Expression and characterization of the human YWK-II gene, encoding a sperm membrane protein related to the Alzheimer βA4-amyloid precursor protein

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The YWK-II cDNA, RSD-2, encoding a sperm membrane protein was isolated from a rat testis cDNA expression library. Using the RSD-2 insert in combination with rapid amplification of cDNA ends (RACE), the corresponding human gene was isolated from a human testis cDNA expression library. The human testis cDNA, HSD-2, is 3654 bp in length and contains an open reading frame of 763 codons. Hydropathicity analysis showed that the deduced polypeptide is a single strand transmembrane protein. The deduced polypeptide has partial homology with the amyloid precursor protein (APP) and high homology with the amyloid precursor homologue, APLP2/APPH. The YWK-II gene was mapped and assigned to human chromosome locus: 11q24-25. Northern blotting of various human tissue RNAs using the HSD-2 cDNA as a probe showed that the gene is transcribed ubiquitously. The cytoplasmic domain of HSD-2 was expressed in Escherichia coli. In-vitro studies showed that the recombinant polypeptide bound to a GTP-binding protein (Gα) and was phosphorylated by protein kinase C and cdc2 kinase. In mammalian F11 cells, the recombinant polypeptide was found to be coupled to Gα. Thus, the YWK-II component has the characteristics of a Gα-coupled receptor and may be involved in Gα-mediated signal transduction pathway. Protein kinase C and cdc2 kinase may regulate this pathway in spermatozoa by phosphorylating the cytoplasmic domain of the YWK-II component.

Key words: cell signalling/G proteins/protein kinase/sperm membrane protein/YWK-II

Introduction

Successful fertilization in mammals requires early and precise recognition and interaction between specific membrane components of the spermatozoa and oocytes. The membrane proteins may also play a role in spermatogenesis (Hecht, 1995). For a better understanding of the molecular mechanisms involved in spermatogenesis and gamete interaction, it is imperative that sperm membrane proteins should be identified and characterized (Yanagimachi, 1994).

In previous studies, a rat testis λgt11 cDNA expression library was screened with a monoclonal antibody (mAb) raised against the human sperm membrane protein, the YWK-II component (Yan et al., 1986, 1990). A cloned cDNA insert, RSD-2 (GenBank accession no. M31322), was isolated. RSD-2 is 1.8 kb in length and contains an open reading frame (ORF), encoding a polypeptide of 191 amino acid residues. A segment of the deduced polypeptide, position 694–763, has 70.6% (48 out of 68 amino acid residues) homology with the transmembrane cytoplasmic domain of the βA4-amyloid precursor protein (APP) found in the brain plaques of patients with Alzheimer’s disease (Yan et al., 1990).

The human YWK-II component is immunolocalized to the equatorial sector of human sperm heads (Yan et al., 1987). Components at this location are considered to participate in the interaction of spermatozoa and oocytes and in the subsequent fusion of the membranes during fertilization (Haneji and Koide, 1987). To determine whether the YWK-II component affects fertility, three oligopeptides corresponding to the extracellular domain of the deduced rat polypeptide were synthesized as multiple antigen peptides (MAP) and assessed as antifertility immunogens (Vanage et al., 1992). Three groups of female rats, 12 rats per group, were each immunized with a single MAP; with a different MAP used in each group. Fertility was determined by rat mating behaviour and by the presence of implanted embryos upon examination. The most effective of the three MAPs, YAL-198, caused infertility in seven out of 12 female rats and subfertility in two animals. These results clearly showed that production of antibodies to the YWK-II component reduced fertility in female rats probably by interfering with sperm–oocyte interactions (Vanage et al., 1992).

In the present study, a human testis cDNA library was screened with the RSD-2 cDNA as a probe and a full-length cDNA was isolated by subsequent 5′ rapid amplification of the cDNA ends (5′ RACE). The locus of the YWK-II gene in human chromosomes was determined, as was its expression in various human tissues. The YWK-II cDNA, HSD-2, was isolated and found to be composed of 3654 bp with an ORF of 763 codons. It is structurally related to APP and to the amyloid precursor homologue, APPH/APLP2 (Sprecher et al.,...
Amino acid sequence analysis of the deduced polypeptide revealed that the cytoplasmic domain contains a consensus sequence (20 amino acids), which is homologous with that found in APP and a C-terminal motif ‘BBXXB’ (B = basic residue; X = non-basic residue). The 20 amino acid segment in APP is capable of activating G proteins (Nishimoto et al., 1993) and possesses potential sites for phosphorylation by heterotrimeric guanine nucleotide binding protein (G protein) signalling system, this segment was expressed in Escherichia coli in the form of a glutathione-S-transferase (GST) fusion protein and also in mammalian cells as an epitope-tagged protein. Evidence is presented to validate the hypothesis that the YWK-II component may function as a signal transducing G protein receptor in the sperm membrane.

