysis buffer at 4°C for 4 h. Beads were washed five times with washing buffer (50 mM Tris [pH 8.5], 1% NP-40, 5 µg/10 ml aprotinin) and collected. The washed pellet was resuspended in electrophoresis sample buffer, heated at 56°C for 15 min and then at 100°C for 5 min, and thereafter centrifuged to eliminate the Sepharose beads. The supernatant was submitted to SDS-PAGE and silver staining (kit AG-25; Sigma).

FLB1 Secretion

Epididymides were excised from mature golden hamsters and defatted. Caput and corpus regions of four epididymides were minced in 1-mm-sized fragments and washed five times with PBS. They were incubated for 30 min in leucine-free MEM medium containing L-glutamine (Gibco BRL, Gaithersburg, MD) plus 10 µg/ml insulin, 5 µg/ml transferrin, 1 µM testosterone, and 1 µM retinoic acid. They were then incubated in 1 ml of the same medium supplemented with 300 mCi of L-4,5-³H-leucine (specific activity 74 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 16 h at 35°C in 5% CO₂ in air. At the end of the incubation the medium was saved and centrifuged at $200 \times g$ for 10 min at 4°C to eliminate any organ fragments. The supernatant was immediately complemented with 5 µl aprotinin, 0.5% NP-40, and 10% glycerol and incubated on a rotator with 15 µl CA6 ascites, first at 20°C for 30 min and then at 0°C for 90 min. Sixty microliters of protein G Sepharose beads was then added, and incubation was performed at 20°C for 30 min and then at 4°C for 2 h. After four washings with PBS containing 50 mM Tris-HCl (pH 8.5), 5 µl aprotinin, and 0.5% NP-40 followed by one washing with 50 mM Tris-HCl (pH 8.5) and 0.5 M NaCl, the antigen-antibody complex was eluted from the beads with 15 µl sample buffer by heating at 56°C for 15 min and then at 100°C for 5 min. After centrifugation at $5600 \times g$ for 5 min, the supernatant was subjected to SDS-PAGE on a 10% acrylamide gel. To remove the free labeled amino acids, the gel was then incubated three times for 30 min with 40% methanol and

10% acetic acid and treated for fluorography with Intensify A and B solutions according to the manufacturer's instructions (NEN Research Products, Boston, MA). The gel was finally exposed to Hyperfilm-MP films (Amersham) at -80°C for 4-6 wk.

Microsequencing

As immunoprecipitation displayed a poor efficiency, FLB1 protein was isolated by two successive electrophoresis procedures. Briefly, protein extracts from ejaculated sperm from proven fertile men (about 4×10^7 spermatozoa) were separated by SDS-PAGE (8% acrylamide gels). Gel fragments corresponding to the 100-kDa region were excised, squashed between two slides, dried by vacuum speed, pooled and solubilized in sample buffer, and submitted to a second SDS-PAGE electrophoresis. The bands obtained were excised again and partially dehydrated in a Speed Vac (Savant, Farmingdale, NY). For each gel, as a control, a lane was transferred onto a nitrocellulose membrane and blotted with CA6 supernatant as explained above.

Microsequencing was performed by J. d'Alayer (Institut Pasteur, Paris). Gel pieces were rehydrated in 0.1 M Tris-HCl (pH 8.8) and 0.3% SDS and digested with endoproteinase Lys-C (Boehringer) at a final concentration of 2 mg/ml for 18 h at 30°C. The supernatant was recovered and the pellet rinsed with 60% acetonitrile. The supernatant and the acetonitrile extract were pooled and submitted to Speed Vac in order to remove the acetonitrile. Peptides were HPLC isolated using a DEAE C:18 reverse-phase column eluted with a 0-50% acetonitrile, 0.1% trifluoroacetic acid gradient. Sequencing was performed on an Applied Biosystems (Foster City, CA) 470 gas phase sequencer. Spectra were recorded on an Applied Biosystems 1000 S detector.

