Extracellular domain of YWK-II, a human sperm transmembrane protein, interacts with rat Müllerian-inhibiting substance

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The YWK-II protein in human spermatozoa is structurally related to the β A4-amyloid precursor protein of Alzheimer disease and has high similarity with amyloid precursor homologues. Antibodies to the YWK-II protein agglutinate human spermatozoa and may be a potential cause of infertility. In the present study, a yeast two-hybrid system (MATCHMAKER Two-Hybrid System 2; Clontech, Palo Alto, CA) was used to screen a rat ovary cDNA library for potential ligands capable of interacting with the YWK-II component. Müllerian-inhibiting substance was found to

interact with the extracellular domain of YWK-II protein. The interaction was confirmed by a binding experiment *in vitro* and surface plasmon resonance assays. The recombinant Müllerian-inhibiting substance can significantly increase the viability and longevity of human spermatozoa after 5 and 22 h of incubation, presumably through binding the YWK-II component on the sperm membrane. The results of this study indicate that the YWK-II sperm membrane protein may function as a receptor for Müllerian-inhibiting substance.

Introduction

Sperm membrane proteins probably play an important role in fertilization. A human sperm membrane protein, YWK-II component, has been isolated and characterized, and the coding gene identified (Yan *et al.*, 1990; Huang *et al.*, 2000). The open reading frame consists of 763 codons encoding a polypeptide characteristic of a single transmembrane protein, established by hydropathicity analysis. The transmembrane and cytoplasmic domains of the YWK-II component and the β A4-amyloid precursor protein (APP) of Alzheimer disease have 70% similarity. The similarity is higher with the amyloid precursor homologues, APPH/APLP2 (Yan *et al.*, 1990; Sprecher *et al.*, 1993). The YWK-II protein and APP can be delineated into three domains: extracellular, hydrophobic transmembrane and cytoplasmic. The extracellular domains of these two proteins are distinctly different. Three peptide segments (YAL-198, YAL-201 and YAL-212) corresponding to a section of the extracellular domain of YWK-II were synthesized as a multiple antigen peptide (MAP) and administered to female rats to assess whether anti-YWK-II antibodies may be a cause of infertility. The YAL-198 induced infertility in seven out of 12 female rats and subfertility in two animals. Sera obtained from infertile rats immunized with YAL-198

contained higher titres of antibodies compared with those obtained from fertile animals, indicating that anti-YWK-II antibodies possess antifertility activity (Vanage *et al.*, 1992).

A study of the molecular properties of YWK-II component revealed that the cytoplasmic domain binds specifically G_o , a GTP-binding protein (Huang *et al.*, 2000), indicating that the YWK-II component can couple with G proteins and may activate the signal transduction system of responsive cells. The two-hybrid system relies on the modulating property of eukaryotic site-specific transcriptional activators to generate signals after the interaction of a protein fused to a DNA-binding domain with another protein fused to a transcription-activating domain (Bartel *et al.*, 1993). A yeast two-hybrid system was used to screen a rat ovary cDNA library. The ligands binding to the extracellular domain of YWK-II were identified. In the present paper, the interaction of the extracellular domain of YWK-II with the carboxy-terminus (74 amino acids) of Müllerian-inhibiting substance (MIS) was investigated.

Materials and Methods

Yeast strain

Saccharomyces cerevisiae strain Y190 was used. The partial genotype of Y190 cells MATa, his3-200, ura3-52, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4 Δ , gal80 Δ , cyh2, LYS2::GAL1_{UAS}-HIS3_{TATA}-HIS3, URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ.

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Fig. 1. Experimental strategy to identify proteins that interact with the extracellular domain of the YWK-II component of spermatozoa using the yeast two-hybrid system. Yeast strain Y190 cells were sequentially transformed with pAS2-1-HSD2 as bait plasmid and pGADGH rat ovary cDNAs. Transformants were selected using appropriate media. His⁺ LacZ⁺ cells were selected and analysed.