Materials and methods

Screening of human testis cDNA library

RSD-2, a truncated cDNA of 1.8 kb (Yan et al., 1990), was labelled by random priming, using a digoxigenin (DIG)-labelling kit (Boehringer Mannheim, Indianapolis, IN, USA) with DIG-dUTP. The labelled RSD-2 was used to screen a human testis expression library (gt11 cDNA library; Clontech Corporation, Palo Alto, CA, USA), according to the manufacturer’s instructions. cDNA was prepared as described previously (Wang et al., 1990) from positive clones and digested with EcoRI (Sambrook et al., 1989) for Southern blot analysis. Sequencing of the cDNA was carried out as described below.

5’ RACE for YWK-II cDNA

The 5’-end of the YWK-II cDNA was prepared using a RACE Kit (Boehringer Mannheim) according to a previously described method (Frohman et al., 1988). mRNA was prepared from human testis as described in a previous report (Liu et al., 1992) and used as template. The specific antisense primer SP1 (5’-GGGTTCAAGTATTCCCTCATTGGT-3’), matching the region 227 bp (848–873 bp) downstream from the 5’-end of the YWK-II cDNA was synthesized and used with the template and reverse transcriptase. The first strand of the cDNA formed was purified and polyadenylated by adding a homopolymeric A-tail to the 3’ end of cDNA (Schaefer et al., 1995) using terminal deoxynucleotidyl transferase and dATP. The synthetic poly(A) segment served as the target for the poly(T)-containing anchor primer. This tiled-cDNA was amplified by using oligo(dT)-anchor primer in combination with a second specific primer SP2 (5’-AGCTGCTTCTGTGAAGTCTTCCAG-3’) matching the region 116 bp (736–759 bp) downstream from the 5’-end of the YWK-II cDNA. To ensure specificity of the reaction, a nested polymerase chain reaction (PCR) was performed with the anchor primer and a third primer SP3 (5’-TTTCCTCTTTGGACACAGAT-3’) matching the region located 9 bp (630–652 bp) downstream from the 5’-end of the YWK-II cDNA. The amplified PCR products were cloned and sequenced.

DNA sequencing and analysis

The cDNA inserts prepared from positive clones were cloned into vector pUC19 and sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the Sequenase™ version 2.0 DNA Sequencing Kit System (US Biochemical Corporation, Cleveland, OH, USA). In sequencing each cDNA, several overlapping fragments were prepared and subcloned. Each fragment was sequenced from both ends. To determine sequence homology with other components, proteins in the Data Bank of the National Center for Biotechnology Information were searched with the use of the Pearson and Lipman Blast program. Amino acid sequence identity was determined with the Blastp and Blastx programs (Wang et al., 1999a).

Multiple tissue Northern blot analysis

Human tissue samples were obtained from the Peking Union Medical Hospital (Beijing, China) and mRNA was prepared according to a previous report (Liu et al., 1992). A 2 µg sample of mRNA was applied to each lane for Northern blot analysis. Multiple tissue Northern blot nylon membranes were purchased from Clontech Corporation. The HSD-2 cDNA probe (1.8 kb from 667–2510 bp) was labelled with [α-32P]-dCTP (Amersham Corporation, Piscataway, NJ, USA) by random priming as described previously (Wang et al., 1999b). The hybridization procedure was performed as described in a previous report (Wang et al., 1999b).

Fluorescence in-situ hybridization

The locus of the YWK-II gene on chromosomes prepared from human blood leukocytes was determined by fluorescence in-situ hybridization (FISH), combined with 4,6-diamidino-phenylindole (DAPI) staining, as described in a previous report (Wang et al., 1999b). The HSD-2 cDNA probe (Wang et al., 1999b) was biotinylated with DATP using the BRL BioNick labelling kit (Life Technologies, Bethesda, MD, USA) according to the manufacturer’s instructions.