Statistical Methods

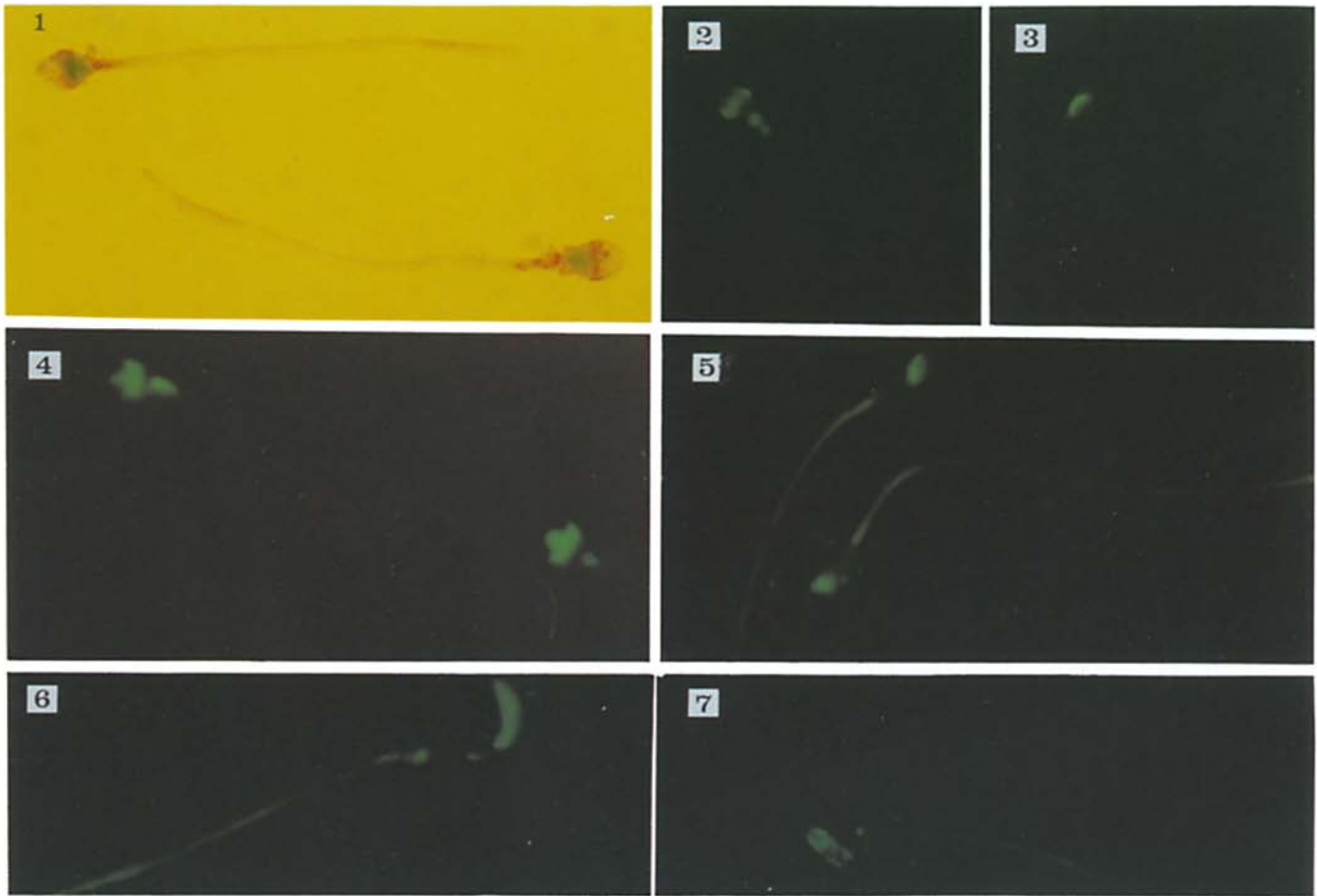
Means of bound sperm were expressed with standard error of the mean (\pm SEM) and were compared through use of Student's *t*-test. Comparisons between proportions were performed with the chi-square test.

RESULTS

The ELISA selected hybridomas that secreted mAbs directed against presumed sperm surface proteins [27]. Among the 17 mAbs of particular interest that recognized proteins of epididymal origin, CA6 mAb was chosen on the basis of

FIG. 1. Immunohistochemical localization of FLB1 in sperm cells (A) and in human testis and epididymis (B) incubated with purified IgG1. A1: Immunoperoxidase staining of human ejaculated spermatozoa. Immunofluorescence staining was performed on human (A2), macaque (A3), rabbit (A4), hamster (A5), rat (A6), and mouse (A7) spermatozoa. B: Testis (1), vasa efferentia (3) and caput (4), corpus (5), and cauda (6) epididymis sections probed with CA6 mAb. B2: Caput epididymis section reacted with a negative hybridoma supernatant. E: epididymal epithelium. L: lumen. 1200 \times (A); 1000 \times (B1, 2, 4, 5); 250 \times (B3, 6).

