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Fertilization antigen-1: cDNA cloning, testis-specific expression, and immunocontraceptive effects

(sperm antigens/anti-sperm antibodies/fertility/contraceptive vaccine)

XIAOLONG ZHU AND RAJESH K. NAZ*

Division of Research, Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo, OH 43699

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ABSTRACT **cDNA encoding for a sperm antigen, designated fertilization antigen (FA-1), was cloned and sequenced from murine testis cDNA-λgt11 expression library using FA-1 mAb. Computer-generated translation analysis of 649-bp cDNA yielded an ORF of 164 amino acids with the first ATG Met start codon at nucleotide 81 and the stop codon TAA at nucleotide 577 and a polyadenylylation tail following the stop codon. The translated protein has a calculated molecular mass of 18.2 kDa and a potential N-linked glycosylation site at amino acids 158–160, besides at least two O-linked glycosylation sites. The hydropathy plot generated from the deduced amino acid sequence indicated it to be a membrane-anchored peptide. Extensive computer search in the GenBank, National Biomedical Research Foundation, and Swiss sequence banks did not identify any known nucleotide/amino acid sequence having homology with FA-1 cDNA or deduced amino acids, indicating it to be a novel protein. Northern blot analysis and reverse transcription–PCR indicated testis-specific expression of FA-1 antigen. The FA-1 cDNA was subcloned into pGEX-2T vector and expressed in glutathione *S*-transferase gene fusion system to obtain the recombinant protein. The recombinant protein specifically reacted with ZP3 of oocyte zona pellucida and its affinity-purified antibodies completely blocked sperm–zona pellucida interaction in mice. These findings suggest that the sperm-specific recombinant FA-1 antigen is an attractive candidate for the development of a contraceptive vaccine.**

Currently, there has been considerable interest in the development of a safe and effective contraceptive vaccine for population control. A contraceptive vaccine based upon spermatozoa is especially encouraging because of the following two reasons. Active immunization of male or female animals of various mammalian species with isologous or autologous spermatozoa results in infertility (1–3). And, the sperm have both autoantigenic and isoantigenic potentials in humans, and the presence of antisperm antibodies is associated with infertility (4, 5). Although whole sperm can produce an antibody response that is capable of inducing infertility, they *per se* cannot be used for the development of a contraceptive vaccine. Sperm have several antigens that are shared with various somatic cells (6–10). A few sperm antigens have been delineated, namely lactate dehydrogenase C_4, PH-20, SP-10, FA-1, FA-2, and CS-1, that are relevant to fertilization in various species of animals (reviewed in ref. 11). The utility of an antigen for the development of a contraceptive vaccine is contingent upon its tissue (sperm)-specificity and involvement in fertilization process.

We have isolated and characterized an antigen, designated fertilization antigen (FA-1), from human and murine testis using a germ-cell specific, but species-crossreactive, mAb that inhibits fertilization in mice and humans (12–15). The FA-1 antigen is a glycoprotein of 23 kDa (monomer) that has a ligand activity for ZP3 of oocyte zona pellucida (16–20) and causes a reduction in fertility of actively immunized female rabbits (21). Interestingly, the FA-1 antigen also is involved in involuntary infertility in humans (22–25).

A large quantity of FA-1 antigen in an homogeneous/recombinant form is required for investigating its role in immunocontraception and involuntary infertility, and for studying structure-function relationship. Initially, FA-1 antigen was purified and characterized using a mAb-immunoaffinity column that yielded enough antigen to investigate its bioefficacy. The present study describes the cloning and sequencing of cDNA encoding for FA-1 antigen from murine testis, its testis-specific expression, and immunocontraceptive effects of the recombinant protein.

METHODS AND MATERIALS

Library Screening and Isolation of cDNA. The mouse testis cDNA-λgt11 expression library (CLONTECH) was screened with FA-1 mAb using the procedure described elsewhere (26, 27). Briefly, the library was plated at a density of $\approx 10 \times 10^3$ plaque-forming units per 100-mm Petri dish with *Escherichia coli* Y1090 as host bacterium. After growth at 42°C for 3.5 hr and induction with 10 mM isopropyl β-D-thiogalactoside, the nitrocellulose membranes were blocked with 3% BSA, and screened with FA-1 mAb (≈ 0.5 μg/ml).

The positive immunoreactive clones were selected and subjected to further analysis. The cDNA insert was eluted from the positive clones by *Eco*RI, subcloned into pBluescript II SK + at *Eco*RI site (Stratagene), and sequenced with dsDNA Cycle Sequencing System (GIBCO/BRL) as described elsewhere (28). The sequencing thermoprofile was: 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, 35 cycles; and the sequencing primers were T3 and T7 promoter primers.

Hydropathy plot of the deduced amino acid sequence of FA-1 cDNA was computed by using the Kyte–Doolittle (29) and Engelman *et al.* methods (30). The search for nucleotide and amino acid sequence homology in GenBank, National Biomedical Research Foundation, and Swiss sequence banks was performed using FASTA and tFASTA search programs (31).