Expression, purification, and cleavage of GST fusion protein

A fragment of Ball/DraI cDNA composed of 357 bp corresponding to a segment (2110–2467 bp) of HSD-2, encoding the cytoplasmic domain (47 amino acids), and a section of the transmembrane domain (13 amino acids) of YWK-II protein was cloned in-frame into the bacterial GST fusion protein expression vector, pGEX-4T-3 (Pharmacia, Piscataway, NJ, USA), constructing the expression plasmid pGEX-4T-HSD-2–350. The recombinant plasmid was sequenced using the ABI auto-sequencer (model No. 377) and used to transform the Escherichia coli strain BL21 (DE3). Positive clones were picked, cultured and treated with 0.1 mmol/l isopropyl-β-d-thiogalactoside (IPTG) to induce expression from the lac operon in the recombinant plasmid. The expressed protein (GST-60AA) was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 12% gel, extracted from the gel, dialysed against 50 mmol/l Tris–HCl, pH 8.0, plus 0.25% NP-40 for 72 h and subsequently against the same buffer without NP-40, for 24 h. The purity of the expressed GST-60AA was determined by SDS–PAGE on a 12% gel followed by staining of the protein bands with Coomassie Blue, and confirmed by Western blotting on a nitrocellulose membrane using goat anti-GST antibody (Pharmacia, Uppsala, Sweden). Protein concentrations were determined using the Bio-Rad Protein Assay Kits (Bio-Rad Laboratories, Hercules, CA, USA). Thrombin cleavage of GST-60AA was performed in phosphate-buffered saline (PBS; 140 mmol/l NaCl, 2.7 mmol/l KC1, 10.1 mmol/l Na2HPO4, 1.8 mmol/l H3PO4, pH 7.3) at room temperature (22–25°C) for 6 h. Bacterial cells, harbouring pGEX-4T-3, expressed GST alone under the same conditions as above. As a control, GST was purified by chromatography on a glutathione–Sepharose column (Pharmacia) according to the manufacturer’s protocol.

Construction of epitope-tagged cytoplasmic domain of YWK-II antigen (HA tag-60AA)

Epitope-tagged cytoplasmic domain of YWK-II (HA tag-60AA) was generated by introducing a 9-amino acid peptide obtained from the
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Co-immunoprecipitation and immunoblotting

Identifying the G protein interacting with GST-60AA

Goat anti-GST antibody (0.1 ml, Pharmacia) was mixed with 0.1 ml of protein-G agarose (CalBiochem, Pasadena, CA, USA). To the mixture was added 2.5 ml TETN buffer [20 mmol/l Tris–HCl, pH 7.6, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% Nonidet P-40 (NP-40)] and the mixture was shaken at 4°C for 4 h. The supernatant was removed, and the sedimented agarose was washed three times with 2.5 ml of TETN buffer, and collected by centrifugation. The washed beads were resuspended in 2.5 ml TETN buffer containing 250 µg GST-60AA or GST, and the suspension was placed on a shaker at 4°C for 48 h. The mixture was centrifuged, and the precipitated beads were washed as above five times, then resuspended in 400 µl buffer D [20 mmol/l HEPES/NaOH, pH 7.4, 1 mmol/l EDTA, 120 mmol/l NaCl, 1.1 mmol/l MgCl2, 2% BSA, 2 mmol/l phenyl methyl sulphonyl fluoride (PMSF), 20 µg/ml aprotinin, 20 µg/ml leupeptin] containing 16 µl of bovine brain total G protein (50% Gs, 20% Gi1, 20% Gi2, 10% Gi3). The suspension was shaken at 4°C overnight. The beads were collected, washed five times with TETN buffer, resuspended in 140 µl of SDS–PAGE sample buffer, boiled for 10 min, and centrifuged. The proteins in the supernatant were separated by SDS–PAGE and subjected to Western blot analysis by incubation overnight at 4°C with specific rabbit anti-G protein α-subunit antibodies (CalBiochem, Pasadena, CA, USA) and subsequently with AP-conjugated goat anti-rabbit IgG. The immunoreacted bands were visualized by staining with nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP) solution.

Identifying the G protein interacting with the product of HA tag-60AA

The transformed F11 cells (expressing HA tag-60AA) were collected, resuspended in a lysis buffer containing 50 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 0.5 mmol/l PMSF, 1 µg/ml aprotinin, 1% NP-40 (Serva), placed on ice, and mixed for 1 h. Cell debris was removed by centrifugation at 12 000 rpm at 4°C for 10 min. Anti-HA mAb 12CA5 (Boehringer Mannheim) (4 µg/ml) was added to the supernatant (cell lysate), and the mixture was incubated at 4°C for 1 h. Protein G-agarose (Boehringer Mannheim; 20 µl) was added and incubated at 4°C on a rocker platform for 1 h. The immunoprecipitated proteins were collected by centrifugation, washed, dissolved in 40 µl of electrophoresis sample buffer, boiled for 10 min, and subjected to SDS–PAGE and immunoblotting analysis. The protein bands were stained with anti-Gα,α antibodies (as described above).

\[\gamma^{[35S]}\text{GTP binding assay}\]