A



B

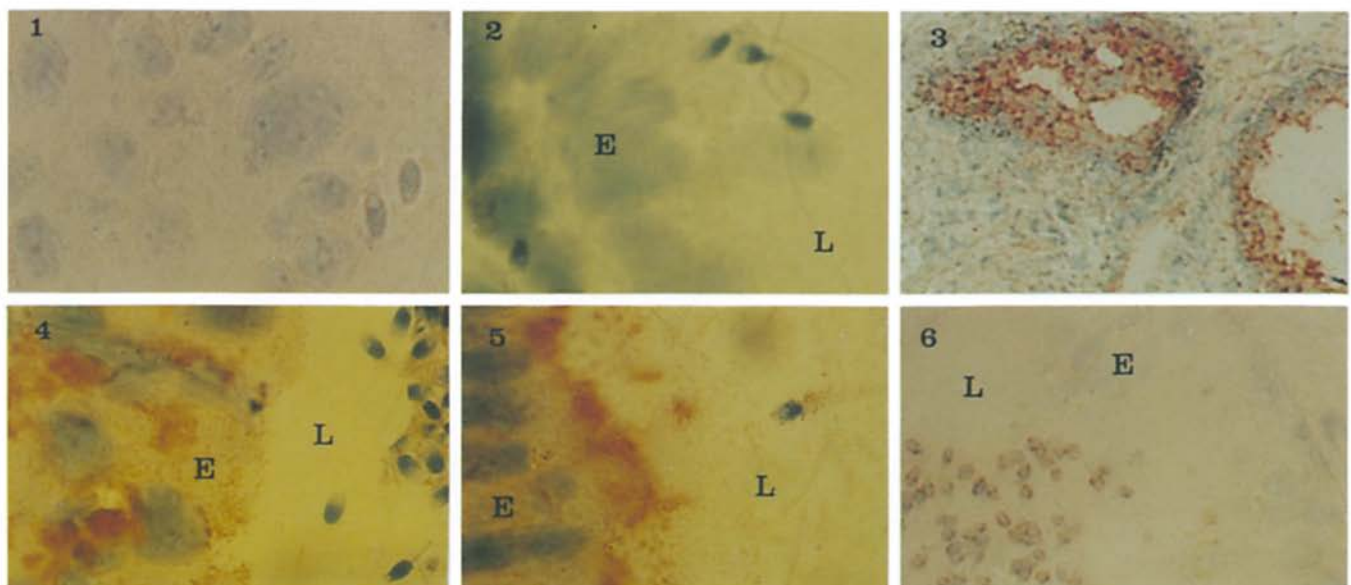


TABLE 3. Effect of pretreatment with CA6 supernatant or CA6 IgG1 on human sperm attachment to human zonae pellucidae and human zona-free oocytes.

	Control	Treated with CA6 supernatant	Treated with CA6 IgG1 (1 mg/ml)
Zonae pellucidae	99.8 ± 6.7 (11)	95.7 ± 5.8 (11) NS	
	20.0 ± 2.9 (15)	22.3 ± 2.4 (15) NS	
	8.5 ± 2.4 (14)	7.8 ± 2.3 (14) NS	
Zona free oocytes	96.8 ± 1.7 (3)	60.0 ± 3.3 (5) **	
	52.6 ± 3.6 (3)	27.6 ± 3.8 (6) **	
	60.7 ± 2.9 (3)		31.3 ± 2.7 (3) *

** $p < 0.001$; * $p < 0.01$; ±, SEM; NS, not significant; (), number of examined oocytes.

and 48.44%, respectively. The oocytes were considered alive, as 32–65% of them were fertilized in control conditions.

In all these experiments, neither CA6 supernatant nor CA6 IgG1 treatment modified the percentages of spermatozoa expressing a forward progressive motility (data not shown).

Biochemical Characterization of Protein FLB1

Proteins from human ejaculated spermatozoa separated by reducing SDS-PAGE and probed either with CA6 super-

natant or with CA6 IgG1 showed a single band at 100 kDa corresponding to protein FLB1, whereas no band was observed in controls (Fig. 2). When probed to the same amount of sperm proteins submitted to SDS-PAGE in nonreducing conditions, CA6 mAb revealed a much weaker band (data not shown). Silver staining of the immunoprecipitation product of human sperm using protein G Sepharose displayed the same protein band of 100 kDa.

Western blots of NP-40 extracts from human epididymis revealed a sharp band of 94 kDa (Fig. 3). In contrast, immunoblots realized with human plasma, testis, ovary, and male and female liver extracts did not reveal any band of either 94 or 100 kDa (Fig. 3). However, CA6 mAb showed several bands between 50 and 75 kDa in extracts from human epidermis, which is known to strongly express cyto-keratins 1 and 10.

As can be seen in Figure 4, a similar protein was evidenced in Western blots of human, macaque, rabbit, hamster, and mouse sperm by CA6 supernatant.

