[Frontiers in Bioscience 11, 2371-2380, September 1, 2006]

Delineation of the functional domains of the extracellular region of YWK-II Protein/APLP2 of sperm membrane

Dazhong Zhuang [1], Yong Wang [1], Chunbo Yang [1], Yuan Qiao [1], Shiying Miao [1], Samuel S. Koide [2], and Linfang Wang [1]

[1] National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan San, Tiao, Beijing, 100005, China, [2] Center for Biomedical Research, Population Council, 1230 York Avenue, New York, New York 10021, USA

TABLE OF CONTENTS

1. ABSTRACT

The sperm membrane protein, designated as YWK-II protein/APLP2, is a member of the amyloid precursor protein (APP) superfamily and is a type I transmembrane protein involved in fertilization. Here, the structure-function of the domains of YWK-II protein was examined. Five segments with overlapping ends encompassing the entire extracellular region of mouse YWK-II gene were prepared, cloned and separately expressed in *E. coli.* The recombinant YWK-II segments were fused with glutathione S-transferase (GST), purified and evaluated for their antifertility activities by measuring their capacity to block *in vitro* mouse sperm-egg interaction. The structural domain(s) involved in the fertilization process was identified. The polypeptide segment corresponding to position 22-207 of YWK-II-763 inhibited the early stage of fertilization when the spermatozoa interacted with zona-free eggs; whereas the polypeptide segment 201-395 (lacking 309-364) of YWK-II-763 blocked sperm-egg membrane fusion. The remaining three segments, 201-395, 389-574 and 517-704 (lacking 613-624) of YWK-II-763, did not influence the *in vitro* fertilization process. The present results suggest that segment 22-308 of YWK-II-763 participates in the binding and fusion of sperm and egg plasma membranes thereby promoting fertilization.

Table 1. Primer sequences

Name	Type	Sequence	Position
Fa	Sense	5'-CTGGGATCCGTAGCCTGGCTGT GCGAGAGAC-3'	57-36
	Anti-sense	5'-CTGGTCGACTCTTTGTCTGAGGGCAGCACAC-3'	622-601
Fb/Fc	Sense	5'-CTCGGATCCGTGTGCTGCCCTCAGACAAAGA-3'	601 - 622
	Anti-sense	5'-CTCGTCGACTTCCTTAGCCTT CTGGAAGCGG-3'	1184 - 1165
Fd	Sense	5'-CTCGGATCCCGCTTCCAGAAGGCTAAGGA-3'	1165-1184
	Anti-sense	5'-CTCGTCGACAACTGGTCCATATCTGCA CGCT-3'	1724 - 1703

2. INTRODUCTION

YWK-II protein, a sperm membrane component, was identified initially as the target antigen to a monoclonal antibody (mAb) raised against extracted proteins of human spermatozoa, possessing potent sperm agglutinating activity (1). The coding cDNA was isolated and its sequence determined. The deduced polypeptide, designated as the YWK-II protein, contained a segment with high homology (70.6%) to the transmembrane-cytoplasmic domain of the βA4-amyloid precursor protein (APP) found in brain plaques of subjects with Alzheimer's disease (2). Subsequently, it has been shown to be homologous with the human placenta amyloid precursor protein homolog (APPH) and rat amyloid precursor-like protein 2 (APLP2), which are apparently species-specific form of the same component. Also the alternative splicing patterns of isoforms of YWK II protein gene are similar to those of the APP gene family members. Both the YWK-II protein and APP have a splicing region homologous to the Kunitz protease inhibitor(KPI) consensus sequence, which has a role in regulation of extracellular protease activity. (3-6).

Among the members of the APP superfamily, β-amyloid precursor protein (APP) is the most prominent component. The role of APP in the pathogenesis of Alzheimer's disease has been underscored by the discovery of mutations within its sequence (7). Potential biological and biochemical functions of APP in neurons, based on the data obtained from *in vitro* studies, are the promotion of cell survival, stimulation of neurite outgrowth and synaptogenesis, modulation of synaptic plasticity, regulation of cell adhesion and protection against a range of metabolic, excitotoxic and oxidative agents (8). Also its wide tissue distribution implicates its importance in cell metabolism. This contention is substantiated by the capacity of the YWK-II protein to interact with Go protein; thereby modulating the intracellular signaling pathway (9). Few experimental studies on the function of YWK-II protein/APLP2 have been reported compared to those of APP. Nonetheless, high homology in their sequences suggests that they have related functional activities. For example, APLP2 exhibits neurite outgrowth-promoting activity comparable to that of APP isoforms (10).

A series of our previous experimental results indicate that YWK-II protein is involved in sperm-egg interaction. YWK-II mAb agglutinates human and rat sperm (11,12) and prevents heterogamete fertilization (13). Passive immunization of female mice with YWK-II mAb causes a significant reduction in the number of zygotes formed compared to controls (14). Also immunization with a polypeptide corresponding to segment 592-607 of rat YWK-II -763 protein effectively reduces fertility in both male and female rats (15). Furthermore, in null-mutated mice, both APP-/- individuals and APLP2-/- ones are fertile while APP and APLP2 double-mutated mice are infertile (16), demonstrating that the YWK-II protein/APLP2 is a critical factor involved with fertility.

The aim of the present study was to clarify the molecular role of YWK-II protein in the fertilization process and to identify the interacting domains involved in sperm-egg interaction. Five nucleotide segments covering the entire extracellular domain of the YWK-II gene were synthesized and expressed in *E. coli.* *Two* segments (Fb, Fc, Figure 1A) spanning the KPI region were synthesized and produced to ascertain the functional role of this well-defined domain. The recombinant polypeptides were isolated, purified, sequenced and examined for their potential in inhibiting fertilization, using an *in vitro* mouse sperm-egg interaction or binding assay. The amino acid sequences of the polypeptides capable of blocking sperm-egg interaction were determined and the functionally active regions of YWK-II protein/APLP2 identified.

3. MATERIALS AND METHODS

3.1. Animals

Eggs were obtained from 6- to 8-week female Kunming mice while testes and spermatozoa were obtained from male Kunming retired breeders.