Binding of \([\gamma^{35S}]\text{GTP} (DuPont NEM, Boston, MA, USA) by bovine brain total G proteins (CalBiochem, Pasadena, CA, USA) was determined using a previously described method (Northup et al., 1982). Binding was initiated by adding 5 mmol/l \([\gamma^{35S}]\text{GTP} (10^{-5} \text{cpm})\). Non-specific binding was <10% of the total binding under identical experimental conditions; however, in the presence of a 1000-fold excess of unlabelled GTP, none of the proteins tested bound \([\gamma^{35S}]\text{GTP}.\) The ratio of bound GTP was derived from the formula:

\[
\text{total bound of }[\gamma^{35S}]\text{-GTP} - \text{non-specific bound }[\gamma^{35S}]\text{-GTP}
\]

\[
\text{Total }[\gamma^{35S}]\text{-GTP}
\]

\([\gamma^{35S}]\text{-GTP} \) was pre-diluted (1:1000) in a buffer of 10 mmol/l Tris–HCl, pH 7.6, 10 mmol/l dithiothreitol (DTT) and frozen at ~70°C. G proteins from bovine brain were reconstituted in phospholipid vesicles using 1 mg/ml phosphatidylethanolamine (Sigma, St Louis, MO, USA) at a final G protein concentration of 100 nmol/l in a buffer containing 20 mmol/l HEPES/NaOH, pH 7.6, 0.1 mmol/l EDTA.

| haemagglutin (HA) sector of influenza virus located at the amino terminus of the cytoplasmic domain. The strategy used for epitope addition was based on oligonucleotide-directed mutagenesis using PCR and the 5′ primer (60 bp): 5′ GGAATTCATGTTGGATTACCC-CTTATGATGTTGACATTGCGATTGCACGGTCATGCT 3′; and the 3′ primer (26 bp): 5′ CAGGATCCTCCCTGGCAATCT 3′. The underlined part encodes the HA 9AA epitope. The HA epitope (YPYDVPDYA) is recognized by the anti-HA mAb 12CA5 (Boehringer Mannheim). The PCR product encoding HA tag-60AA and consisting of 230 bp was subsequently cloned into the eukaryotic expression vector pcDNA3.1Myc-HisA (a gift of Dr Lu Hai Wang, The Mount Sinai Medical College, NY, USA) by EcoRI/BamHI (Boehringer Mannheim) directional insertion (with the EcoRI site introduced at the 5′ end of the 5′-primer, and BamHI site at the 5′-end of the 3′ primer). The structure of the recombiant vector, pcDNA3.1A-230, was verified by sequence analysis. | Expression of the cytoplasmic domain of YWK-II antigen (HA tag-60AA) in F11 cells | Co-immunoprecipitation and immunoblotting

| The cDNA fragment encoding the cytoplasmic domain of YWK-II component was amplified by PCR using HSD-2 cDNA as the template. The 5′ primer (described above) was 60 bp in length and contained the following: (i) an EcoRI site; (ii) the initiating codon, ATG; (iii) the sequence encoding the 9AA HA epitope; and (iv) the sequence containing the BalI site located 17 bp downstream on HSD-2 cDNA. The 3′ primer, a 26 bp oligonucleotide, contained a BamHI site and an 18 bp sequence whose complementary sequence contained the stop codon TAG in the ORF of HSD-2 cDNA. Following PCR, a 230 bp fragment was generated, excised with both EcoRI and BamHI, and then ligated directionally into the eukaryotic expression vector pcDNA3.1Myc-His. The resulting recombinant expression plasmid, pcDNA3.1A-230, was transfected into F11 cells (gift of Dr L.Nishimoto, Department of Pharmacology, Keio University, Japan). These cells are hybridomas prepared by the fusion of a primary rat DRG neuron and a mouse neuroblastoma cell N18TG2, over-expressing Go and maintained and cultured in the high glucose medium, Dulbecco’s modified Eagle’s medium (DMEM)/F12 (50/50 v/v, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS). These cells were transfected with pcDNA3.1A-230, using the new type of Lipofectin-DOTAP (Boehringer Mannheim). To select stable transfected cells, genetecin (G418, 1 mg/ml; Gibco) was added to the culture medium. Antibiotic-resistant colonies of transfected cells were picked after 2–3 weeks of incubation and expanded into cell lines that were maintained in a medium containing 0.2 µg/ml G418. The transfected cells were assayed for the expressed protein (HA-tag-60AA) by immunofluorescence using mAb 12CA5, as the primary antibody, and FITC-conjugated rabbit anti-mouse immunoglobulin G (IgG) as the second antibody (as described below). | Immunofluorescence and confocal laser scanning microscopy