Plasmid construction

A segment (226 amino acids) of HSD2 encoding the extracellular domain contiguous to the transmembrane domain of the deduced YWK-II polypeptide was amplified by PCR and cloned into the GAL4 binding domain of the yeast shuttle vector pAS2-1, yielding pAS2-1-HSD2 to generate a bait plasmid (GAL4 fusion). The cloned DNA inserts were sequenced to ensure that no errors had been introduced during PCR and that the HSD2 sequences were contained within the same reading frame as GAL4 encoding sequence.

Transformation and library screening

Yeast interaction trap selections were performed (Fig. 1). Yeast transformants were generated by following a modified lithium acetate transformation protocol and grown at 30°C using the appropriate synthetic media. Yeast Y190 cells harbouring the pAS2-1-HSD2 were transformed using a rat ovary cDNA library containing the GAL4 transactivation domain in pGADGH (Clontech). Y190 cells possessed both *lacZ* and *HIS3* reporter genes. The transformants were plated on a complete synthetic medium lacking tryptophan, leucine and histidine, but containing 2% glucose and

25 mmol 3-aminotriazole l⁻¹, an inhibitor of the *HIS3* enzyme. The addition of this inhibitor was necessary to suppress background growth of yeast cells that may result from leaky transcription of the *HIS3* gene in the absence of any GAL4 function. Large (> 2 mm) colonies surviving the selection step were picked and assayed for β-galactosidase activity using a filter assay by observing the formation of a blue colour in the presence of the chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosides). Plasmids were obtained from colonies grown on the nutrient (His⁻) plates and showed positive reaction for β-galactosidase activity. The isolated plasmids were transformed into *Escherichia coli* HB101. Yeast Y190 cells were re-transformed with the candidate plasmids either alone or with pAS2-1 or pLAM5 (Clontech) containing the GAL4 DNA binding domain fused to a fragment of laminin to ascertain whether only positive plasmids are transactivated in the presence of pAS2-1-HSD2. Colonies were again assayed for β-galactosidase activity (Bartel *et al.*, 1993). Positive controls included Y190 cells transformed with pCL1, containing the full-length GAL4, and co-transformed with pVA3-1 and pTD1-1, containing the GAL4 BD fused to p53 encoding sequences and GAL4 AD fused to SV40 large T-antigen-encoding sequences. pCL1, pVA3-1 and pTD1-1

Fig. 2. The three sectors of the YWK-II component of spermatozoa used as bait proteins.

were obtained from Clontech. Plasmids from positive colonies, which were transactivated in the presence of pAS2-1-HSD2, were isolated and sequenced. A search for homology in the amino acid sequences of proteins deposited in the database was performed using the BLAST network service.

Identification of the active segment of 'bait protein'

HSD2 (Fig. 2) was delineated into three sectors to determine the segment of 'bait protein' that binds to the partner protein: (1) an alternatively spliced segment in the mid-portion composed of 63 amino acids; (2) a segment of 75 amino acids corresponding to the C-terminus, containing the 16 amino acid peptide, a potential antifertility immunogen; and (3) the remaining 97 amino acid segment of the N-terminus. All three sectors were fused individually to GAL4 BD in pAS2-1, generating three smaller bait proteins. Subsequently, the positive plasmid was co-transformed with each of these smaller bait proteins transformed into yeast cells. The culture media were assayed for β -galactosidase activity to verify and quantify the two-hybrid interactions.

Construction of protein expression clones

The NcoI/SalI fragment of the pAS2-1-HSD2 encoding the 226 amino acid polypeptide (HSD226) of HSD2 was ligated into pET-30a(+) (Novagen, Madison, WI). The 222 bp positive cDNA insert in the AD/library plasmid pGADGH (clone 17#) encodes a polypeptide identical to the segment, corresponding to amino acid residues 303–376 of rat MIS (Fig. 3) (Haqq *et al.*, 1992). This segment was excised and cloned into the pET-30a(+). The same polynucleotide segment encoding rMIS(303–376) was also ligated into pGEX-4T-3 (Amersham Pharmacia Biotech, Uppsala). The nucleotide sequences of the cloned inserts were validated by analysis.

