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A sperm component, HSD-3.8 (SPAG1), interacts with G-protein beta 1 subunit and activates extracellular signal-regulated kinases (ERK)

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1. ABSTRACT

HSD-3.8 cDNA (accession number AF311312) encodes a human sperm component. A 0.7kb fragment (HSD-0.7) containing three immunological epitopes of HSD-3.8 cDNA was prepared and expressed in *E. coli.* Immunization of female rats with the recombinant HSD-0.7 proteins induced infertility. A cDNA fragment encoding the C-terminal 144 amino acids of human G-protein beta 1 subunit (Gβ1-C144) was screened by yeast two-hybrid, when HSD-0.7 segment was used as a bait. Recombinant His6-tagged-Gβ1-C144 protein was expressed in *E.coli* BL21 and Anti-Gβ1 serum was raised with purified Gβ1-C144. HA-tagged HSD-0.7 and FLAG-tagged Gβ1 plasmids were constructed and co-transfected into human embryonal kidney 293 cells. Two proteins were localized at superimposable sites in the cytoplasm, and they formed a complex when 500 μmol/L GDP existed. Overexpression of HSD-0.7 activated the G-protein-mediated extracellular signal-regulated kinases (ERK1/2); however, the truncated fragments of HSD-0.7, which lacked either TPR domain or P-loop, lost the ability to activate the ERK1/2 pathway. Further study revealed that the activation of ERK1/2 was protein kinase C (PKC) rather than Ras dependent. These results provide evidence that HSD-3.8 present in spermatocytes and sperm may participate in spermatogenesis and fertilization process by activating the PKC-dependent ERK1/2 signal transduction pathway.

2. INTRODUCTION

Human sperm DNA-3.8 (HSD-3.8), a testis-specific gene isolated from human testis cDNA library, is the target antigen of anti-sperm antibodies found in the serum from an infertile woman(1). It was designated as Sperm Associated Antigen 1 (approved gene symbol: SPAG1) by HUGO Gene Nomenclature Committee. Anti-HSD-3.8 antibodies possess potent sperm agglutinating activity(1). The deduced HSD-3.8 polypeptide contains sequences corresponding to three tetratricopeptide repeat (TPR) regions, a phosphate binding (P)-loop domain, and several phosphorylation sites(2, 3). TPR has been shown to participate directly in protein-protein interaction, while the P-loop domain has the capacity to bind GTP or ATP and manifest GTPase or ATPase activities(3). A 0.7 kb fragment, designated as HSD-0.7, was the C-terminal of HSD-3.8 and it contained the TPR motif and the P-loop domain of HSD-3.8. Immunization with the HSD-0.7 polypeptide caused infertility in female rats(1). HSD-0.7 polypeptide was found to bind with GTP specifically and possess GTPase activity *in vitro*, suggesting that a probable function of HSD-3.8 is to act as a signal-transducing G-protein or be coupled to a G-protein, capable of inducing GTP/GDP conversion(1). Heterotrimeric G-proteins consist of a Gα-subunit and a Gβγ-dimer. The Gα-subunit possesses intrinsic GTPase activity. Under physiologic conditions, the Gβγ-dimer associates with the Gα-subunit and the Gα-subunit binds the guanine nucleotide, GDP. It is known that G-protein-coupled receptors (GPCRs) are activated by a wide variety of stimuli; whereby following receptor activation, there is an exchange of GDP for GTP by the G-protein resulting in the dissociation of the Gα-subunit and the Gβγ-dimer. Both the Gα-subunit and dimer trigger diverse intracellular signal cascades(4).

Evidence supporting the thesis that GPCRs activates MAPK has recently been reviewed(5, 6). The mechanism of activation of specific MAPK cascades may vary depending on the cell type as being GPCRs or G-protein-dependent(7). The known molecular activators of the MAPK pathway are the GTP-binding proteins e.g., Ras and Raf, both having GTPase activities. The conversion of the active/inactive state depends on the exchange of GTP for GDP. Heterotrimeric G proteins also play important roles as signal transducing components in various mammalian sperm functions(8). The sperm-induced egg activation has several features in common with G protein-coupled receptor signal transduction mechanisms(9). Previous studies showed that stimulation of ERK1/2 requires the participation of the Gβγ-dimer; whereas Gβ or Gγ subunits individually are inactive(10-12).