| For experiments using permeabilized cells, stable transfected F11 cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. The cells were permeabilized by suspending in PBS containing 0.2% NP-40, 0.5% bovine serum albumin (BSA) at room temperature for 15 min and subsequently in the same medium containing mAb 12CA5 (1 µg/ml) at 4°C for 40 min. The second antibody (FITC-conjugated rabbit anti-mouse IgG) diluted 1:50 was applied in the same buffer. The expressed HA tag-60AA in transfected F11 cells was detected by confocal laser scanning microscopy (Meridian Instruments Inc, Okemas, MI, USA), according to manufacturer’s instructions. | |
1 mm/l DTT. The final incubation mixture for the determination of bound \( [\gamma^{35}S] \)-GTP contained 10 mmol/l of reconstituted G proteins.

**In-vitro phosphorylation assay**

Protein kinase C (PKC) and cdc2 kinase were purchased from CalBiochem Corporation (Pasadena, CA, USA) and cyclin B from New England Biolabs (Boston, MA, USA). The substrate protein or peptide (GST-60AA or the 0.8 kb thrombin cleavage product; 40 \( \mu \)mol/l) was incubated with PKC (1 \( \mu \)l) in a reaction volume of 30 \( \mu l \) containing 50 mmol/l Tris–HCl, pH 7.5, 0.5 mmol/l CaCl\(_2\), 15 mmol/l MgCl\(_2\), and freshly added 0.15 mmol l-\( \alpha \)-phosphatidyl-L-serine (PS) and 0.06 mg/ml 1,2-dioleoyl-rac-glycerol (DG). The protein or peptide (40 \( \mu \)mol/l) was incubated with cdc2 kinase (1 \( \mu \)l) in a reaction volume of 30 \( \mu l \) containing 50 mmol/l Tris–HCl, pH 7.5, 10 mmol/l MgCl\(_2\), 1 mmol/l EGTA, and 1 mmol/l DTT. The reaction was initiated by adding 2 \( \mu l \) of 2 mCi/ml \( [\gamma^{32}P] \)-ATP (3 Ci/mmol; Amersham Corporation), and the mixture was incubated for 60 min at 30°C. The reaction was stopped by adding concentrated SDS-stop solution. The radio-labelled peptides or proteins were collected, and separated by SDS–PAGE, and autoradiography was performed overnight at –70°C using an intensifying screen and Kodak X-Omat AR film.

**Results**

**Characterization of the cDNA coding the human sperm membrane protein YWK-II**

A human testis cDNA expression library was screened with RSD-2 as probe, yielding five positive clones which were sub-cloned (Figure 1A). The cDNAs were isolated and sequenced. There were two regions of deletion at codons 613–624 and 562–624, indicative of splicing sites. The cDNA was extended at the 5’ end by RACE. The full cDNA, HSD-2, consisted of 3654 bp with an ORF of 2289 bp encoding 763 amino acids and a 3’ non-coding region of 1365 nucleotides but lacked a 5’ non-coding region (Figure 1B). Hydropathy analysis indicated that it was encoded as single strand transmembrane protein.

**Chromosome assignment of YWK-II**

The locus of the YWK-II gene in human chromosomes was established by FISH (Figure 2A) in combination with DAPI staining (Figure 2B), and assigned to chromosome 11q24-25. A diagram of FISH mapping of HSD-2 on chromosome 11 is depicted in Figure 2C.

**Tissue distribution**

Northern blot analysis of human tissue mRNAs showed that the YWK-II gene was transcribed in all tissues examined. Two transcripts of YWK-II were detected: a major 4.4 kb mRNA (probably containing a longer non-coding segment or by splicing), and a minor 3.6 kb mRNA. In the pancreas, however, the 3.4 kb mRNA was found to be the major transcript (Figure 3). Similarly, other workers (Sprecher et al., 1993) found two major transcripts of APPH in various human tissues, a major mRNA of 3.8 kb and a less abundant mRNA of 3.0 kb.