Northern Blot Procedure. RNA was extracted from various mouse tissues ($n = 11$) by RNA STAT-60 method (TEL-TEST,

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Abbreviations: FA-1, fertilization antigen; RT–PCR, reverse transcription–PCR.

Data deposition: The sequence reported in this paper has been deposited in the GenBank databank (accession no. U95114).

*To whom reprint requests should be addressed at: Division of Research, Department of Obstetrics and Gynecology, Medical College of Ohio, P.O. Box 10008, Toledo, OH 43699-0008. e-mail: RNAZ@GEMINI.MCO.EDU.

Friendswood, TX) (32). The RNA was treated with RNase-free DNase (Stratagene), phenol-extracted, and ethanol-precipitated, and the poly(A)$^+$ RNA was prepared by using oligo(dT)- cellulose (GIBCO/BRL) (33). Two micrograms of poly(A)$^+$ RNA from each tissue was separated on a 1.2% denaturing agarose/formaldehyde gel and transferred onto nitrocellulose membranes by upward capillary transfer for 12–16 hr and permanently bound to the membranes by UV crosslinking (33). The membranes were prehybridized (56°C, 15 min) with QuickHyb solution (Strategene), then incubated (56°C, 2 hr) with ^{32}P-labeled FA-1 cDNA probe, washed, and exposed to x-ray film for 24 hr to 3 weeks. The probe eluted from pBluescript vector by *Eco*RI digestion was prepared by the random hexamer method (GIBCO/BRL).

After the membrane was stripped off the FA-1 probe, it was rehybridized (65°C, 2 hr) with β-actin probe (CLONTECH).

Reverse Transcription–PCR (RT–PCR). Total RNA isolated from various tissues (n = 11) was treated (twice) with RNase-free DNase, followed by phenol extraction and ethanol precipitation as described above (33). Two micrograms of the poly(A)$^+$ RNA from each tissue was mixed with 0.5 μg (0.5 mg/ml) of oligo(dT)$_{15}$ primer and 4 μl of 5× buffer (250 mM Tris·HCl, pH 8.3/375 mM KCl/15 mM $MgCl_2$), heated to 65°C, and cooled slowly to 37°C. To this reaction mixture 0.5 μl (38 units/μl) of rRNAsin RNase inhibitor, 2 μl of 100 mM DTT, 1 μl of 10 mM dNTPs, and 2 μl (400 units) of Moloney murine leukemia virus reverse transcriptase were added. The reaction components were mixed, incubated at 37°C for 60 min, and then stored at −20°C. All reagents were of analytical grade and obtained from GIBCO/BRL. Two microliters of the resulting cDNAs was amplified by PCR (Amplitron II, Dubuque, IA) for 30 cycles (94°C for 45 sec, 55°C for 30 sec, 72°C for 90 sec) by the FA-1-specific or β-actin primers.

The FA-1-specific primers of 31-mer were mapped to the initiation and termination of translation sites, respectively; sense primer: 5′-ATGACAGAGGCTGATGTGAATCCGA-AGCCTA-3′, and antisense primer: 3′-CCAGTTACTATTA-TAATTGTACTACATCTCGTT-5′. This primer set is expected to amplify a 495-bp fragment.

β-Actin-specific primers of 29-mer were based on the conserved regions between rat and human β-actin cDNAs sequences; sense primer: 5′-GGGGATCCGTACATGGCTGG-GGTGTTGAA-3′, and antisense primer: 3′-CCCCTAGGC-ATGTACCGACCCCACAACTT-5′ (34). This primer set is expected to amplify a 256-bp fragment.

PCR-amplified products were analyzed by agarose gel electrophoresis containing ethidium bromide along with *Hin*dIII-digested λ DNA markers. After gel electrophoresis, DNAs were transferred to nitrocellulose membrane for Southern blot procedure to examine the specificity of the amplified fragments. For Southern blot procedure, the membranes were prehybridized (65°C, 15 min) with QuickHyb solution, and then incubated (65°C, 2 hr) with ^{32}P-labeled probe (FA-1 or β-actin). The membranes were washed and exposed to x-ray film for 24 to 48 hr.

Expression of Recombinant FA-1 Protein. Based on the results of *in vitro* transcription using the Riboprobe system and translation using the rabbit reticulocyte lysate system (Promega) (data not shown), and the sequence of FA-1 cDNA in pBluescript vector, the pGEX-2T vector was selected (Pharmacia). The vector was digested by *Eco*RI and dephosphorylated by calf intestinal alkaline phosphatase, and the insert cDNA was eluted from pBluescript vector by *Eco*RI. The vector and insert DNAs were mixed at 1:5 molar ratio in a ligation reaction mixture containing 1 mM ATP and incubated at 10°C overnight. The ligation reaction was used directly to transform the competent *E. coli* JM105 cells (Promega). The authenticity and right orientation of the FA-1 cDNA were confirmed by sequencing the recombinant clones using pGEX sequencing primers (Pharmacia).