3.2. Materials

All restriction enzymes and buffers, Taq enzyme, Klenow enzyme (fragment), and T4 DNA ligase were purchased from Boehring-Mannheim (Germany) or Promega Co. (USA). Micro Fast Track kit was obtained from Invitrogen (USA), Superscript Preamplification system from GIBCO-BRL (USA), Advantage-HF PCR kit from Clontech (USA), pGEX-3X and pGEX-4T-3 vectors, *E. coli* BL21, *E. coli* DH5α and Bulk GST Purification Module were obtained from Pharmacia Biotech (Hong Kong, China). Isopropyl-β -D-thiogalactopyranoside (IPTG), egg white lysozyme, phenylmethylsulphonyl fluoride (PMSF), aprotinin, bovine pancreatic trypsin, α -N-benzoyl-L-arginine-p-nitroanilide (BAPNA) were purchased from Sigma Chemical Co. (USA).

The oligonucleotides were synthesized by the Sangon Bioengineering Ltd. (Shanghai, China)(Table 1). The numbers enclosed in parentheses correspond to the position of the nucleotides numbered from the beginning

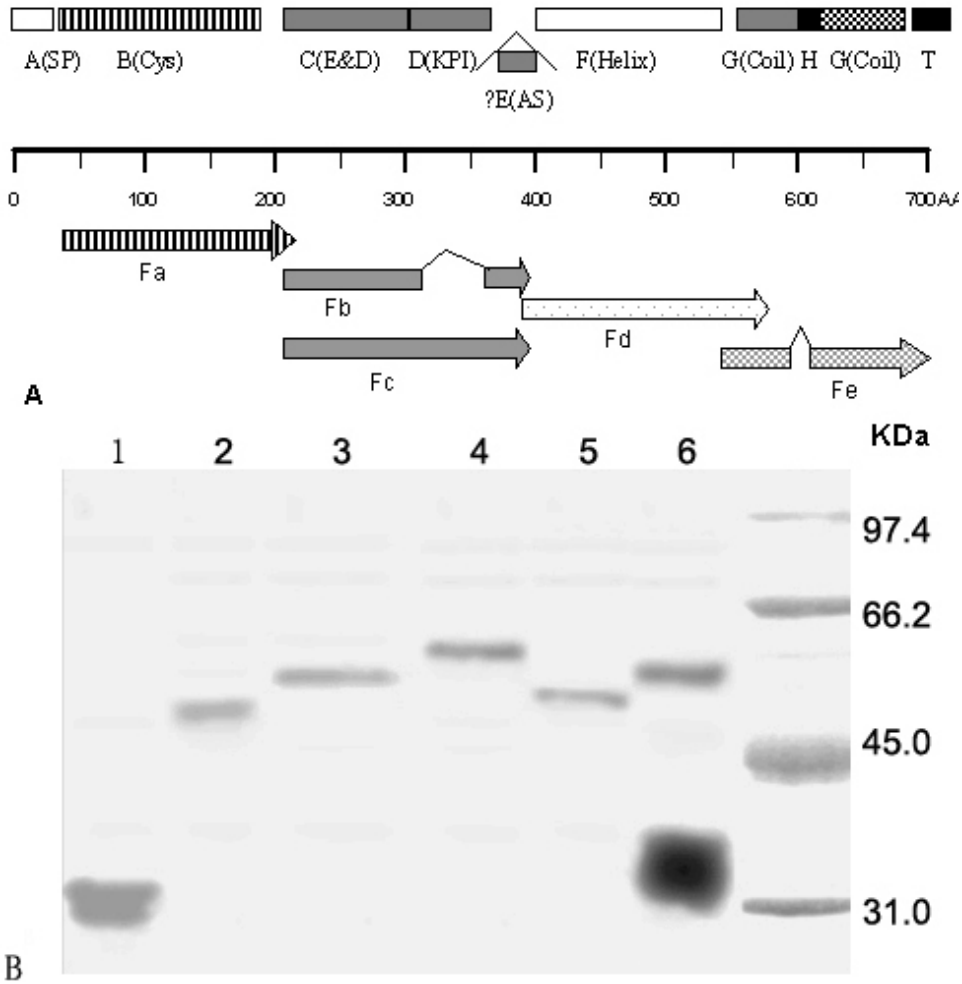

Figure 1. Extracellular region of deduced YWK-II protein. A. Nucleotide sequence of YWK-II cDNA. The expressed segments of the extracellular region of YWK-II protein correspond to the following domains of YWK-II. A: signal peptide (SP); B: cysteine-rich domain (Cys); C (E&D): negative-charged amino acid-rich domain; D: Kunitz protease inhibitor (KPI) domain; E: possible alternative splicing domain (AS); F: (Helix) α-helix-rich domain; G:. (Coil) coil-rich domain; H: second alternative splicing domain; and T: transmembrane domain. Fa: segment 22-207 of YWK-II-763; Fb: segment 201-395 of YWK-II-763 lacking 309-364; Fc: 201-395 of YWK-II-763, including 309-364; Fd: 389-574 of YWK-II-763; Fe: 517-704 of YWK-II-763, lacking 613-624. B. SDS-PAGE pattern of recombinant polypeptides of YWK-II protein purified by glutathione-affinity chromatography. Lane 1, GST; lane 2,GST-Fa ; lane 3, GST-Fb; lane 4, GST-Fc; lane 5, GST-Fd; lane 6, GST-Fe .. Molecular sizes of standard protein markers are given on the right.

of the open reading frame of mouse YWK-II- 763. Each primer contained a specific sequence of six nucleotides sensitive to the restriction endonucleases BamHI (sense primers) or SalI (antisense primers) and three additional ones at the 5'-end for protection. To amplify the two segments (Fb, Fc) spanning the KPI domain, only one primer was designed which corresponded to the sequence of the conserved region, synthesized, and used with both segments.

3.3. Vectors and bacterial expression

mRNA was isolated from the testes of male mice using the Micro Fast Track kit. Single stranded cDNA was synthesized using Superscript II reverse transcriptase and oligo(dT)12-18 from Superscript Preamplification system. The cDNAs were template sources for the polymerase chain reaction (PCR) using Advantage-HF PCR Kit. The annealing temperatures were 66°C for Fa primers, 64°C for Fb, Fc, 67°C for Fd, respectively. The polymerizing temperature was 68°C. After 30-cycle amplification, the PCR products were heated at 95°C for 20 min. One adenosine was added to the 3'-end of each chain using the Taq enzyme. Then the tailed products were inserted into the pGEM-T vectors.