**Expression, purification, and cleavage of GST fusion protein**

A high level of expression of the GST fusion protein (GST-60AA) occurred in IPTG-treated *E.coli* transformed with pGEX-4T-HSD-2–360. The recombinant protein was collected and analysed by SDS–PAGE (Figure 4). A protein band corresponding to the expected molecular size (33 kDa) of the expressed fusion protein (GST-60AA) was detected in the IPTG-induced cells (Figure 4, lane 3) and was not expressed in uninduced bacteria (Figure 4, lane 2). The expressed protein GST-60AA, separated by SDS–PAGE, was verified as the 33 kDa band (Figure 5, lane 5). When *E.coli* cells, harbouring the expression vector pGEX-4T-3, were induced with IPTG, a 29 kDa protein was produced. This protein was collected, purified by chromatography on a GSH-coupled Sepharose 4B, analysed by SDS–PAGE, and identified as GST (Figure 5, lane 3). The purified GST-60AA (Figure 6, lane 2) and GST (Figure 6, lane 1) were found to be homogenous upon analysis by SDS–PAGE. The respective bands were identified by positive staining with anti-GST antibody. Cleavage of GST-60AA with thrombin, yielded two fragments: GST (29 kDa) and a 0.8 kDa peptide containing the cytoplasmic domain of YWK-II antigen (Figure 5, lane 4). Treatment of GST with thrombin resulted in a slightly smaller polypeptide by the removal of the expression vector polycloning site encoding a segment consisting of 16 amino acid residues (Figure 5, lanes 1 and 3).

**Effect of cytoplasmic domain (GST-60AA) of YWK-II component on \( [\gamma^{35}S] \)-GTP binding by G proteins**

The GST-60AA, containing the cytoplasmic domain of YWK-II antigen, significantly increased the binding of \( [\gamma^{35}S] \)-GTP by bovine brain G proteins, whereas the stimulatory activity of GST was minimal (Figure 7). The binding ratios were 80–90 compared with 10–20 (Figure 7A) respectively. The

![Diagram](figure.png)
Expression and characterization of the YWK-II gene

Figure 1. Legend on facing page.
Figure 2. Fluorescence in-situ hybridization (FISH) mapping of YWK-II gene (HSD-2) and 4,6-diamidino-phenylindole (DAPI) staining of metaphase chromosomes of peripheral blood leukocytes. (A) FISH signals on metaphase chromosomes from blood leukocytes. Note FISH signals at 11q24-25 are indicated by arrows; (B) chromosomes stained with DAPI to identify chromosome 11; and (C) diagrammatic representation of FISH mapping of HSD-2 in chromosome 11.

Figure 3. Northern blot of YWK-II mRNAs prepared from human tissues. (A) Lane 1 = heart; lane 2 = brain; lane 3 = placenta; lane 4 = lung; lane 5 = liver; lane 6 = skeletal muscle; lane 7 = kidney; lane 8 = pancreas. (B) Lane 1 = spleen; lane 2 = thymus; lane 3 = prostate; lane 4 = testis; lane 5 = ovary; lane 6 = small intestine; lane 7 = colon; lane 8 = peripheral lymphocytes. Samples contained 2.0 µg mRNA per lane. Note the staining of two transcripts at 4.4 and 3.6 kb.

facilitating potential of GST-60AA was concentration dependent (Figure 7B). At 66 µmol/l, the binding activity was increased ~4-fold compared with that observed at 1 µmol/l. Furthermore, GST-60AA alone did not bind [γ-35S]-GTP. Thus, the cytoplasmic domain of YWK-II possessed the potential to facilitate [γ-35S]-GTP binding by G proteins in vitro.

In-vitro interaction of cytoplasmic domain of YWK-II (GST-60AA) with Go
GST-60AA was incubated with bovine brain total G proteins and immunoprecipitated with anti-GST antibodies. The immunoprecipitated proteins were collected and analysed by immunoblot using anti-Giα3/Giα3 and anti-Giα3 antibodies. A 39 kDa band, corresponding to Gi protein, was stained with anti-Giα3/Giα3 antibody (Figure 8, lane 3); however, no staining occurred when GST was used as control (Figure 8, lane 2). These findings showed that the 60 amino acid segment of the cytoplasmic domain of YWK-II possessed the potential to bind Gi, whereas GST did not.

YWK-II antigen (HA tag-60AA) expressed in F11 Cells
When F11 cells were transfected with pcDNA 3.1 Myc-HisA and examined by fluorescence cell microscopy, >99% of the cells emitted fluorescence. In contrast, <1% of the control cells showed fluorescence (data not shown). These results show conclusively that the cDNA fragment coding the 60 amino acid segment of the cytoplasmic domain is expressed in the transfected F11 cells.
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**Figure 4.** Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from *Escherichia coli* BL21 transfected with GST-60AA. Lane 1 = low molecular mass standard; lane 2 = lysates of bacterial cells harbouring pGEX-4T-HSD-2–360, cultured in the absence of isopropyl-β-D-thiogalactoside (IPTG); lane 3 = lysates of bacterial cells harbouring pGEX-4T-HSD-2–360, induced with 0.1 mmol/l IPTG for 4 h.