Expression and purification of recombinant proteins

All recombinant protein expression plasmids were transformed into the *E. coli* strain BL21. The DNA insert in the pET-30a(+) was expressed as His₆-tagged fusion proteins and the insert in the pGEX-4T-3 was expressed as glutathione S-transferase (GST) fusion protein. The His₆-tagged HSD226 and His₆-tagged rMIS(303–376) were

purified using Ni-NTA resin (Novagen) according to the manufacturer's protocol. GST-rMIS(303–376) was purified on a glutathione Sepharose 4B (Amersham Pharmacia Biotech) column as reported by Frangioni and Neel (1993). The purified fusion proteins were dialysed overnight against PBS (138 mmol NaCl l⁻¹, 2.7 mmol KCl l⁻¹, 10 mmol Na₂HPO₄ l⁻¹, 1.8 mmol KH₂PO₄ l⁻¹, pH 7.4). Total protein concentrations were determined by the Lowry (1951) method.

Binding experiment in vitro

Purified His₆-tagged HSD226 (2.8 nmol) in 100 μ l PBS was loaded onto a glutathione Sepharose 4B column (column volume 50 μ l) prebound with GST (3 nmol) or GST-rMIS(303–376) (2.5 nmol). Each column was then washed with 50 column volumes of PBS. The columns were eluted with 100 μ l elution buffer, 50 mmol Tris-HCl l⁻¹, pH 8.0, 10 mmol reduced glutathione l⁻¹. The eluted proteins were analysed by SDS-PAGE and the separated proteins stained with Coomassie brilliant blue.

Surface plasmon resonance assays

All surface plasmon resonance (SPR) assays were performed using IAsys (Affinity Sensors, Cambridge, UK). Purified GST-rMIS(303–376) was immobilized in a carboxymethyl dextran-coated cuvette (Affinity Sensors), activated by treatment with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide according to the manufacturer's instructions. With each cycle of the experiment, 50 μ l His₆-tagged HSD226 in PBS-T (138 mmol NaCl l⁻¹, 2.7 mmol KCl l⁻¹, 10 mmol Na₂HPO₄ l⁻¹, 1.8 mmol KH₂PO₄ l⁻¹, 0.05% (v/v) Tween 20) was added over the chip. The chip was regenerated after each cycle by washing with 50 μ l of 1 mol formic acid l⁻¹ three times and

17#	78	RASNTRLALDPGALASFPQGLVNLSDPVALGRLLDGEEP	116
MIS	303	RASNTRLALDPGALASFPQGLVNLSDPVALGRLLDGEEP	341
17#	117	LLLLSPAAATVGEPMLRLHSPTSAPWAAAGLARRVA	151
MIS	342	LLLLSPAAATVGEPMLRLHSPTSAPWAAAGLARRVA	376

Fig. 3. Alignment of related segments of the deduced polypeptides of positive plasmid 17# and rat Müllerian-inhibiting substance (MIS). Within a stretch of 74 amino acids, 17# plasmid polypeptide and rMIS share 100% sequence identity.

Fig. 4. Purified recombinant fusion proteins analysed by SDS-PAGE. Ten or twelve per cent polyacrylamide gels were used and proteins were stained with Coomassie brilliant blue. The fusion proteins were analysed: His₆-tagged HSD226 (lane 1, 10% gel), His₆-tagged rat Müllerian-inhibiting substance (MIS)(303–376) (lane 2, 12% gel), GST- rMIS(303–376) (lane 3, 10% gel).

PBS-T three times. The response of the GST-rMIS(303–376)-coated chip with 50 μ l PBS-T was fixed as the baseline. Data were analysed using IAsys FASTfit software. Six concentrations (11.4, 8.57, 5.71, 2.86, 1.14 and 0.57 μ mol l⁻¹) of His₆-tagged HSD226 were used to calculate the K_D value of His₆-tagged HSD226 binding to GST-rMIS(303–376).