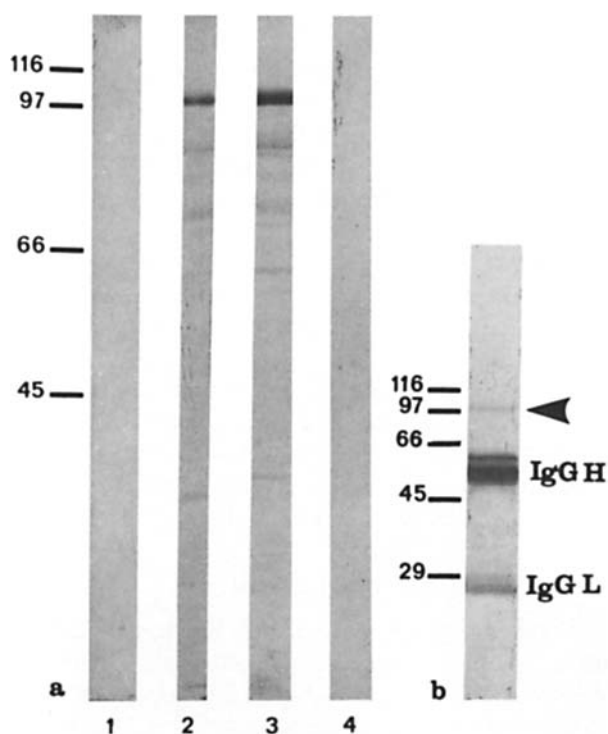


FIG. 2. Identification of FLB1 protein. a) Immunoblotting of human sperm proteins separated by SDS-PAGE on a 10% acrylamide gel. Lane 1 was control blotted with a negative hybridoma supernatant; lanes 2 and 3 were blotted with CA6 supernatant and purified IgG1 (1 mg/ml), respectively; lane 4 was control blotted with an irrelevant mouse IgG1 (1 mg/ml). The amount of protein applied was 25 µg/lane. Lane b is the immunoprecipitation pattern obtained by separation of the immune complex by 10% SDS-PAGE and silver staining. The arrow indicates the position of immunoprecipitated FLB1. IgG H, IgG heavy chain; IgG L, IgG low chain. Standards of known molecular masses are indicated on the left.

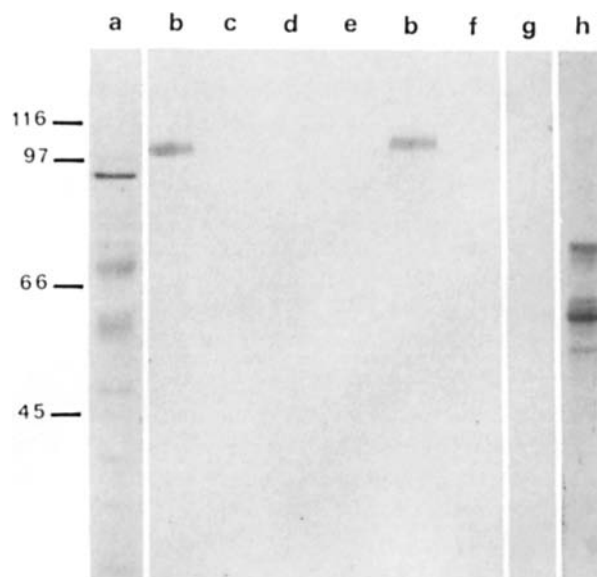


FIG. 3. Immunodetection of FLB1 in human organ protein extracts separated by 10% SDS-PAGE and incubated with CA6 supernatant. a) Caput epididymidis; (b) ejaculated sperm; (c) male liver; (d) female liver; (e) ovary; (f) testis; (g) plasma; (h) epidermis. The amount of protein applied was 25 µg/lane. The molecular masses of standards are indicated on the left.