The plasmid pGEM-T-Fa (T-vector containing the PCR product amplified with primers Fa) was cut with *AvaI* and *SalI*. The 560 bp fragment with the *AvaI* cleavage site was blunted and cloned into the prokaryotic expression vector

pGEX-4T-3, prepared by digesting with *EcoRI* and *XhoI* and filling the *EcoRI* cut end by catalysis with Klenow fragment to generate pGEX-Fa plasmid expressing the segment 22-207 of YWK-II protein. The Fb, Fc and Fd oligonucleotide fragments were transferred to pGEX-4T-3 vectors with the aid of *BamHI* and *SalI* endonuclease sites located at the ends of their primers and the multiple cloning site of pGEX-4T-3 vector, generating pGEX-Fb, pGEX-Fc and pGEX-Fd. They expressed segments 201-395 (lacking 309-364), 201-395 and 389-574 fragments of YWK-II protein, respectively.

The oligonucleotide, corresponding to the open reading frame of 1549-2071 of mouse YWK-II/APLP2, was obtained by digesting human YWK-II cDNA vector with *HincII* and *BalI*. The 523 bp fragment was inserted into pGEX-3X vector linearized with *SmaI*. The plasmid containing the correctly oriented insert was designated as pGEX-Fe, expressing segment 517-704 (lacking 613-624) of YWK-II-763 protein. The correctness of the regions was confirmed by nucleotide sequence analysis.

To express the GST-fused YWK-II fragments, pGEX-Fs (Fa, Fb, Fc and Fd), were transformed in *E. coli* BL21 and pGEX-Fe in *E. coli* DH5α. The temperature of the culture was lowered and maintained at 25°C after the addition of IPTG to a final concentration of 0.3 mmol/L and the induction time limited to 3 h.

3.4. Purification of fusion proteins

Glutathione S-transferase (GST) fusion proteins, partially or completely solubilized in the cytosol of *E. coli*, were purified by adsorption to glutathione-agarose beads according to the protocol of the Pharmacia Bulk GST purification module. The fusion proteins located in the inclusion bodies were initially solubilized and renatured. The transformed *E. coli* was induced and collected. Egg white lysozyme was added and the cells subjected to supersonication. After washing in Tris-HCl buffer (pH 8.0) containing 1% Triton X-100 and 3 mmol/L EDTA, the inclusion bodies were dissolved in 0.01 mol/L NaOH containing 3 mmol/L 2-mercaptoethanol. L-arginine was added to a final concentration of 0.5 mol/L. The solution was incubated with vigorous shaking for 1 h at 37°C and dialyzed at 4°C in phosphate-buffered solution (pH 7.5) for 36 h and the medium replaced every 6 h. The insoluble debris was removed by centrifugation, and the GST-fusion protein purified by glutathione-affinity chromatography. The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the appropriate protein bands were collected (17).

3.5. Measurement of protease inhibition activity

The protease inhibition activity was quantified by measuring its potential to inhibit trypsin-catalyzed hydrolysis of BAPNA according to the method described in the publication by Heald *et al.* (18). BAPNA solution was added to the assay systems containing 2 μg/mL trypsin and serially diluted GST-Fc up to a final concentration of 0.39 mmol/L. After incubating for 30 min at 25°C, the amount of *p-nitroaniline* formed was determined by measuring absorbance at 405 nm.

3.6. Isolation of sperm

Sperms were collected from the cauda epididymis of male retired breeders. The caudae were cut into small pieces and suspended in modified Kreb-Ringer bicarbonate salt solution (mKR) to release the motile sperm, which were collected and capacitated by incubating under CO_2 atmosphere.

3.7. Preparation of egg and isolation

Female mice were superovulated by administering im 10 IU of pregnant mare serum gonadotropin (PMSG), followed 50 h later with an injection of 10 IU of human chorionic gonadotropin (hCG). After 13 h the mice were sacrificed and the egg-cumulus complexes recovered from the oviducts. Cumulus cells were dispersed from the eggs by treating with 0.1% hyaluronidase in mKR. The zona pellucidae (ZP) were solubilized by incubating in acid Ringer's solution (pH 2.5).

3.8. Collection of ZP proteins

The acid Ringer's solution containing 6 ZPs/μL was neutralized with NaOH, clarified by centrifugation and the supernatant stored at -80°C.

3.9. Assessment of acrosomal status

The state of the acrosomes in spermatozoa was determined by staining with Coomassie brilliant blue (19). Spermatozoa were fixed with 5% formaldehyde in phosphate-buffer saline (PBS), washed in PBS, placed on glass slides and dried. The cells were stained with 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid. The slides were rinsed with PBS, covered and examined by light microscopy.

3.10. Sperm-zona binding and sperm penetration assays

Cumulus-free eggs were placed in 10 μL droplets containing the fusion proteins or GST and incubated for 15 min prior to *in vitro* insemination. Spermatozoa suspended in mKR medium were incubated for 30 min. Aliquots of the fusion proteins or GST were added and incubated for another 15 min. Spermatozoa were added to the corresponding droplets containing eggs. After coincubation for 5 or 30 min, the eggs were washed three times with mKR medium and fixed in 4% formalin for sperm-zona binding assay; or eggs with bound spermatozoa were transferred to a new droplet of medium containing the same kind and concentration of proteins and incubated for another 1 or 10 h. Spermatozoa bound to the outer layer of ZP were removed by incubation in acid Ringer's solution. The eggs were fixed and those used for the sperm-binding assay were examined using phase-contrast microscopy to score the number of spermatozoa bound to the ZP. Eggs used in the penetration assay were stained with Hoechst 33258 and examined by fluorescence microscopy to score sperm heads located within the ZPs or as enlarged sperm heads and pronuclei. Data were presented as mean±SEM. T-test was used to determine the significance of the differences.

3.11. Sperm-egg binding and fusion assay

Zona-free eggs were incubated with the fusion proteins or GST for 15 min in droplets under mineral oil.