**Figure 5.** Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of expressed GST-60AA and GST treated with thrombin. Lane 1 = glutathione-S-transferase (GST) cleaved with thrombin; lane 2 = pre-stained SDS-PAGE standards, low molecular weight (19–104 kDa); lane 3 = GST, purified by GSH affinity chromatography; lane 4 = GST-60AA cleaved with thrombin; lane 5 = GST-60AA purified by SDS-PAGE; lane 6 = low molecular mass standard (14–97 kDa).

**Cytoplasmic domain of YWK-II component (HA tag-60AA) binds Go in F11 cells**

The binding of Go by the cytoplasmic domain of YWK-II was determined by analysis of F11 cells transfected with HA tag-60AA. When the lysates of the transfected F11 cells were treated with anti-HA antibody, HA-60AA was found to co-precipitate with Go (Figure 9, lane 2). This finding established that YWK-II component and Go form a complex, or are coupled within the F11 cells.

**Phosphorylation of the cytoplasmic domain of YWK-II antigen in vitro**

GST-60AA (33 kDa) and the thrombin-cleaved 0.8 peptide (containing the cytoplasmic domain of YWK-II) were found to be phosphorylated in vitro when treated with PKC and cyclin B/cdc2 (Figure 10A,B, lanes 1 and 2). With GST as the substrate, however, cleavage with thrombin did not yield phosphorylated fragments (Figure 10A,B, lane 3). The present results show that the cytoplasmic domain of the YWK-II antigen contains sites for phosphorylation.

**Discussion**

In the present study, a human testis expression library was screened with RSD-2 as a probe and the positive clones were sequenced. The sequence was completed with 5'RACE, yielding a full-length cDNA, HSD-2, which encodes the YWK-II component. HSD-2 consists of 3654 bp with an ORF of 2289 bp encoding a polypeptide of 763 amino acids. This protein consists of an extracellular domain, including the Kunitz inhibitor, a transmembrane region (23 amino acids), and a short cytoplasmic domain (47 amino acids). The transmembrane–cytoplasmic segment of rat YWK-II and APP have ~70% homology (Yan et al., 1990). Recent reports suggest that APP is a member of a family of conserved genes encoding membrane proteins (Yan et al., 1990; Vidal et al., 1992; Wasco et al., 1992, 1993; Hanes et al., 1993; Sprecher et al., 1993; Sandbrink et al., 1994a; Slunt et al., 1994). Several isoforms of APP have been identified (Sandbrink et al., 1994b, 1996) and attributed to alternative splicing of inserts encoding the Kunitz protease inhibitor domain and a divergent region on the amino terminal side of the transmembrane domain. RSD-2, isolated from a rat testis expression cDNA library, encodes the YWK-II component and has been considered to be a member of the APP gene family (Vidal et al., 1992; Wasco et al., 1992, 1993; Hanes et al., 1993; Sprecher et al., 1993; Sandbrink et al., 1994a). The present results show that HSD-2, isolated from a human testis expression library, is highly...
homologous with RSD-2, to APLP2 (a member of the APP gene family), and to APPH cloned from a human placenta expression library (Sprecher et al., 1993). The YWK-II gene was mapped to chromosome 11 and assigned to 11q24-25 in proximity to the locus of APLP2/APPH (11q23-25) (Von der Kammer et al., 1994), whereas the APP gene maps to human chromosome 21 (Selkoe, 1994). Other homologous genes have been isolated from various mammalian species expression libraries, e.g. rat APLP2 and murine CDE1-binding protein (Vidal et al., 1992; Hanes et al., 1993; Sandbrink et al., 1994a). YWK-II protein, APPH, rat APLP2, and murine CDE1-binding protein appear to be species or tissue specific forms of the same protein, the function of which remains to be determined. Northern blot of tissue RNAs showed that two alternative transcripts are transcribed in a wide variety of human tissue. The cytoplasmic domain of YWK-II contains a 20 amino acid consensus sequence, SHGIVEVDPM-

LTPEERHLNK, which acts as a binding site for G proteins and may stimulate GTP binding by G proteins. This segment also contains two phosphorylation sites, i.e. TPEER for cdc2, and RKRQYGT for PKC which is situated between the consensus sequence and the transmembrane domain.