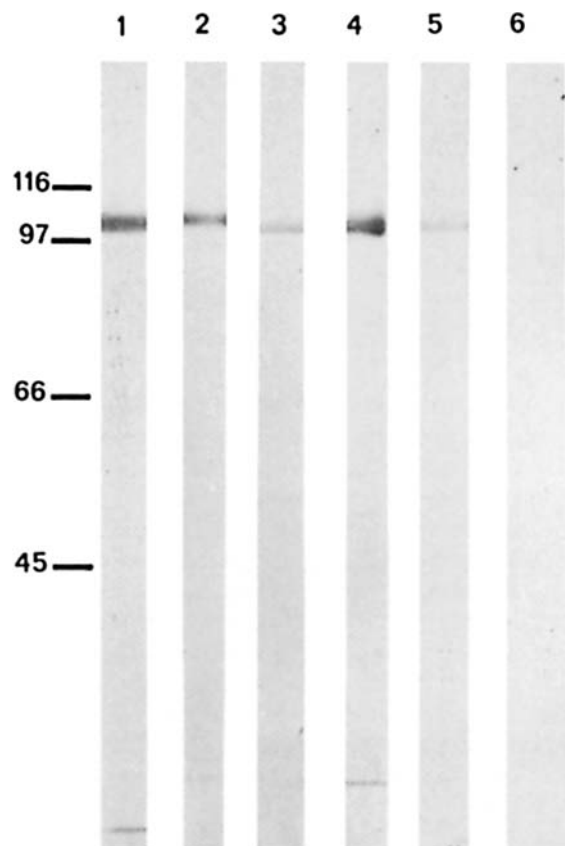


FIG. 4. Comparison of immunoblots of FLB1 sperm protein from men and animals, incubated with CA6 supernatant. Proteins were extracted from ejaculated sperm for the human (1), macaque (2), and rabbit (3) and from aspirated sperm from cauda epididymidis of the hamster (4) and mouse (5). Lane 6 was incubated with a negative hybridoma supernatant. The amount of protein applied was 25 μ g/lane. Proteins were separated on 10% SDS-PAGE. The molecular masses of standards are indicated on the left.

Western blotting of human ejaculated sperm proteins treated with 9.5 M urea and separated by 2D electrophoresis is shown in Figure 5a. The CA6 supernatant revealed one spot of 100 kDa with a pI of 5.3 and a doublet of 47 kDa with slightly different pIs (5.8 and 5.9). Figure 5b shows the results obtained with sperm protein extracts treated with a saturating concentration of urea: only the doublet of 47 kDa was observed.

FLB1 Secretion

When proteins secreted into the medium by hamster epididymis segments incubated with 3 H-leucine for 16 h were immunoprecipitated with CA6 ascites, a single band at 47 kDa was revealed after SDS-PAGE (Fig. 6). The mean leucine incorporation efficiency in the incubation medium from three independent experiments was 0.65%.

Amino Acid Sequencing

The results of isolation of FLB1 are indicated in Figure 7. After the second round of SDS-PAGE, the isolated protein

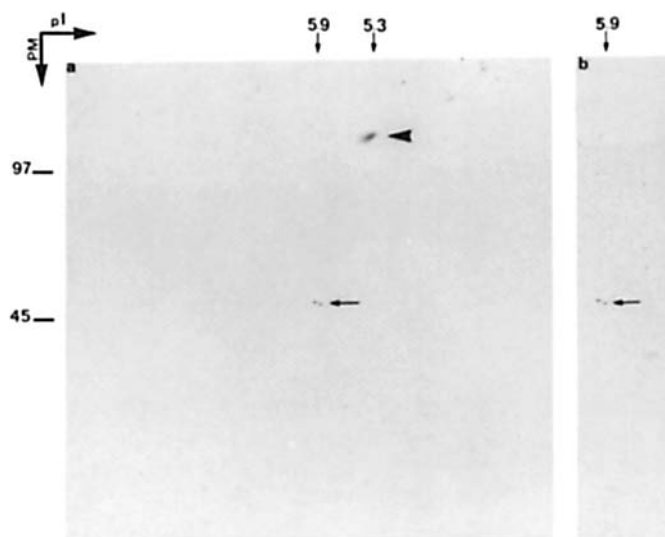


FIG. 5. Immunoblot of FLB1 from human sperm after 2D electrophoresis and incubation with CA6 supernatant. Samples were treated with 9.5 M urea (a) or with a saturating urea concentration (b). The isoelectric points of FLB1 spots are indicated at the top of the figure, and molecular masses of standards are indicated on the left.

displayed a positive reaction when blotted with CA6 supernatant (lane c).

The HPLC profile of FLB1 obtained after proteolytic digestion by the endoproteinase Lys C revealed 43 peaks. Four were analyzed, one of them consisting of three unresolved peaks. Results are summarized in Figure 8. The amino acid sequence analysis revealed a total homology of peptide 14a with the human cytokeratin 1 described by Johnson et al. [37], while peptide 14b was only 87% homologous. Peptides 14a and 14b were 77.8% and 100% similar in sequence, respectively, to the cytokeratin 1 described by Steinert et al. [38]. Peptide 14c revealed no homology with any known protein. Peptide 25 was homologous to human cytokeratin 10 [39, 40]. Peptide 17 was 100% and 40% similar to the cytokeratins 10 described by Rieger and Franke [39] and Zhou et al. [40], respectively. Peptide 27 was only 76.9% homologous to both cytokeratins 10.

DISCUSSION

The objective of the present study was to identify human proteins involved in sperm maturation and gamete interaction processes. CA6 antibody was selected out of a library of mAbs directed against human ejaculated sperm for its ability to react with a sperm antigen synthesized by the epididymis and to modify sperm binding to hamster oocytes. This protein was referred to as FLB1. The present paper reports further results on its physiological function and biochemical nature.