Expression of the recombinant FA-1 protein was conducted according to the manufacturer's protocol (Pharmacia) (35). Bacteria containing the plasmid with right insert were cultured overnight at room temperature with vigorous shaking. The culture then was diluted (1:100) and continued until the OD_{600} reached 0.5–2. The culture was induced with isopropyl β-D-thiogalactoside (0.1 mM final concentration), and incubation was continued for an additional 6 hr. The bacteria were centrifuged, washed, and sonicated. The supernatant containing the recombinant FA-1 glutathione *S*-transferase fusion protein was incubated with glutathione conjugated to Sepharose 4B beads. The unbound proteins were washed off the immunocolumn, and the recombinant FA-1 protein was released from the fusion protein bound to the beads, by treatment with thrombin. The protein was further purified using an anti-FA-1 mAb immunocolumn to remove thrombin (13). The purified protein was analyzed in SDS/PAGE and Western blot procedures.

SDS/PAGE–Western Blot Procedure. The purity and authenticity of the purified recombinant protein were analyzed by using SDS/PAGE and Western blot procedure (12, 13). Briefly, ≈20–40 μg of the purified recombinant protein was run under nonreducing condition in the slab SDS/PAGE (5–15% gel) before and after treatment with 1.5 M NaCl solution. For Western blot procedure, the recombinant protein was electrophoretically transferred from the gel to nitrocellulose membrane, the blotted strips were incubated with FA-1 mAb (≈1 μg/ml), and the reacted bands were localized as described elsewhere (12, 13).

Interaction Between the Recombinant FA-1 Protein and ZP3 of Oocyte Zona Pellucida. Six- to 10-week-old B6D2F1 female mice were superovulated by i.p. injection of 7 units of pregnant mare serum gonadotropin, followed 48 hr later by i.p. injection of 7 units of human chorionic gonadotropin (12–14). Ova were collected 16–18 hr after human chorionic gonadotropin injection. Cumulus cells were removed with hyaluronidase, the ova were washed (three times), and the zonae were heat-solubilized in sodium carbonate buffer (0.001 M, pH 9.0) at 60°C for 1 hr as described elsewhere (36). The solubilized zona pellucida preparation was separated in SDS/PAGE (5–15%) and transferred to nitrocellulose membrane for Western blot procedure. The recombinant protein was biotinylated and separated from unbiotinylated protein following the manufacturer's protocol (ECL protein biotinylation module, Amersham). The Western blot was incubated with biotinylated protein (≈1.5 μg/ml), washed, allowed to react with streptavidin-horseradish peroxidase (1:1,500), washed again, incubated with substrate, and exposed to x-ray film for 1–5 min. The biotinylated BSA was used as a control in these experiments.

Antibodies to Recombinant FA-1 Antigen. Sexually mature virgin female rabbits (random-bred New Zealand White strain) were immunized systemically by two injections simultaneously, one subcutaneously and one intramuscularly, against purified recombinant FA-1 protein as described elsewhere (21). The first injection consisted of 200 μl of solution containing 100–125 μg of the protein emulsified with an equal volume of Freund's complete adjuvant. Two weeks after the primary injection, the rabbits were injected at each site weekly for 3 weeks with the same amount of the antigen mixed with incomplete Freund's adjuvant. Two weeks after the last injection, the animals were bled at various pre- or postimmunization stages to collect sera, the antibodies affinity-purified using protein A column, and the antibody activity was determined by ELISA (12–14). Animals injected with adjuvant emulsified with PBS alone served as controls.

Sperm–Ova Binding Assay. Mice were superovulated, and ova were collected as described above. Cumulus cells were removed by treatment with hyaluronidase (12–14). Cauda epididymides were removed from a male mouse, snipped, and

placed in 500 μl of Tyrode solution (Sigma). After 20 min, sperm were collected and diluted in Tyrode medium (1×10^6 sperm per ml). One hundred fifteen microliters of the sperm suspension was incubated (37°C, 30 min) with 5 μl of the antibodies, and then 5–10 ova were added into each drop to examine the sperm binding to zona pellucida. After 30 min of sperm–ova coincubation, the ova were washed to remove the loosely bound sperm, and the number of sperm bound to zona pellucida counted.

Statistical Analysis. Significance of differences was analyzed by using unpaired Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Nucleotide and Deduced Amino Acid Sequences of FA-1 cDNA. Immunological screening of murine testis cDNA-λgt11 expression library using FA-1 mAb gave three positive signals on duplicate filters after screening over 10^7 recombinant clones. These three isolates were subjected to further screening. One of these clones gave intense positive signal with FA-1 mAb and not with myeloma control antibody of the same class and isotype specificity (IgG_{2a}), on repetitive immunological analysis. The phage DNA of this positive clone was purified, digested with *Eco*RI, and the agarose gel electrophoretic analysis established the insert size to be 0.7 kb. The insert was subcloned into pBluescript II SK + at *Eco*RI site and sequenced. The complete nucleotide sequence of the 0.7-kb FA-1 cDNA was obtained by complete sequencing of both strands. Each strand was sequenced several times, and their alignments provided a 649-bp cDNA sequence (Fig. 1*A*). Computer-generated translation analysis of the cDNA yielded an ORF of 164 amino acids with the first ATG Met start codon at nucleotide 81 and the stop codon TAA at nucleotide 577. The cDNA has an 82-bp 5′ and a 75-bp 3′ noncoding region and a polyadenylylation tail following the stop codon.