Determination of the effect of His₆-tagged rMIS(303–376) on the viability and longevity of human spermatozoa

Human ejaculates were obtained from seven healthy adults between the ages of 24 and 36 years. Semen samples were collected into sterile containers by masturbation after a period of sexual abstinence of 3–5 days and allowed to liquefy for 40 min in an incubator at 37°C. Semen baseline parameters were determined based on the World Health Organization protocol and criteria (WHO, 1994). BWW medium (2 ml) containing 3.5 mg BSA ml⁻¹ (Boehringer, Mannheim) was added to each round bottomed sterile tube. The swim-up procedure was performed: a 0.5 ml sample of liquefied semen was added to the tube, and the tube was sealed with Parafilm and placed in an incubator at 37°C for 1 h. The supernatants were aspirated gently and the sperm specimens were pooled. The spermatozoa were washed twice with BWW and collected by centrifugation at 300 g for 5 min at room temperature. The sperm concentration was adjusted to 20 $\times$ 10⁶ spermatozoa ml⁻¹. A sperm viability assay was performed according to the method of Siow *et al.* (1998) which involved an eosin–nigrosin exclusion test (WHO, 1994). The sperm suspension was divided into two fractions and 0.5 μ g His₆-tagged rMIS(303–376) in PBS was added to one fraction and an equal volume of PBS was added to the other fraction to serve as a control. Both fractions were incubated at 37°C. Sperm viability and longevity were evaluated at 0, 5 and 22 h of incubation. At each time-point, 100 spermatozoa were counted and scored as being live (exclusion of eosin) or dead (sperm permeated with eosin). The percentages of

viable spermatozoa between the experimental and control groups were compared and the significance of the differences determined statistically using Student's *t* test.

Results

Interaction of YWK-II with Müllerian-inhibiting substance in a yeast two-hybrid system

A yeast two-hybrid system transformed with rat ovary cDNA library was screened for components that bind to the extracellular domain of YWK-II. An ovarian protein was found attached to the YWK-II probe. The extracellular domain of YWK-II fused to the GAL4 binding region was bound to a target protein fused to the GAL4-activating domain. The binding was established by measuring the transcriptional activities of the two reporter genes, *HIS3* and *lacZ*. Both genes contained upstream the GAL4 binding sites. Among the approximately 2.1 $\times$ 10⁶ transformants, 860 colonies were found growing on the plates lacking tryptophan, leucine and histidine, although the size of the colonies varied over a wide range. Occurrence of the transformation was verified by measuring the β -galactosidase activity of the cells. Among the His⁺ colonies, 127 isolates gave positive reactions. Plasmids were isolated from 102 yeast colonies and transformed into *E. coli* HB101 that were selected nutritionally for the *LEU2* gene. The process was repeated, and the cloned plasmids were subjected to PCR amplification. The insert was identified by restriction endonuclease digestion map analysis. On the basis of the results, 31 colonies were selected and their plasmids isolated and reintroduced into the Y190 cells, either alone or together with pAS2-1-HSD2, or in combination with control plasmids, encoding other DNA-binding domain hybrids. After these rigorous selection steps, 24 plasmids were established as authentic specimens.

The nucleotide sequences of the plasmid segments were determined and their encoded polypeptides deduced. The amino acid sequences of the deduced polypeptides were compared with those proteins deposited in the database using the BLASTP program. One of the clones, 17# contained an insert identical to the carboxy-terminus (74 amino acids) of the open reading frame of rat MIS (Fig. 3). Moreover, only the mid-portion of the bait protein, consisting of alternatively spliced region of 63 amino acids, designated HSD2-2, interacted with MIS (Fig. 2). Quantitative measurement of β -galactosidase activity was performed to determine the affinity of the bait protein–MIS interaction. The affinity of MIS with the mid-portion segment (HSD2-2) of the bait protein and with the entire YWK-II was 30.4 and 8.22 expressed as β -galactosidase units, respectively, showing that the mid-segment was the reactive site.