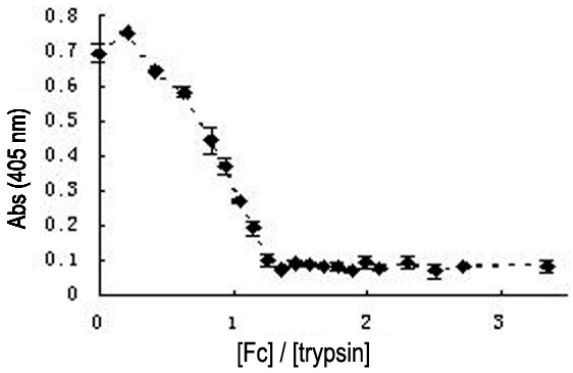

Figure 2. *In vitro* inhibition of trypsin activity by GST-Fc. The proteinase inhibitory activity was quantified by measuring its capacity to inhibit trypsin-catalyzed hydrolysis of BAPNA. Abs: absorbency; Fc: 201-395 of YWK-II-763, including 309-364.

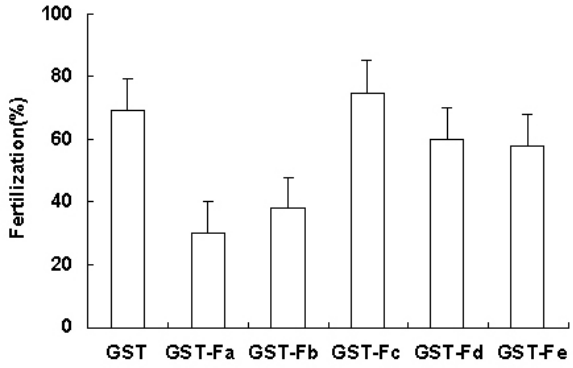

Figure 3. Effects of GST and GST-fusion proteins on the fertilization of zona-intact mouse eggs by spermatozoa. The respective proteins were added to both sperm capacitation and fertilization assay systems. The fertilization rate is given as percent of fertilized eggs. Protein concentrations are 500 μg/mL in each group. GST: glutathione S-transferase; Fa: segment 22-207 of YWK-II-763; Fb: segment 201-395 of YWK-II-763 lacking 309-364; Fc: 201-395 of YWK-II-763, including 309-364; Fd: 389-574 of YWK-II-763; Fe: 517-704 of YWK-II-763, lacking 613-624.

Spermatozoa were incubated for 1 h. Fusion proteins or GST were added and incubated for another 15 min. These spermatozoa were used in the *in vitro* fertilization assay. For the binding assay, the sperm-egg coincubation time was 5 or 30 min; whereas for the fusion assay, following a 30-min incubation, the eggs with bound spermatozoa were transferred to fresh droplets without fusion proteins or GST for another 3-hour incubation After fixation, the eggs were stained with Hoechst 33258. Sperm heads attached to the surface of the eggs were scored as the number bound. The fluorescence images of enlarged sperm heads were recorded as undergoing fusion. The data were presented as mean ± SEM. T-test was used to analyze the significance of the differences.

4. RESULTS

4.1. Expression and purification of GST-fusion fragments of YWK-II protein

Five oligonucleotides corresponding to specific segments of the extracellular region of mouse YWK-II cDNA were synthesized and expressed in *E. coli*. These segments covered the entire length of the extracellular region and designated as GST-Fa (segment 22-207/mouse YWK-II-763), GST-Fb (201-395, lacking 309-364)/mouse YWK-II-763), GST-Fc (201-395), GST-Fd (389-574) and GST-Fe (517-704, lacking 613-624/human YWK-II-763) (Figure 1A). All the expressed polypeptides were soluble products located in the cytosol of the bacteria except GST-Fa, which was formed in association with inclusion bodies. Fa was solubilized with 0.01 M NaOH and renatured with L-arginine. The solubilized Fa and other fusion peptides, including GST-Fb, GST-Fc, GST-Fd and GST-Fe, expressed in the cytosol were isolated by glutathione-affinity chromatography. The fusion polypeptides were examined by SDS-PAGE. Their apparent molecular weights (Mr) were 45, 48, 55, 47 and 50 kDa, for GST-Fa, GST-Fb, GST-Fc, GST-Fd and GST-Fe, respectively, which are in agreement with their theoretical molecular sizes (Figure 1B).

4.2. GST-fusion polypeptides renaturation and purification

All the GST-fusion peptide segments were expressed in *E. coli* BL21 except GST-Fe which was produced in E. coli DH5α. To enhance the production of soluble GST-fused polypeptides, the cultures were maintained at 25°C after the addition of IPTG and the induction time limited to 3.0 h. Under these conditions, GST-Fb was produced in a completely soluble state while portions of GST-Fc, GST-Fd and GST-Fe were produced in a soluble form in the cell cytosol.

4.3. Proteinase inhibitory activity of Fc

Fc contained a 56 amino acid segment (309-364), coding a domain homologous to KPI. The proteinase inhibitory activity of Fc was determined to evaluate whether the prokaryote-expressed protein in the bacterial cytosol retained the native conformation and possessed biological activity (Figure 2). The recombinant Fc completely inhibited the proteolytic activity of trypsin when tested at the molecular ratio of 1.3:1 (Fc: trypsin).

4.4. Effects of the recombinant polypeptides on fertilization

To determine whether the YWK-II protein is involved in the fertilization process, the effects of the fusion polypeptides on the *in vitro* fertilization rate were assessed. Among the recombinant polypeptides tested in an *in vitro* fertilization system, GST-Fa and GST-Fb reduced the fertilization rates by 56.5%($P<0.001$) and 52.2%($P<0.001$), respectively; whereas GST-Fc, GST-Fd and GST-Fe had no effect (Figure 3).

4.5. Inhibition of sperm binding to plasma membrane of the eggs by GST-Fa

The acrosome reaction in spermatozoa was induced by treating mouse sperms with solubilized zona

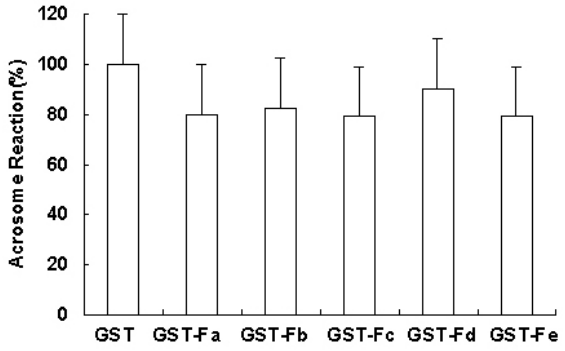

Figure 4. Effects of GST and GST-fusion proteins on ZP-induced acrosome reactions. Sperms were incubated for 30 min to induce capacitation, followed by incubation with GST or GST-fusion proteins (GST: glutathione S-transferase; Fa: segment 22-207 of YWK-II-763; Fb: segment 201-395 of YWK-II-763 lacking 309-364; Fc: 201-395 of YWK-II-763, including 309-364; Fd: 389-574 of YWK-II-763; Fe: 517-704 of YWK-II-763, lacking 613-624.) for 15 min. An aliquot of ZP suspension (pH 7.4) was added to the suspension of spermatozoa to a final concentration of 3 ZP/μL. After incubating for 15 min, the spermatozoa were collected, fixed and stained with Coomassie blue. The number of acrosome-reacted sperms was scored and recorded as percentage of total counted.