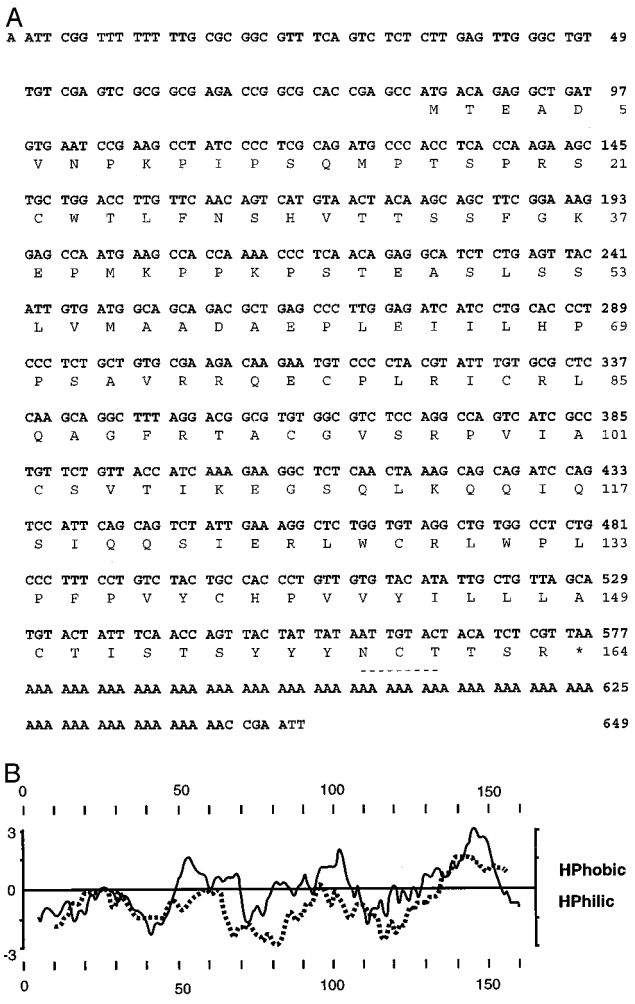

FIG. 1. (*A*) Nucleotide (nt) and deduced amino acid (aa) sequences of FA-1 cDNA. The mouse testis cDNA-λgt11 expression library was screened with the FA-1 mAb. The positive immunoreactive clone containing 0.7-kb insert was eluted from λgt11 by *Eco*RI, subcloned into pBluescript II SK + at *Eco*RI site, and sequenced with dsDNA Cycle Sequencing System using T3 and T7 promoter primers. (*B*) Hydropathy plot of the deduced amino acid sequence of FA-1 cDNA. It was computed by using the Kyte–Doolittle (29) (solid line) and Engelman *et al.* (30) (dotted line) methods.

The translated protein has a calculated molecular mass of 18.2 kDa and an isoelectric pH of 8.5. Among the charged group amino acids, there are 10 Arg, 6 Lys, and 3 His representing the positively charged amino acids; and 6 Tyr, 9 Cys, 8 Glu, and 2 Asp representing negatively charged amino acids. A strong potential N-linked glycosylation site is present at aa 158–160, besides at least two O-linked glycosylation sites.

Hydropathy plot, based on the deduced amino acid sequence, indicated a slightly hydrophilic N-terminal region that has a tendency toward hydrophobicity, a central core with several hydrophilic domains, and a strong C-terminal hydrophobic domain (Fig. 1*B*). Both methods, namely Kyte–Doolittle (29) and Engelman *et al.* (30), indicated the similar computation. Extensive computer search in the GenBank, National Biomedical Research Foundation, and Swiss sequence banks did not identify any known nucleotide/amino acid sequence having homology with FA-1 cDNA or deduced amino acid.

Tissue-Specific Expression. Tissue-specific expression of FA-1 was examined by Northern blot analysis and RT–PCR. Northern blot analysis using poly(A)$^+$ RNA isolated from 11 tissues indicated a strong signal only in the testis lane at 1.35 kb, when the membrane was probed with FA-1 cDNA (Fig. 2*A*). No other tissue showed any hybridization with the probe

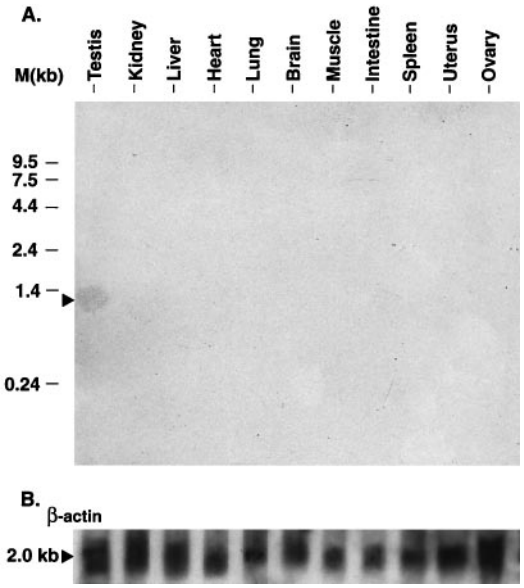

FIG. 2. (*A*) Northern blot of various tissues mRNAs probed with FA-1 cDNA. Two micrograms of poly(A)$^+$ RNA isolated from each tissue was separated on a 1.2% denaturing agarose/formaldehyde gel and transferred to nitrocellulose membrane. The membrane was prehybridized with QuickHyb solution, incubated (56°C, 2 hr) with ^{32}P-labeled FA-1 cDNA probe, washed, and exposed to x-ray film for 24 hr to 3 weeks. (*B*) After the membrane was stripped of the FA-1 probe, it was rehybridized (65°C, 2 hr) with ^{32}P-labeled β-actin probe, washed, and exposed as above.