Recombinant protein expression and purification

The 226 amino acid fragment of HSD2 used as bait in the yeast two-hybrid system was cloned into pET-30a(+) and

Fig. 5. SDS-PAGE analysis of His₆-tagged HSD226 bound to glutathione S-transferase (GST)–rat Müllerian-inhibiting substance (MIS)(303–376). Purified His₆-tagged HSD226 was loaded onto a glutathione Sepharose 4B column prebound with GST or GST–rMIS(303–376). The bound proteins were eluted with buffer containing reduced glutathione, collected and analysed by SDS-PAGE. Lanes 1–3: control buffer; lanes 1 and 5: GST; lanes 2 and 4: GST–rMIS(303–376); lanes 3–5: His₆-tagged HSD226.

expressed as a His₆-tagged fusion protein. The His₆-tagged HSD226, expressed in a soluble form, was purified using Ni-nitrilo-tri-acetic acid (NTA) resin under native conditions. The fusion protein migrates as a single band on SDS-PAGE (lane 1, Fig. 4). The 222 bp positive cDNA insert in the AD/library plasmid pGADGH (clone 17#) encoding the rMIS(303–376) was cloned into pET-30a(+) and pGEX-4T-3. The major part of His₆-tagged rMIS(303–376) was expressed as inclusion bodies and a lesser amount in the soluble form, which was purified under native conditions. However, GST–rMIS(303–376) was expressed mainly as inclusion bodies and purified by the method of Frangioni and Noel (1993).

Direct interaction of the extracellular domain of YWK-II with rMIS(303–376)

The interaction of the extracellular domain of YWK-II with rMIS(303–376) in the yeast two-hybrid system was confirmed by the binding experiment *in vitro*. Purified His₆-tagged HSD226 was loaded onto a glutathione Sepharose 4B column prebound with GST or GST–rMIS(303–376). The bound proteins were eluted with reduced glutathione and subsequently analysed by SDS-PAGE and protein staining. His₆-tagged HSD226 was bound to GST–rMIS(303–376) (lane 4, Fig. 5) but not to GST (lane 5, Fig. 5).

Fig. 6. Surface plasmon resonance assay of the binding of His₆-tagged HSD226 to glutathione S-transferase (GST)–rat Müllerian-inhibiting substance (MIS)(303–376). (a) Response curves with various concentrations of His₆-tagged HSD226; (b) plot of pseudo-first order rate constant (k_{on}) values versus His₆-tagged HSD226 concentration. From the linear plot of k_{on} values at various protein concentrations, the association and dissociation rate constants, k_{ass} and k_{diss} , are the slope and y intercept, respectively. The equilibrium constant, $K_D = 6.57 \mu\text{mol l}^{-1}$ was derived as the ratio of $k_{diss}:k_{ass}$.

Kinetics of interaction between His₆-tagged HSD226 and rMIS(303–376)

The kinetics of the interaction of His₆-tagged HSD226 with rMIS(303–376) were measured by SPR analysis (Fig. 6). Six different concentrations of His₆-tagged HSD226 were used. The association rate, k_{ass} , and the dissociation rate, k_{diss} , were obtained as the intercept and from the gradient of the plot of k_{on} versus ligand concentrations (Fig. 6), using the software FASTfit. The uncorrected values were as follows: k_{diss} (intercept) = 0.0121873 s^{-1} ; k_{ass} (gradient) = $1854.54 \text{ s}^{-1} \text{ mol}^{-1}$; K_D (k_{diss}/k_{ass}) = $6.57 \mu\text{mol l}^{-1}$.