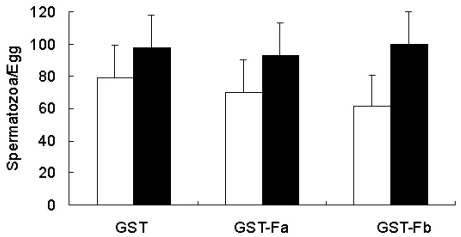

Figure 5. Effect of GST-Fa and GST-Fb on sperm binding to zona pellucidae of mouse eggs. A suspension of 2 X 10^6 spermatozoa and zona-intact eggs were incubated for 5 or 30 min. The eggs were collected, washed, fixed, and examined under a light microscope. The number of bound sperm per egg was scored and analyzed. GST: glutathione S-transferase; Fa: segment 22-207 of YWK-II-763; Fb: segment 201-395 of YWK-II-763 lacking 309-364.

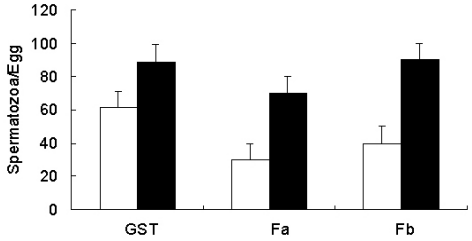

Figure 6. Effect of GST-Fa and GST-Fb on sperm binding to ZP-free eggs. A suspension of 2 X 10^6 sperms was incubated with zona-free eggs for 5 and 30 min. The eggs were collected, washed, fixed and the number of bound sperm scored by examination under a light microscope. GST: glutathione S-transferase; Fa: segment 22-207 of YWK-II-763; Fb: segment 201-395 of YWK-II-763 lacking 309-364.

proteins. None of the GST-fusion polypeptides tested influenced the zona-induced acrosome reaction (Figure 4).

However, GST-Fb reduced the early-stage binding (5 min) of sperm to ZP ($P<0.001$), while all segments failed to influence the late-stage binding (30 min) (Figure 5).

On the other hand, with zona-free eggs, GST-Fa and GST-Fb inhibited the early-stage binding of sperm to the egg. Fertilization rates were 48.4% ($P<0.001$) and 69.4% ($P<0.001$) of that found with GST control, respectively. Late-stage binding, however, was not affected ($P>0.05$) (Figure 6.). The present results suggest that the Fa domain participates in the binding of sperm to the plasma membrane of zona-free eggs while the Fb fragment is involved in the binding of sperm to either the ZP or the plasma membrane of zona-free eggs.

4.6. Blockage of sperm-egg membrane fusion by GST-Fb

The recombinant polypeptides were assayed for anti-fertility potency, using the fertilization system containing zona-free eggs (Figure 7). Only GST-Fb exhibited significant inhibition at concentrations of 62.5 μg/mL and higher. At concentrations of 500 μg/mL, it depressed the fertilization rate to about 10% of the control (Figure 7A). It can be concluded that GST-Fb blocked sperm binding to plasma membrane of eggs and reduced markedly the ability of sperm to fertilized zona-free eggs *in vitro*.

4.7. YWK-II protein on the plasma membrane of oocytes accounted for sperm-egg membrane fusion

Since YWK-II protein was located on spermatozoa and eggs (20), their relative importance in sperm-egg membrane fusion was examined. Capacitated spermatozoa and zona-free eggs were incubated separately with purified GST-Fa, GST-Fb or GST for 15 min. The various treated eggs and spermatozoa were washed in fresh mKR medium and incubated with the untreated eggs or spermatozoa. Spermatozoa pretreated with GST-Fb showed a 68% reduction in their fertilizing potential compared to GST-treated (control) (Figure 8). A slight decrease in the fertilizability of GST-Fb-treated eggs occurred although the effect was not statistically significant. The present finding shows that spermatozoa are sensitive to the effects of extraneous GST-Fb which probably interferes with the action of the *in situ* YWK-II protein.

5. DISCUSSION

The present results show that specific domains of the extracellular region of YWK-II protein may be involved in sperm-egg interaction. GST-Fa, prepared from the inclusion bodies, contains 12-cysteine residues and blocks the early-stage of sperm binding to the plasma membrane of intact eggs, indicating that this recombinant polypeptide is functional, active and possesses the proper conformation. GST-Fb, containing the domain rich in acid amino acids, however, effectively inhibits the fusion of sperm to zona-free eggs but not with intact eggs. Hence, the segment

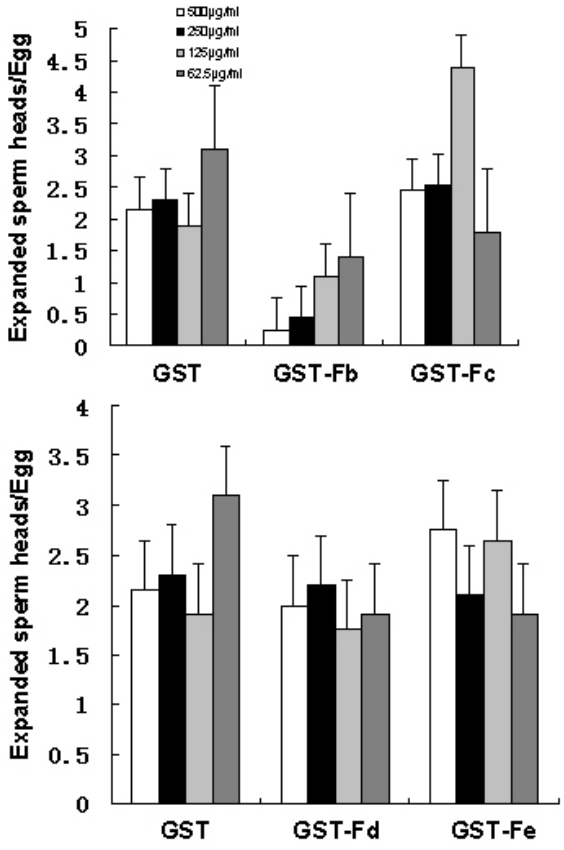

Figure 7. Effect of the GST and GST-fusion proteins on the fertilization rate of zona-free eggs. GST and GST-fusion proteins: (A) GST-Fb and GST-Fc and (B) GST-Fd and GST-Fe, were tested at various concentrations for their potential to inhibit the *in vitro* fertilization of zona-free eggs. The fertilization rates are given as percentage of fertilized eggs. GST: glutathione S-transferase; Fb: segment 201-395 of YWK-II-763 lacking 309-364; Fc: 201-395 of YWK-II-763, including 309-364; Fd: 389-574 of YWK-II-763; Fe: 517-704 of YWK-II-763, lacking 613-624.