in any area even when the membrane was exposed to x-ray film up to 3 weeks. When the membrane was reprobed with β-actin cDNA, all the tissues showed a positive signal of equal intensity (Fig. 2*B*).

Similar results were obtained in RT–PCR. PCR amplification of the cDNAs, reverse-transcribed from chromosomal DNA-free RNAs isolated from 11 different tissues, yielded the expected band of 495 bp only in the testis and in no other tissue (Fig. 3*A*). This band got specifically hybridized with the FA-1 cDNA in the Southern blot analysis (Fig. 3*B*). Again, using primers based on β-actin sequence, the reverse-transcribed cDNA from all the tissues got amplified by PCR, yielding the expected 256-bp band (Fig. 3*C*) that was specifically hybridized with β-actin cDNA probe in the Southern blot analysis (Fig. 3*D*).

Recombinant FA-1 Protein. To obtain the recombinant FA-1 protein for immunobiological analysis, the glutathione *S*-transferase gene fusion system was used. The FA-1 cDNA was isolated from pBluescript vector by *Eco*RI and subcloned into pGEX-2T vector. The sequence of cDNA sublconed into pGEX-2T vector demonstrated sense orientation and identical sequence that matched completely with the original FA-1 cDNA sequence. The appropriate clone expressed a protein of the expected ≈18-kDa molecular size as seen in the SDS/PAGE analysis (Fig. 4*A*, lane a). The expressed recombinant protein has a tendency to polymerize. However, treatment of the recombinant protein with 1.5 M NaCl changed the polymeric forms into monomer, showing only a single band of ≈18 kDa (Fig. 4*A*, lane b). The recombinant protein, both polymers (Fig. 4*A*, lane a) and monomer (lane b), were specifically recognized by the FA-1 mAb (Fig. 4*B*) and not by the myeloma control antibody of the same concentration, class, and isotype specificity (Fig. 4*C*) as FA-1 mAb in the Western blot procedure.

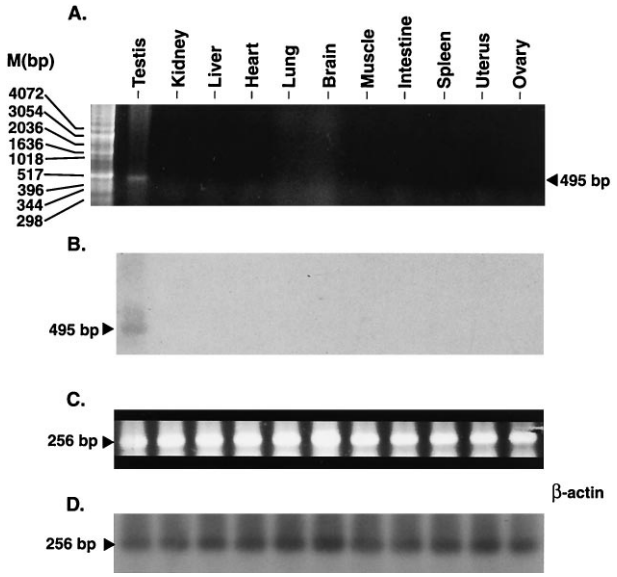

FIG. 3. RT–PCR pattern of various murine tissue RNAs. Two micrograms of whole RNA from each tissue, free of chromosomal DNA contamination, were reverse-transcribed in a total volume of 20 μl. Two microliters of the resulting cDNA was amplified by PCR for 30 cycles using the FA-1-specific or β-actin-specific primers. The FA-1-specific primer set was expected to amplify a 495-bp fragment. (*A*) Ethidium bromide-stained electrophoretic gel pattern of the PCR products amplified by using FA-1 primers. (*B*) Hybridization pattern of the amplified products with ^{32}P-labeled FA-1 cDNA on the Southern blot. The β-Actin-specific primer set was expected to amplify a 256-bp fragment. (*C*) Ethidium bromide-stained electrophoretic gel pattern of the PCR-amplified products. (*D*) Southern blot hybridization pattern with ^{32}P-labeled β-actin probe.