To determine the potential function of these motifs during spermatogenesis and fertilization, the DNA segment encoding 60 amino acids in the cytoplasmic domain of YWK-II component was expressed in E.coli and in eukaryotic cells. E.coli BL21 (DE3) transformed with pGEX-4T-HSD-2–360, produced GST-60AA. The recombinant product was shown to bind Go protein of bovine brain in vitro. When HA tag-60AA was expressed in F11 cells which are known to produce an abundance of Go, the expressed product was found to be complexed with Go.
These findings suggest that the YWK-II component may be a Go-coupled receptor. In general, this family of receptors has a common membrane-spanning conformation of seven transmembrane loops which may be involved in signal transduction by receptor–G protein communication. G-protein receptors with a single transmembrane region can activate G protein-mediated pathway in both intact cells and broken cell membranes (Liang and Garrison, 1991; Nishimoto et al., 1993; Gong et al., 1995; Okamoto et al., 1995; Sun et al., 1995). Nishimoto et al. (1993) discovered that these two types of receptors have a region that interacts with G proteins. The binding structure has the following common characteristics: (i) two basic residues at the N-terminus; and (ii) a C-terminal motif of BBXXB or BBXB (where B is a basic residue, and X is a non-basic residue). The cytoplasmic domain of YWK-II component contains a 20 amino acid region (SHGIV-X is a non-basic residue). The cytoplasmic domain of YWK-II component contains one basic residue at its C-terminus; and (ii) a SHGIV-XISHTPEERHLNK) adjacent to the transmembrane domain (13 amino acids) and the entire cytoplasmic domain (47 amino acids); while in the study with APP, only the short 20 amino acid peptide was assessed. These findings suggest that the binding of G proteins by YWK-II probably resides in the 20 amino acid segment of the cytoplasmic domain.

The coupling of YWK-II component and Go may play a physiological role in sperm function. The resulting metabolic pathways in spermatozoa may involve calcium channels and protein kinase activities. The acrosome reaction in spermatozoa is regulated by calcium influx (Breitbart and Spungin, 1997). The interaction of spermatozoa with the oocyte during fertilization can trigger the intracellular binding of Go and the release of the subunit Goα, thereby activating phospholipase C (PLC) (Kikuchi et al., 1986; Haugen et al., 1992) which will trigger the opening of calcium channels and promote calcium influx. Northern blot analysis of germ cells shows that Goα mRNA is highly expressed in rat pachytene spermatocytes (Paulssen et al., 1991; Haugen et al., 1992). The YWK-II component as a Goα-coupled receptor may also influence germ cell differentiation at this stage of spermatogenesis. Moreover, haploid germ cells of rat testis contain a novel splice variant of the Go protein (Haugen et al., 1992). Although the YWK-II protein occurs in all tissues examined, it may initiate a sperm-specific signal pathway through this novel splice variant of Goα in germ cells.

The phosphorylation sites for PKC and cdc2 kinase are Ser655 and Thr668 respectively, located within the cytoplasmic domain of APP695 (Gandy et al., 1988; Suzuki et al., 1994). The YWK-II component also contains potential phosphorylation sites and was shown to be a substrate for PKC and cdc2 kinase. Furthermore, Suzuki et al. (1994) recently showed that two 11 amino acid peptides (–RKRQYGTISHG– and –DPMLTPEERHLNK–) are substrates for kinases. Each peptide contains one of the two phosphorylation sites and corresponds to a segment of the cytoplasmic domain of APPH/APLP2. The APPH/APLP2 cDNA prepared from human placenta is homologous with the cDNA encoding the YWK-II component. Since Thr in these peptides is the site of phosphorylation by PKC and cdc2 kinase, Thr is the probable site of phosphorylation in YWK-II component. It is noteworthy that rapid phos-

Figure 10. In-vitro phosphorylation of the cytoplasmic domain of YWK-II component: GST-60AA, by (A) protein kinase C or (B) cyclin B/cdc2. Lane 1 = phosphorylated GST-60AA cleaved with thrombin; lane 2, phosphorylated intact GST-60AA; and lane 3 = GST cleaved with thrombin.
phorylation of G protein-coupled receptors accompanies stimulus-driven desensitization of the β adrenergic receptor (Hausdorff et al., 1990; Lefkowitz, 1993). Two types of kinases are known to mediate protein phosphorylation: one group consists of second messenger kinases, e.g. PKC and PKA, and the other group is G protein-coupled receptor kinases. Accordingly, phosphorylation of the cytoplasmic domain of YWK-II by PKC may trigger the conversion of the non-active form of Gβ in the cytosol to an active form associated with the plasma membrane. In addition, the resulting phosphorylated component may lose its ability to bind Gα thereby acting as a negative feedback mechanism.

In conclusion, the YWK-II component is a member of the APP family, has the characteristics of a Gβα-coupled receptor, and is a substrate for PKC and cdc2 kinase. It may mediate the Gα signal transducing pathway, and may participate in germ cell differentiation and in sperm metabolism.

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References

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