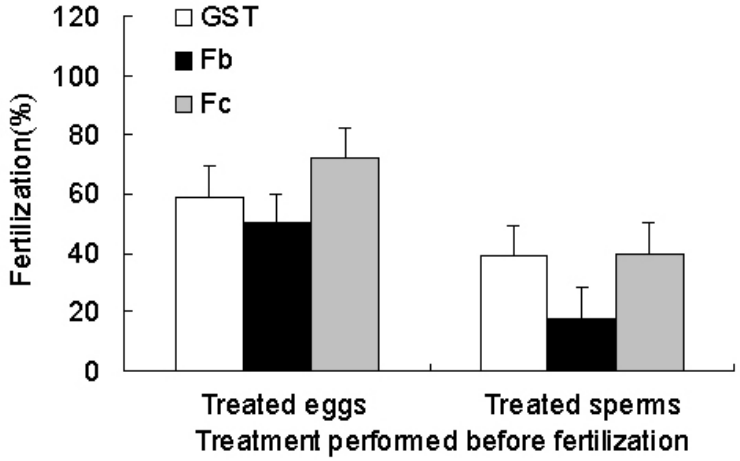

Figure 8. Effects of GST-Fb, GST-Fc and GST on the fertilizability of zona-free eggs and on the fertilizing potential of spermatozoa. Capacitated spermatozoa and zona-free eggs were incubated separately with purified GST-Fb, GST-Fc or GST for 15 min. The treated spermatozoa or eggs were washed in fresh mKR medium and incubated with the respective untreated eggs or spermatozoa. The fertilization rates are given as percentage of fertilized eggs. GST: glutathione S-transferase; Fb: segment 201-395 of YWK-II-763 lacking 309-364; Fc: 201-395 of YWK-II-763, including 309-364.

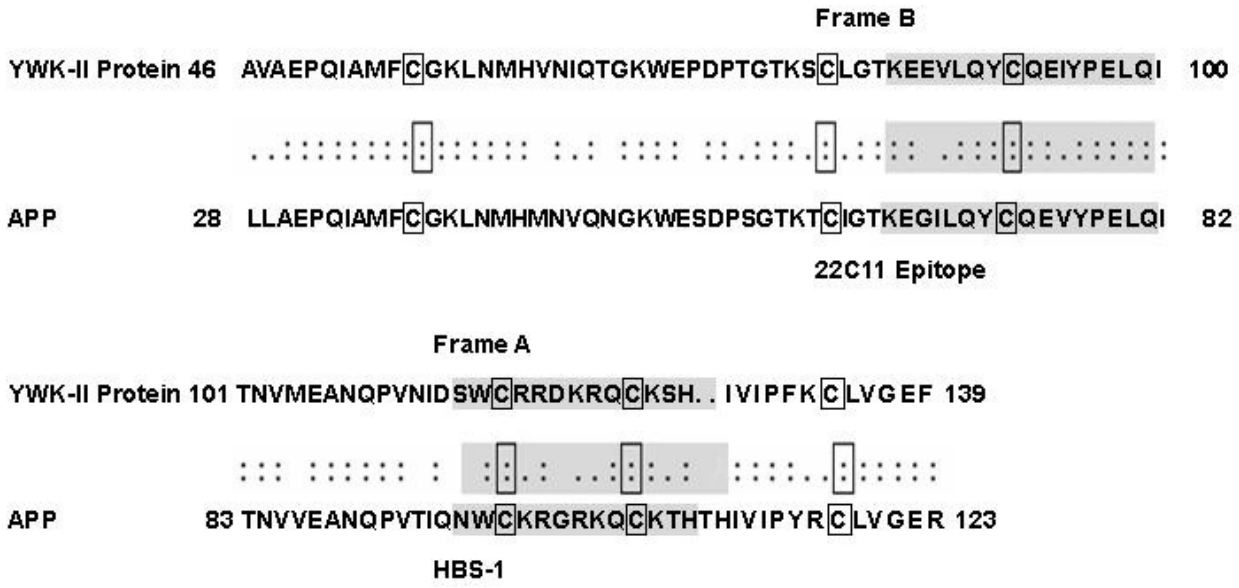

Figure 9. Amino acid sequences of the neurite outgrowth-promoting domain of APP and YWK-II protein containing the cysteine-rich growth factor-like region. Lower Frame A: heparin-binding (HBS) domain-1. Upper Frame B: 22C11 epitope.

composed of Fa and Fb participates in the fertilization process. On the other hand, the remaining three segments, Fc, Fd and Fe, did not influence any of the biological events involved in fertilization, namely, the acrosome reaction (Figure 4), the fertilization rate using zona-intact eggs (Figure 3) or the sperm-zona-free egg interaction (Figure 7).

It has been reported that APLP2 stimulates neurite outgrowth of embryonic chick sympathatic neurons, similar to the action of APP (10). It should be noted that since polypeptide Fa encompasses three domains, this section could account for the neurite-outgrowth-promoting activity of all the isoforms of APP. Since segment 114-126 of YWK-II protein corresponds to segment 96-108 of APP, it has the potential to promote neurite outgrowth by binding with heparin sulfate proteoglycans (HSPG) (21). This fragment contains a cluster of basic residues, the consensus sequence BBXB (B: basic amino acid, X: neutral amino acid) and two cysteine residues on both sides of the basic amino acid cluster (22) (Figure 9, frame A). The second segment, 84-99 of YWK-II protein has a high degree of similarity (93.7%) to segment 66-81 of APP, and to the epitope in 22C11 (23) (Figure 9, upper frame). The third segment 46-139, overlapping the other two sites mentioned above, has high similarity (88.5%) to and also shares the same three-dimensional structure with the corresponding segment 28-123 of mouse APP (Figure 9). This finding implies that Fa derived from YWK-II protein possesses the same structural characteristics as cysteine-rich growth factors (24), suggesting that these three segments of APP can act as a cytokine and affect target cells having the appropriate receptors or counterparts.