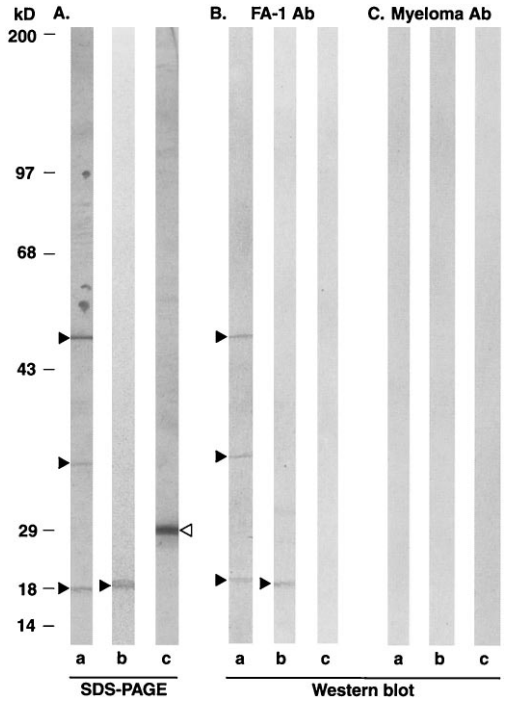

FIG. 4. SDS/PAGE pattern of FA-1 recombinant protein and its immunoreactivity in the Western blot procedure. The subcloning of FA-1 cDNA into pGEX-2T vector at *Eco*RI site and the expression and purification of recombinant protein are described in *Methods and Materials*. Twenty to 40 μg of the nonreduced recombinant protein was run before (lane a) and after (lane b) treatment with 1.5 M NaCl (lane b) in SDS/PAGE and either stained with silver nitrate (*A*) or electrophoretically transferred to nitrocellulose membrane for Western blot analysis using FA-1 mAb (*B*) or myeloma control antibody (*C*). The glutathione *S*-transferase protein (20 μg) was used as control in these experiments (lane c).

Involvement of Recombinant FA-1 Protein in Fertilization. The recombinant FA-1 protein showed binding with ZP3 on the Western blot involving solubilized-zonae pellucidae of murine oocytes (Fig. 5). To further investigate the involvement of recombinant FA-1 in fertilization, antibodies were raised in female rabbits. Affinity-purified polyclonal antibodies against recombinant antigen were specific and recognized the native as well as recombinant FA-1 protein, as seen with the FA-1 mAb depicted in Fig. 4. The antibodies predominantly reacted with the postacrosomal, mid-piece, and tail regions of the sperm, as has been previously published for the FA-1 mAb, in the indirect immunofluorescence technique. Incubation of sperm with these antibodies completely blocked sperm-zona pellucida binding in mice without affecting motility of sperm (Table 1). There was no effect of the antibodies on percent motility of sperm [untreated control, 75 ± 5%; anti-FA-1 antibodies (polyclonal/mAbs), 74 ± 7%; control/preimmune immunoglobulins/myeloma control, 76 ± 4%; $P > 0.05$].

DISCUSSION

We described here the cloning and sequencing of cDNA encoding for the murine sperm FA-1 antigen. The authenticity of the clone is indicated by its strong reaction with the FA-1 mAb and not with the myeloma control antibody of same class and isotype specificity as the FA-1 mAb. Also, the molecular size of the deduced amino acid peptide, that is 18.2 kDa, is in agreement with the published molecular mass of the carbohydrate-free cognate FA-1 antigen isolated from murine testes (13). The native FA-1 antigen is a glycoprotein of 23 kDa in its monomeric form and has ≈18.8% carbohydrates (13). Analysis of the deduced amino acid sequence revealed one potential

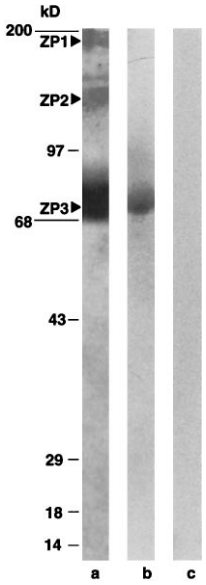

FIG. 5. Interaction between the recombinant FA-1 protein and ZP3 of oocyte zona pellucida. The solubilized-zona pellucida preparation from murine oocytes was separated in SDS/PAGE (5–15%), and then transferred to nitrocellulose membrane for Western blot procedure. The recombinant protein was biotinylated and separated from unbiotinylated protein following the manufacturer's protocol. The Western blot was incubated with biotinylated protein (≈1.5 μg/ml), washed, allowed to react with streptavidin-horseradish peroxidase (1:1,500), washed again, incubated with substrate, and exposed to x-ray film for 1–5 min. The solubilized zona pellucida preparation showed three bands (ZP1, ZP2, and ZP3, respectively) in SDS/PAGE (lane a). The recombinant FA-1 protein specifically recognized ZP3 on the Western blot (lane b). The biotinylated BSA used as a control did not react with any band on the Western blot (lane c).

N-linked and at least two O-linked glycosylation sites. The glycosylation during posttranslational modification at these positions could account for the difference in the molecular size between the cognate FA-1 antigen (≈23 kDa) and the deduced amino acid sequence/recombinant protein (≈18.2 kDa). The presence of polyadenylylation tail following the stop codon indicates the end of the cDNA.

The several hydrophilic domains in the central region and a strong C-terminal hydrophobic domain, as predicted from the hydropathy plot based on the deduced aa sequence, indicate FA-1 antigen to be a membrane-anchored protein. Lack of homology with any known nucleotide/amino acid sequence in the GenBank, National Biomedical Research Foundation, and Swiss banks, indicate it to be a novel protein.