Effect of the His₆-tagged rMIS(303–376) on viability and longevity of human spermatozoa

Mean sperm volume of the fresh ejaculates from the

Fig. 7. Effect of His₆-tagged rat Müllerian-inhibiting substance (MIS)(303–376) on the viability and longevity of spermatozoa. The viability value is the percentage (mean $\pm$ SD) of initial motile spermatozoa counted at 0 h. Spermatozoa were incubated with His₆-tagged rMIS(303–376) (■), or only in BWW (□). Statistical analysis was performed by Student's *t* test, $P < 0.01$ at 5 h, $P < 0.001$ at 22 h.

seven donors was 4.5 ± 0.42 ml (range 3–6 ml). The mean fresh sperm concentration was $195.9 \pm 13.9 \times 10^6$ spermatozoa ml⁻¹ (range 145–246 spermatozoa ml⁻¹) and the mean percentage of motile spermatozoa was $72.3 \pm 2.9\%$ (range 63.0–83.9%). The effect of MIS on the viability and longevity of human spermatozoa *in vitro* was determined (Fig. 7). Significant increases in the viability and longevity of spermatozoa were evident at 5 and 22 h of incubation, as determined by Student's *t* test (two-tailed comparison of means for paired samples).

Discussion

Yeast two-hybrid systems have been used to identify many novel interactions of proteins as they provide a simple but reliable transcriptional assay for detecting specific protein–protein interactions in yeast. Although many novel associations are predicated by the two-hybrid library screens and may reflect biological associations of two proteins *in vivo*, the functional co-relevance of two proteins scored as interacting in the two-hybrid system is unclear. The basis for the occurrence of false positives needs to be clarified. Some strategies have been developed to identify the causes (Serebriiskii, 2000). In the present study, 24 positive clones were identified and sequenced, and a search for homologous sequences was performed using the BLAST network service. Among these sequences, nine are new expressed sequence tags (ESTs) and known ESTs that are difficult to evaluate since the kind of proteins, if any, they encode is unknown. The other sequences are common extraneous contaminants that occur with the two-hybrid systems: three mitochondrial proteins, one zinc finger protein, two heat shock proteins, three ribosomal proteins and one cytoskeletal protein (for additional information, see the

Golemis Lab Web Site, www.fccc.edu:80/research/labs/golemis). In addition, two segments of *S. cerevisiae* chromosome II were identified that were probably introduced when the library was constructed. The two cytoplasmic proteins, rat neurabin (Burnett *et al.*, 1998) and rat Bat3 (Ozaki *et al.*, 1999), are apparently false positive since the extracellular domain of YWK-II protein is used as bait protein. Rat MIS is considered to be a genuine reactant because it is a hormone capable of interacting with the YWK-II protein, a putative receptor. MIS is a 140 kDa homodimeric glycoprotein secreted by fetal Sertoli cells during the development of mammalian testis (Blanchard and Josso, 1974; Donahoe *et al.*, 1977). The hormone promotes regression of the embryonic Müllerian ducts (Donahoe *et al.*, 1982), known to be the primordial structures of the female reproductive system. This activity of MIS assures the phenotypic development of the male reproductive tract. The gene coding rat MIS is composed of five exons encoding the precursor protein composed of 553 amino acids, which contains a leader sequence of 24 amino acids (Haqq *et al.*, 1992). A failure to express MIS activity by the embryos at the critical time during the differentiation of the gonadal anlage to testis will result in the postnatal retention of the Müllerian structures. Although MIS was believed initially to be produced only by the testes and is essential during fetal and early neonatal life, subsequent studies show that this hormone is also produced by ovarian granulosa cells (Vigier *et al.*, 1984; Takahashi *et al.*, 1986), indicating that MIS is not a male-specific substance. A continuous production of MIS by the ovary and testis seems to occur postnatally, to the extent that it attains detectable concentrations in the blood of adult men and women (Hudson *et al.*, 1990; Lee *et al.*, 1996). In men, the highest MIS concentration (50–70 ng ml⁻¹) occurs during the perinatal period and is maintained until adulthood (Hudson *et al.*, 1990). Pre-adolescent females begin to produce measurable amounts of circulating MIS equivalent to that of adult males; however, this decreases to undetectable amounts at the onset of menopause (Hudson *et al.*, 1990; Lee *et al.*, 1996). The findings that MIS is secreted by the ovary in women and is being produced throughout adulthood in men indicate that this hormone plays a functional role in the development of multiple organ systems in addition to its action as promoter of Müllerian duct regression.