FLB1 has been localized to the equatorial region of human, macaque, and rabbit ejaculated sperm and of mouse, rat, and hamster spermatozoa aspirated from cauda epididymis.

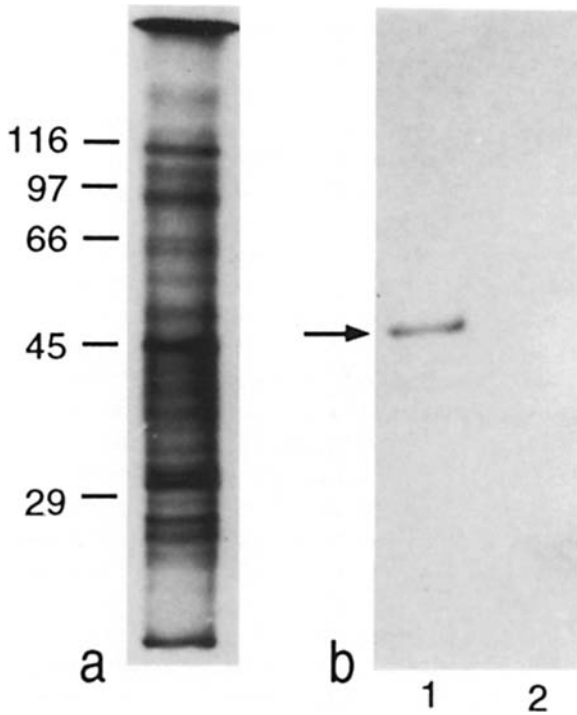


FIG. 6. Autoradiography of radiolabeled proteins from epididymis fragments after incubation for 16 h with ³H-leucine. a) Proteins extracted from the epididymis fragments; (b1) protein immunoprecipitated from the incubation medium of epididymis fragments. Labeled proteins (8000 cpm) were added to lane a; 16 000 cpm of labeled proteins from incubation medium was submitted to immunoprecipitation, and the total product of protein A Sepharose bead elution was applied to lane b1. Lane b2 was control, with incubation medium free of epididymis fragments, ascites, and protein A Sepharose beads. Samples were run on 10% SDS-PAGE. The autoradiogram was exposed for 40 days; it is representative of three distinct experiments. The positions of standards of known molecular masses are indicated on the left.

dymidis. Intact and permeabilized human sperm displayed the same pattern. In fact, CA6 supernatant and purified CA6 IgG1 were both shown to significantly decrease human sperm binding to zona-free hamster and human oocytes. As observed in numerous studies, individual sperm parameters probably accounted for variable results in the observed percentages of bound sperm. However, the rates of inhibition of sperm-oocyte binding observed after pretreatment with either CA6 supernatant or purified CA6 IgG1 were comparable for all sperm samples. The inhibition of human sperm-oocyte binding was lower probably owing to the use of 2-day-old oocytes that failed to fertilize in an IVF program. Results should be improved by the use of larger-sized classes of oocytes. Furthermore, the inhibitory effect of CA6 mAb was confirmed by an evaluation conducted by D. Anderson for the sperm antigen database of the Third WHO Sperm Antigen Workshop [41]; this evaluation showed that CA6 reduced the hamster egg penetration rate by human spermatozoa by 78%. That we could not observe a complete inhibition of sperm binding to oocytes is to be considered together with the difficulty of detecting the protein

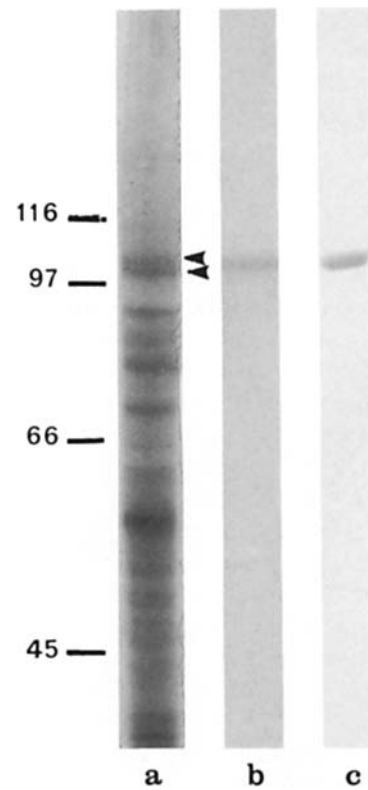


FIG. 7. Isolation of FLB1 protein by two rounds of 8% SDS-PAGE. Coomassie blue-stained gel of total sperm protein extract (lane a) and of the excised 100-kDa band (lane b) and immunoblotting of the excised band incubated with CA6 supernatant (lane c). Arrows indicate the points of excision of the 100-kDa protein band at the end of the first migration. The positions of standards of known molecular masses are indicated on the left.