Table 1. Effect of antibodies to recombinant FA-1 antigen on sperm–zona pellucida binding in mice

Treatment	Amount, μg/120 μl	Number of ova tested*	Sperm bound per egg
Antibody to recombinant FA-1	5	52	0‡
	10	105	0‡
Anti-FA-1 mAb	10	48	2 ± 0.2
Preimmune immunoglobulins	5	38	40 ± 6
	10	41	39 ± 7
Control rabbit immunoglobulins†	5	51	43 ± 6
	10	62	38 ± 9
Myeloma control immunoglobulins	10	53	41 ± 7
Untreated control	—	102	42 ± 8

*Pooled from 3–5 different experiments performed on various days using sperm and oocytes from different groups of mice.
†Control rabbit immunoglobulins were collected from rabbits immunized with Freund's adjuvant alone.
‡Versus untreated/preimmune/control rabbit immunoglobulins/myeloma control immunoglobulins, $P < 0.0001$; others were nonsignificant, $P > 0.05$.

FA-1 antigen was expressed only in the testis as studied by expression at the mRNA level in the Northern blot analysis and sensitive RT–PCR procedure. No hybridization signal was observed in any other tissue except testis, even when the Northern blot was exposed up to 3 weeks. Lack of hybridization with other tissues was not due to problems with RNA integrity or insufficient loading. The integrity and equal loading of RNA from various tissues was confirmed by hybridization with the β-actin probe. The results obtained in RT–PCR/Southern blot hybridization procedure were specific because: (*i*) RNA samples amplified by PCR without previous reverse transcription did not demonstrate the expected band of 495 bp ruling out chromosomal DNA contamination; (*ii*) RNA samples treated with RNase and subsequently amplified by PCR did not show any band (data not shown); and (*iii*) the amplified product observed after PCR in the testis sample was of the expected size that was specifically recognized by the cDNA probe in the Southern blot hybridization procedure. The present findings on the tissue specificity of FA-1 antigen observed at the mRNA level are in agreement with these observed previously at the protein level. FA-1 mAb (12) as well as polyclonal antibodies (15, 21) against the native FA-1 antigen react only with sperm/testicular cells and not with any somatic cell/tissue.

The purified recombinant protein expressed in the glutathione *S*-transferase gene fusion system also showed the molecular mass of ≈18 kDa, as expected from the deduced amino acid sequence. The recombinant protein was specifically recognized by the FA-1 mAb, confirming the authenticity of cDNA and the recombinant protein. The recombinant antigen has a tendency to polymerize, as was also seen by the native FA-1 antigen (12, 13). The forces causing polymerization are mainly of ionic nature and can be disrupted by using 1.5 M NaCl. Several hydrophilic domains in the core region and a strong C-terminal hydrophobic region seem to be involved in polymerization of the antigen. As seen in other systems, the membrane proteins have a general tendency to aggregate, especially in the presence of detergents (37, 38).

The recombinant protein specifically recognized the ZP3 component of the zona pellucida of the oocytes. Various studies have shown that the zona pellucida of murine oocytes is composed of three components namely ZP1, ZP2, and ZP3, and the ZP3 molecule acts as a receptor for recognition and binding with complementary ligand/receptor on the sperm surface (39, 40). The recognition/binding of the ZP3 by the recombinant molecule indicate that the FA-1 is a complementary receptor molecule on the sperm surface for recognition/binding to the zona pellucida of the oocyte. Similar results were observed with the native human FA-1 antigen for binding to the ZP3/zona pellucida of the human oocyte (16–20). These findings on interaction of ZP3 with recombinant FA-1 on Western blot also indicate that the recognition/binding may involve protein-protein interaction, recognizing the linear sequences of the complementary epitopes of two interacting cells (sperm/oocyte).

The involvement of FA-1 antigen in fertilization process was further confirmed by inhibition of the sperm-zona interaction by its antibodies. Antibodies raised against the purified recombinant antigen, that specifically recognized the recombinant as well as cognate FA-1 antigen on the Western blot, completely blocked sperm binding to the zona pellucida of the murine oocyte. There was no apparent effect of the antibodies on sperm motility. mAb against the cognate FA-1 antigen also does not affect percent motility, although affect various motility characteristics such as linearity, amplitude of lateral head displacement and beat frequency, the parameters contributing to hyperactivation that is a integral part of capacitation

preceding the acrosome reaction and sperm binding to the zona pellucida (11, 17). These effects of antibodies further indicate that the FA-1 antigen may be a receptor on the sperm for recognition/binding to the zona pellucida. Besides sperm–zona interaction, it also seems to be involved in capacitation/acrosome reaction of sperm that is prerequisite for fertilization.

The utility of an antigen in the development of a contraceptive vaccine is contingent upon its sperm-specificity and involvement in the fertilization process. The recombinant FA-1 antigen fulfills both of these criteria. Besides these criteria, its involvement in human infertility makes it an attractive candidate for immunocontraception. The cloned FA-1 cDNA also will help to gain further insights into the transcriptional control of testis-specific genes during spermatogenesis, and for studying the species-specificity and molecular mechanisms involved in sperm activation (capacitation/acrosomal exocytosis) and sperm-zona interaction (recognition/binding).