MIS in human seminal plasma may influence sperm vitality (Fallat *et al.*, 1996). In healthy men categorized on the basis of WHO parameters as producing normal semen, the MIS concentration in seminal plasma ranges from 0.5 to 3.6 ng ml⁻¹. Seminal plasma ranges are significantly higher in teratozoospermic men (0.5–17.8 ng ml⁻¹) (Fallat *et al.*, 1996). MIS tends to increase the longevity of motility and viability of fresh and cryopreserved spermatozoa (Siow *et al.*, 1998). Similar results were obtained in the present study using a segment of MIS. These effects of MIS may be mediated by its binding to specific receptors on the sperm membrane. Examination by transmission electron

microscopy shows that MIS binds mainly to the surface of the sperm head, whereas practically no binding sites are found on the tail. In addition, spermatozoa from normal fertile men possess a larger number of MIS binding sites than those from subfertile men (Fallat *et al.*, 1998). Since YWK-II is a transmembrane protein located at the equatorial sector of the human sperm head and exhibits the properties of a receptor (Yan *et al.*, 1990; Sprecher *et al.*, 1993), the extracellular domain of YWK-II protein may act as the specific receptor for MIS. The present study shows that a segment of rMIS(303–376) binds to the 226 amino acids sector of the extracellular domain of YWK-II, determined in yeast cells using an *in vitro* system. The heterogeneous system used in the present study is acceptable but not ideal, since the binding segments of both YWK-II protein and MIS are highly homologous between the human and rat forms, that is, 91 and 79%, respectively (data obtained using the BLAST network service). Taking all these results together, it is reasonable to hypothesize that the YWK-II protein is the receptor of MIS. Although the affinity between His₆-tagged HSD226 and GST-rMIS(303–376) is low compared with the interaction of other ligands and their respective receptors, the bonding between MIS and YWK-II in their native conformation *in vivo* may be stronger. Furthermore, their affinity may be high should MIS acts as a dimer, thereby triggering dimerization of the receptor.

MIS is also known to be involved in ontogenic regulation, mediated through its binding to cell membrane receptors (Lee and Donahoe, 1993; Teixeira *et al.*, 1996). MIS blocks protein phosphorylation in A-431 cells by inhibiting phosphorylation of epidermal growth factor (EGF) receptor mediated by tyrosine kinase (Coughlin *et al.*, 1987). In addition, MIS is involved in the ontogenic regulation of fetal lung development by modulating EGF receptor phosphorylation (Catlin *et al.*, 1990, 1991). However, both the induction of capacitation and the occurrence of the obligatory acrosomal reaction during sperm–egg interaction involves protein phosphorylation mediated by tyrosine kinase (Furuya *et al.*, 1993; Yanagimachi, 1994; Burks *et al.*, 1995). Should the relationship between MIS binding and inhibition of protein phosphorylation in A-431 cells and fetal lung cells be a general cellular event, the interaction of MIS with YWK-II protein on the sperm surface may promote longevity and viability of motile spermatozoa, prevent capacitation or block the acrosomal reaction during fertilization. The cytoplasmic domain of YWK-II protein is coupled to the GTP-binding protein G_o (Huang *et al.*, 2000). Thus the binding of YWK-II protein with MIS would stimulate the G_o-mediated signal transduction pathway via its intracellular domain and influence sperm metabolism. The link between this pathway and protein phosphorylation by tyrosine kinase needs to be established.

In summary, the finding that YWK-II sperm membrane protein interacts with MIS indicates that it may function as the receptor for the hormone. Further studies need to be performed to elucidate the mechanism by which the two

molecules working co-operatively can sustain the viability and the fertilizing potential of spermatozoa.

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