The counterpart of APP may be heparan glycoproteins since the neurite outgrowth promoting sequence is attributed to the heparin-binding site (HBS). Fa contains other HBS in segment 147-182, encompassing all the heparin binding properties, which corresponds to segment 131-166 of APP (25). On the other hand, sperm heads of a variety of species possess HBS on their surface including human, bull, monkey and rabbit (26,27). Furthermore, glycosaminoglycans and HBS together have been associated with (1) the promotion of capacitation and the induction of the acrosome reaction in spermatozoa obtained from bulls, boars, hamsters and humans (28-31); (2) conversion of proacrosin into acrosin (32); and (3) penetration of zona-free hamster eggs by human spermatozoa (33,34). The findings that the YWK-II protein is located on the acrosomal region of mouse spermatozoa and that HBS in Fa of YWK-II protein is involved with the binding of mouse sperm to egg suggest that the glycosaminoglycans on the surface of spermatozoa may participate in the fertilization process. The fact that the YWK-II protein is also located in the plasma membrane of mature oocytes suggests its involvement in oocyte metabolism and participation in the fusion with sperm.

Fa contains several interesting sequences listed above; whereas Fb contains only one domain of YWK-II protein, the negative-charged amino acid rich segment. This domain is characterized by the extension of two regions with 8 to 18 acid amino acids arranged in tandem. This kind of region with acidic amino acids arranged in a high density locus appears in many G-protein binding receptors with seven transmembrane domains. Although the function of the region is unclear, they might be involved in binding to positive-charged molecules. The occurrence of two negative-charged amino acids assembled region suggests that these two loci interact with their counterparts. Although GST-Fc contains the entire Fb segment and the KPI domain, it did not influence sperm-egg membrane fusion in contrast to GST-Fb. These differences in the effects of GST-Fb and GST-Fc can be attributed to the

proposition that YWK-II-695 without the KPI domain forms a new molecular binding domain at the spliced locus. This domain does not exist in GST-Fc. Another explanation is that GST-Fb without KPI acquire a new conformational structure that enhances the interaction between the YWK-II protein fragment (201-339) and its counterparts. This interaction may also exists between GST-Fc and its counterparts, but presumably by a comparatively weak affinity. In fact, previous studies on the affinity of various APP isoforms for the extracellular matrix proteins show that the binding of heparan sulfate proteoglycans and to laminin (at 4°C) by APP-695 is stronger than that of APP-770 or APP–751 (35,36). These differences can be attributed to the influence of the KPI domain on the conformation of APP isoforms. In conclusion, the marked *in vitro* inhibition of fertilization by GST-Fa and GST-Fb suggests that segment 22-308 participates in sperm-egg interaction and deletion of the KPI domain may modulate this activity.

6. ACKNOWLEDGEMENTS

This study was supported by grants from Special Fund for Major State Basic Research Project, National Natural Sciences Foundation of China, National High Technology Research and Development Plan of China, State Ministry of Science and Technology Program. The experimental study was performed in compliance with the laws of the People's Republic of China. The authors thank Professors D.Y. Chen, Q.Y. Sun and X.F. Song of The State Key Laboratory of Reproductive Biology, Beijing, for their valuable assistance with the fertilization studies. Drs Dazhong Zhuang and Yong Wang contributed equally to the manuscript.

7. REFERENCES

1. Y. Yan., L. Wang & S. S. Koide: Properties of a monoclonal antibody interacting with human sperm. *Arch Androl* 8, 245-254 (1987)

2. Y. Yan., Y. Bai, L. Wang, S. Miao & S. S. Koide: Characterization of cDNA encoding a human sperm membrane protein related to A4 amyloid protein. *Proc Natl Acad Sci USA* 87, 2405-2408 (1990)

3. Sprecher C. A., F. J. Grant, G. Grimm, P. J. O'Hara, F. Norris, K. Norris & D. C. Foster: Molecular cloning of the cDNA for a human amyloid precursor protein homolog: evidence for a multigene family. *Biochemistry* 32, 4481-4486 (1993)

4. Wasco W., S. Gurubhagavatula, M. D. Paradis, D. M. Romano, S. S. Sisodia, B. T. Hyman, R. L. Neve & R. E. Tanzi: Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid β protein precursor. *Nature Genet* 5, 95-100 (1993)

5. Sandbrink R., C. L. Masters & K. Beyreuther: Complete nucleotide and deduced amino acid sequence of rat amyloid precursor-like protein 2 (APLP2/APPH); two amino acids length difference to human and murine homologues. *Biochim Biophys Acta* 1219, 167-170 (1994)

6. Sandbrink R., C. L. Masters & K. Beyreuther: Similar alternative splicing of a non-homologous domain in βA4-amyloid protein precursor-like proteins. *J Biol Chem* 269, 14227-14234 (1994)

7. Goate A. M.: Monogenetic determinants of Alzheimer's disease: APP mutations. *Cell Mol Life Sci* 54, 897-901 (1998)

8. Mattson M. P.: Cellular action of β-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol Rev* 77, 1081-1132 (1997)

9. P. Huang, S. Miao, H. Fan, Q. Sheng, Y. Yan, L. Wang & S. S. Koide: Expression and characterization of the human YWK-II gene, encoding a sperm membrane protein related to the Alzheimer βA4-amyloid precursor protein. *Mol Hum Reprod* 6, 1069-1078 (2000)

10. Cappai R., S. S. Mok, D. Galatis, D. F. Tucker, A. Henry, K. Beyreuther, D. H. Small & C. L. Masters: Recombinant human amyloid precursor-like protein 2 (APLP2) expressed in the yeast *Pichia pastoris* can stimulate neurite outgrowth. *FEBS Lett* 442, 95-98 (1999)

11. Y. Yan, L Wang, SM Mitsudo & SS Koide: Characterization of an antisperm monoclonal antibody inducing human sperm agglutination. In: Immunological Approach to Contraception and Promotion of Fertility. Eds: GP Talwar, NY (1986)