peptide 14a	D	V	D	G	A	Y	M	T	K				
cytokeratin 1 (37)	D	V	D	G	A	Y	M	T	K				
cytokeratin 1 (38)	D	V	D	A	A	Y	M	N	K				
peptide 14b	A	E	A	E	S	L	Y	K					
cytokeratin 1 (37)	A	E	A	E	S	L	Y	Q					
cytokeratin 1 (38)	A	E	A	E	S	L	Y	Q					
peptide 14c	L	P	Y	V	A	K							
peptide 17	E	W	Y	E	K								
cytokeratin 10 (39)	E	W	Y	E	K								
cytokeratin 10 (40)	E	R	Y	D	G								
peptide 25	D	A	E	A	W	F	N	E	K				
cytokeratin 10 (39)	D	A	E	A	W	F	N	E	K				
cytokeratin 10 (40)	D	A	E	A	W	F	N	E	K				
peptide 27	N	Y	S	T	Y	Y	N	T	I	D	D	L	K
cytokeratin 10 (39)	D	Y	S	K	Y	Y	K	T	I	D	D	L	K
cytokeratin 10 (40)	D	Y	S	K	Y	Y	K	T	I	D	D	L	K

FIG. 8. Amino acid sequences of the fragments of FLB1 proteolytic digestion and comparison with human cytokeratins 1 and 10. Numbers in brackets are references.

on Western blots after SDS-PAGE in nonreducing conditions. It appears that the configuration of the mature protein makes the access of its reactive epitope to the mAb more difficult. Nevertheless, the observations that CA6 mAb modified neither the percentage of acrosome-reacted spermatozoa nor sperm binding to human zonae pellucidae argue for its specific physiological effect.

The presence of FLB1 was shown in the epithelium of vasa efferentia and caput and corpus epididymidis, the vasa efferentia and caput regions being the most strongly stained fragments. The epithelium of the cauda segment was not stained, and FLB1 was specifically localized on spermatozoa. Such a decreasing gradient in protein production in successive segments of the epididymis has been described in mice [1, 2], rats [3], and humans also [22].

The hypothesis that FLB1 is primarily synthesized by the epididymal epithelium, then secreted into the luminal space before being taken up by sperm, was supported by the presence of an immunoprecipitated protein in the incubation medium of hamster caput and corpus epididymidis. FLB1 can be defined as a sperm-coating protein of epididymal origin. The secretion of proteins by the human epididymis and their association with spermatozoa have already been ascertained by two groups using polyclonal antibodies that recognized the same antigens on the sperm surface and in epididymis and fluid in one case and in the epididymis incubation medium in the other [23, 24].

Western blotting evidenced a 100-kDa protein when applied to either crude protein extracts or the immunoprecipitation product of human ejaculated sperm proteins. Western blotting of 2D electrophoresis in the presence of a saturating urea concentration showed that the 100-kDa protein was resolved in two subunits of 47 kDa. This result was confirmed by the presence of an immunoprecipitated protein band of 47 kDa secreted in the incubation medium of hamster caput and corpus epididymidis fragments. Therefore, it seems that FLB1 is secreted in its monomeric form. Additional studies are needed to resolve this issue. On the other hand, CA6 mAb reacted with a 94-kDa protein in human epididymis extracts. This apparent discrepancy suggests that the protein is submitted to further modifications at the sperm surface. Since preliminary experiments have shown that FLB1 extracted from ejaculated sperm harbors several glycosylation sites, it would be of interest to elucidate the effect of specific deglycosylation on its apparent molecular mass. A differential glycosylation of rat β -D-galactosidase in epididymal fluid from different segments has been recently described [42].

The similarity between human, macaque, and rodent sperm proteins detected by CA6 mAb shows that a comparable component is operating in all these species. In return, results from Western blotting of different human organ extracts favor the organ specificity of FLB1, which could be evidenced neither as a dimer nor as a monomer. On the other hand, CA6 mAb revealed several bands ranging

from 50 to 70 kDa in extracts of epidermal epithelium, which has been shown to express cytokeratins 1 and 10 [43]. Nevertheless, epidermis extract did not display any band corresponding to FLB1. Taken together, this observation and further results from immunosequencing suggest that FLB1 and several cytokeratins share a common epitope that is recognized by CA6 mAb.

The molecular masses of the five proteins identified by Tezon et al. [22] from culture medium of epididymal tubules ranged from 13.9 to 69 kDa, with no equivalent around 50 kDa. In turn, in another study, a polyclonal antibody directed against sperm surface proteins was shown to react with a 48-kDa protein from human epididymis incubation medium [24]. The short incubation time may account for the weak staining of this protein, and the protein may be related to FLB1. Nevertheless, the involvement of these proteins in the gamete recognition process was not assessed in either of these two studies. On the other hand, surface antigens of human sperm recognized by antisera from immune infertile and vasectomized patients have been described [44]. Clinical data suggested that they interfere with sperm function, but none of them had molecular mass comparable to the dimeric form of FLB1.