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1. Edwards, R. G. (1964) *Nature (London)* **203,** 50–53.
2. Menge, A. C. (1970) *J. Reprod. Fertil.* **10,** 171–185.
3. Menge, A. C & Naz, R. K. (1988) *Am. J. Reprod. Immunol.* **18,** 17–20.
4. Naz, R. K., Deutsch, J., Phillips, T. M., Menge, A. C. & Fisch, H. (1989) *Biol. Reprod.* **40,** 163–173.
5. Naz, R. K. & Menge, A. C. (1994) *Fertil. Steril.* **61,** 1001–1013.
6. Kerek, G. (1974) *Int. J. Fertil.* **19,** 181–191.
7. Chaffee, J. K. & Schachner, M. (1987) *Dev. Biol.* **63,** 173–184.
8. Jorgensen, O. S. & Moller, M. (1983) *Dev. Biol.* **100,** 275–286.
9. Mathur, S., Goust, J. M., Williamson, H. O. & Fundenberg, H. H. (1981) *Am. J. Reprod. Immunol.* **1,** 113–118.
10. Naz, R. K. & Menge, A. (1990) *Human Reprod.* **5,** 511–518.
11. Naz, R. K., Sacco, A., Singh, O., Pal, R. & Talwar, G. P. (1995) *Hum. Reprod. Update* **1,** 1–18.
12. Naz, R. K., Alexander, N. J., Isahakia, M. & Hamilton, D. (1984) *Science* **225,** 342–344.
13. Naz, R. K., Phillips, T. M. & Rosenblum, B. B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5713–5717.
14. Naz, R. K. & Mehta, K. (1989) *Biol. Reprod.* **41,** 533–542.
15. Naz, R. K. & Bhargava, K. K. (1990) *Mol. Reprod. Dev.* **26,** 175–183.
16. Naz, R. K., Sacco, A. G. & Yurewicz, E. C. (1991) *J. Reprod. Immunol.* **20,** 43–58.
17. Naz, R. K., Ahmad, K. & Kumar, R. (1991) *J. Cell Sci.* **99,** 157–165.
18. Naz, R. K., Brazil, C. & Overstreet, J. (1992) *Fertil. Steril.* **57,** 1304–1310.
19. Naz, R. K. & Wolf, D. P. (1994) *J. Reprod. Immunol.* **27,** 111–121.
20. Kadam, A. L., Fateh, M. & Naz, R. K. (1995) *J. Reprod. Immunol.* **29,** 19–30.
21. Naz, R. K. (1987) *J. Reprod. Immunol.* **11,** 117–133.
22. Naz, R. K. (1987) *J. Clin. Invest.* **80,** 1375–1383.
23. Bronson, R. A., Cooper, G. W., Margalioth, E. J., Naz, R. K. & Hamilton, M. (1989) *Fertil. Steril.* **52,** 457–462.
24. Naz, R. K., Chaudry, A. & Witkin, S. S. (1990) *Am. J. Obstet. Gynecol.* **163,** 610–613.
25. Naz, R. K., Ahmad, K. & Menge, A. C. (1993) *J. Clin. Invest.* **92,** 2332–2338.
26. Huynh, T. V., Young, R. A. & Davis, R. W. (1985) *in DNA Cloning: A Practical Approach*, ed. Clover, D. M. (Oxford Univ. Press, London), pp 49–78.
27. Javed, A. A. & Naz, R. K. (1992) *Gene* **112,** 205–211.
28. Zhu, X., Wittner, M., Tanowitz, H. B., Cali, A., Weiss, L. M. (1993) *J. Infect. Dis.* **168,** 1570–1575.
29. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
30. Engelman, D. M., Steitz, T. A. & Goldman, A. (1986) *Annu. Rev. Biophys. Biophys. Chem.* **15,** 321–353.
31. Perlman, D. & Halvorson, H. O. (1983) *J. Mol. Biol.* **167,** 391–409.
32. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
33. Naz, R. K., Kumar, G. & Minhas, B. S. (1994) *J. Assist. Reprod. Genet.* **11,** 208–216.
34. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
35. Chung, C. T., Niemela, S. L. & Miller, R. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2172–2175.
36. Drell, D. W., Wood, D. M., Bundman, D. & Dunbar, B. S. (1984) *Biol. Reprod.* **30,** 435–444.
37. Javaid, J. I. & Winzler, R. J. (1974) *Biochemistry* **13,** 3635–3642.
38. Segrest, J. P., Jackson, R. L., Andrews, E. P. & Marchesi, V. T. (1971) *Biochem. Biophys. Res. Commun.* **44,** 390–395.
39. Ringuette, M. J., Chamberlin, M. E., Baur, A. W., Sobieski, D. A. & Dean, J. (1988) *Dev. Biol.* **127,** 287–295.
40. Yanagimachi, R. (1988) in *Physiology of Reproduction*, eds. Knobil, E. & Neill, J. (Raven, New York), pp 135–185.