12. Haneji T. & S. S. Koide: Identification of antigen in rat spermatogenic cells interacting with antihuman sperm monoclonal antibody. *Biol Reprod* 37, 467-477 (1987)

13. L Wang, Y. Yan, S. Miao & SS Koide: Immunobiology of human sperm. In: New Horizons in Spermatozoa Research. Eds: H Mohri, Tokyo (1987)

14. Y. Yan, P. Liang, L. Wang, S. Miao & S. S. Koide: Inhibition of zygote formation and embryo development in mice immunized with monoclonal anti-sperm autibodies. *Proc CAMS PUMC* 5, 9-13 (1990)

15. Vanage G., Y. Lu, J. P. Tam & S. S. Koide: Infertility induced in rats by immunization with synthetic peptide segments of a sperm protein. *Biochem Biophys Res Commun* 183, 538-543 (1992)

16. von Koch C. S., H. Zheng, H. Chen, M. Trumbauer, G. Thinakaran, L. H. van der Ploeg, D. L. Price & S. S. Sisodia: Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. *Neurobiol Aging* 18, 661-669 (1997)

17. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of the bacteiophage T4. *Nature* 227, 680-684 (1970)

18. Heald S. L., R. Tilton, L. J. Hammond, A. Lee, R. M. Bayney, M. E. Kamarck, T. V. Ramabhadran, R. N. Dreyer, G. Davis, A. Unterbeck & P. P. Tamburini: Sequential NMR resonance assignment and structure determination of the Kunitz-type inhibitor domain of the Alzheimer's β-amyloid precursor protein. *Biochemistry* 30, 10467-10478 (1991)

19. Moller C. C., J. D. Bleil, R. A. Kinloch & P. M. Wassarman: Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Dev Biol* 137, 276-286 (1990)

20. D. Zhuang, Y. Qiao, X. Zhang, S. Miao, S.S. Koide & L. Wang: YWK-II Protein/APLP2 in Mouse Gametes:Potential Role in Fertilization. *Mol Reprod Dev* 73, 61-67 (2006)

21. Small D. H. , V. Nurcombe, G. Reed, H. Clarris, R. Moir, K. Beyreuther & C. L. Masters: A heparin-binding

domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J Neurosci* 14, 2117-2127 (1994)
22. Cardin A. D. & H. J. Weintraub: Molecular modeling of protein- glycosaminoglycan interactions. *Arteriosclerosis* 9, 21-32 (1989)
23. Hilbich C., U. Monning, C. Grund, C. L. Masters & K. Beyreuther: Amyloid-like properties of peptides flanking the epitope of amyloid precursor protein-specific monoclonal antibody 22C11. *J Biol Chem* 268, 26571-26577 (1993)
24. Rossjohn J., R. Cappai, S. C. Feil, A. Henry, W. J. McKinstry, D. Galatis, L. Hesse, G. Multhaup, K. Beyreuther, C. L. Masters & M.W. Parker: Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein. *Nat Struct Biol* 6, 327-331 (1999)
25. Clarris H. J., R. Cappai, D. Heffernan, K. Beyreuther, C. L. Masters & D. H. Small: Identification of heparin-binding domains in the amyloid precursor protein of Alzheimer's disease by deletion mutagenesis and peptide mapping. *J Neurochem* 68, 1164-1172 (1997)
26. Delgado N. M., R. Reyes, L. Huacuja, H. Merchant & A. Rosado: Heparin binding sites in the human spermatozoa membrane. *Arch Androl* 8, 87-95 (1982)
27. Handrow R. R., S. K. Boehm, R. W. Lenz, J. A. Robinson & R. L. Ax: Specific binding of the glycosaminoglycan ^{3}H-heparin to bull, monkey and rabbit spermatozoa *in vitro*. *J Androl* 5, 51-63 (1984)
28. Lenz R. W., G. D. Ball, J. K. Lohse, N. L. First & R. L. Ax: Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and *in vitro* fertilization. *Biol Reprod* 28, 683-690 (1983)
29. Reyes R., A. Carranco, O. Hernandez, A. Rosado, H. Merchant & N. M. Delgado: Glycosaminoglycan sulfate as acrosomal reaction-inducing factor of follicular fluid. *Arch Androl* 12, 203-209 (1984)
30. Meizel S. & K. O. Turner: Glycosaminoglycans stimulate the acrosome reaction of previously capacitated hamster sperm. *J Exp Zool* 237, 137-139 (1986)
31. Delgado N. M., R. Reyes, J. M. Mora-Galindo & A. Rosado: Size-uniform heparin fragments as nuclear decondensation and acrosome reaction inducers in human spermatozoa. *Life Sci* 42, 2177-2183 (1988)
32. Parrish R. F., T. J. Wincek & K. L. Polakoski: Glycosaminoglycan stimulation of the *in vitro* conversion of boar proacrosin into acrosin. *J Androl* 1, 89-95 (1980)
33. Vasquez J. M., M.A. Winer, R. L. Ax & W. R. Boone: Correlation of human spermatozoa heparin binding with the zona-free hamster egg *in vitro* penetration assay. *Am J Obstet Gynecol* 160, 20-26 (1989)
34. Lalich R. A., S. Vedantham, N. McCormick, C. Wanger & G. S. Prins: Relationship between heparin binding characteristic and ability of human spermatozoa to penetrate hamster ova. *J Reprod Fertil* 86, 297-302 (1989)
35. Narindrsorasak S., D. E. Lowery, R. A. Altman, P. A. Gonzalez-DeWhitt, B. D. Greenberg & R. Kisilevsky: Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins. *Lab Invest* 67, 643-652 (1992)
36. Narindrasorasak S., R. A. Altman, P. A. Gonzalez-DeWhitt, B. D. Greenberg & R. Kisilevsky: An interaction between basement membrane and Alzheimer amyloid precursor proteins suggests a role in the pathogenesis of Alzheimer's disease. *Lab Invest* 72, 272-282 (1995)

Key Words: YWK-II Protein, APLP2, Sperm, Extracellular region

Send correspondence to: Linfang Wang, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing, 100005, People's Republic of China, Tel: 8610-6524-0803, Fax: 8610-6524-0529, E-mail: wanglf@ms.imicams.ac.cn

http://www.bioscience.org/current/vol11.htm