In point of fact, at present, only a few polyclonal or monoclonal antibodies against human sperm proteins have been shown to impair fertilization [45–53]. When tested, the origin of the corresponding antigen was shown to be the testes except for the antigen described by Batova et al. [48]. Two of these antibodies have been shown to act at the ZP level [51–52], while four others inhibited sperm-oocyte binding and/or egg penetration [46–53]. Their mechanism of action in the gamete recognition process has not been elucidated. However, cloning of corresponding genes is in progress or, in the case of several of them, has already been achieved [54–56]. Furthermore, a homologue of the guinea pig protein PH-20, whose corresponding antibody inhibits sperm adhesion to the ZP, has been revealed in humans [57]. To date, only one human protein of epididymal origin that is clearly involved in fertilization has been identified [48]. Although it was not tissue specific, its corresponding mAb exhibited sperm-immobilizing activity and inhibited sperm binding to human ZP. Genes coding for several epididymis-specific proteins have been isolated more recently [58–61]. Recent data suggest that one of these proteins is involved in human sperm binding to ZP [62].

The partial homology of FLB1 with human cytokeratins 1 and 10 obtained from microsequencing was puzzling. If we take into account all the analyzed peptides, only a partial homology with both cytokeratins was observed, ruling out the possibility of contamination. More than 30 cytokeratins have been identified in humans. They are all built on the same plan, which includes a central α -helical domain of highly conserved length and secondary structure-forming coils flanked by two end domains, highly variable in amino acid sequence, that are thought to confer on the cy-

cytokeratins their functional specificity. They resolve into acidic type I and neutral-basic type II groups depending on the charge of their α -helical domain, and they associate in pairs to form one of the five kinds of intermediate filaments. A given epithelium is characterized by the specific pattern of its keratin doublets depending on its type and stage of development and differentiation. Cytokeratins are resistant to classical buffers, and only solubilization in high concentrations of urea, guanidium hydrochloride, or denaturing detergents results in the dissociation of denatured monomers [63]. Cytokeratins participate in the epithelium architecture and are supposed to be involved in cell-cell interaction through the binding of their end domains to desmosomes. Cytokeratins 1 (67 kDa, type II) and 10 (57 kDa, type I) are selectively coexpressed by suprabasal epidermal cells when committed to terminal differentiation [43, 38]; and only cytokeratins 5, 7, 8, 18, and 19 have been detected in epididymal epithelium by immunocytochemistry [64]. The partially homologous sequences that we have analyzed correspond to gene regions of the core domains of keratins 1 and 10 with the exception of peptide 17, whose corresponding sequence is located just before the core region. The observation that FLB1 is formed of two monomers that can be separated only in the presence of denaturing detergent or a high urea concentration is concordant with the solubilization characteristics of cytokeratins. What seems original is the association and the secretion of two subunits of the same molecular mass and very similar isoelectric points. To date, none of the described cytokeratins has been shown to be secreted and all the described pairs of keratins are made of one acid (average pI 5.4) and one neutral-basic (average pI 7.4) protein.

In conclusion, several convergent observations, i.e., microsequencing results, the dissociation of FLB1 into two dimers with high urea concentrations, and the fact that CA6 mAb cross-reacts with human epidermis proteins within the molecular mass range of cytokeratins, suggest that FLB1 is a protein related to the cytokeratin family. Even though FLB1 shares part of its amino acid sequence with cytokeratins 1 and 10, it is likely to be a new protein that harbors specific characteristics. Since the role of cytokeratins in the cell architecture of epithelia is not yet elucidated, it is obvious that additional studies are needed for an understanding of the functional role of FLB1 in fertilization. It is noteworthy that the expression of an additional type of intermediate filament has recently been shown to correlate with invasive cell ability during the process of tumor metastasis [65].

To our knowledge, this is the first report of an epididymis-specific human protein that shares common antigenic determinants with an analogous sperm protein from several mammals, including rodents and macaques, and contributes to the association of the spermatozoon with the oocyte plasma membrane. These findings strongly suggest that epididymal maturation also occurs in humans, as has been indicated by previous reports [20, 21, 66]. The iden-

tification and characterization of human epididymal antigens involved in fertilization should improve our understanding of the mechanisms of sperm maturation and gamete recognition in mammals. They should provide an opportunity to test precisely the hypothesis that some infertile men may suffer also from a deficiency of epididymal protein synthesis.

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