In the present study, the association of HSD-0.7 fragment and C-terminal 144 amino acids of human G-protein beta 1 subunit (Gβ1-C144) was validated *in vivo*. The two proteins were found to interact in the yeast two-hybrid system. The present study was conducted to determine the capacity of HSD-0.7 to activate G-protein-mediated MAPK. We reported here that the HSD-0.7 bound Gβ1 subunit in the presence of GDP. Moreover, in COS-7 cells, HSD-0.7 activates the extracellular signal-regulated kinases (ERK) via a $p21^{Ras}$-independent, but PKC mediated pathway. The ERK1/2 activation caused by HSD-0.7 depends on the concurrence of TPR region and P-loop.

3. MATERIALS AND METHODS

3.1. Construction, expression and purification of recombinant proteins encoded by pET30a (+)-Gβ1-C144

The cDNA encoding Gβ1-C144 was amplified by PCR, inserted into pET30a (+), and transformed in *E.coli* BL21. The transformed bacteria were incubated for 3 h with 0.2 mmol/L isopropyl-β-D-thiogalactoside (IPTG) to induce production of the target protein. The treated cells were sonicated and the resulting lysate separated into the soluble and residue fractions. The His6-tagged-Gβ1-C144 protein expressed by pET30a (+)-Gβ1-C144 was purified from the transfected cells according to the protocols for pET vectors (Novagen), using His Bind Metal Chelation Resin.

3.2. Raising polyclonal antibodies

Polyclonal antiserum to the prey protein Gβ1-C144 was raised in New Zealand rabbits by injecting s.c. 300-400μg of the recombinant protein at 2-week intervals, three times. Two weeks after the last injection, blood was collected, serum prepared and stored at -70°C. Antibody titres of the antiserum were determined by enzyme-linked immunosorbent assay (ELISA) and designated as anti-Gβ1-C144 antiserum.

Similarly, polyclonal antiserum to the HSD-0.7 was raised in Balb/c mouse by injecting HSD-0.7 protein purified in previous work(1).

3.3 .Preparation of testis extract and ovary extract

Specimens of human tissues were obtained from the Peking Union Medical College Hospital, Beijing, China. Informed consent was obtained from patients in accordance with the regulations governing clinical investigation established by the Institutional Review Board of PUMCH. Ovariectomy was performed as treatment for metastatic breast cancer and orchiectomy for prostatic cancer. Samples (0.3 grams) of human testes or ovaries were homogenized in 1.5 ml of Buffer A (50 mmol/L Tris-Cl, pH 7.4, 1 mmol/L EDTA) to which a cocktail of protease inhibitors (4 μg/mL each of pepstatin, aprotinin, leupeptin and PMSF, Sigma) was added. The homogenate was centrifuged at 15000 rpm for 20 min at 4°C. The supernatants were used and designated as testis extract and ovary extract, respectively.

3.4. Western blotting

Proteins in the tissues extracts were separated by SDS-PAGE using a 10% gel and transferred electrophoretically for 1.5 h at 80v onto a PVDF membrane (Millipore). After washing in a Tris-Cl-buffered saline containing Tween 20 (TBS-T, pH7.4), the membrane was treated with a blocking solution: TBS-T solution containing 5% non-fat milk, 1% goat antiserum and 0.02% Tween 20, at ambient temperature for 2 h. The membrane was incubated overnight with anti-Gβ1-C144 antiserum as the

primary antibodies at 1:1000 dilution at 4°C, washed in TBS-T, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at 1:5000 dilution for 2 h, washed in TBS-T again and finally, incubated with Enhanced Chemiluminescence (ECL) reagents (Sigma) and exposed to X-ray film (Kodak). The films were scanned and all the data were entered into SPSS statistical software (SPSS, Inc., Chicago, Illinois) and analyzed using two-sample t-test.

3.5. Construction of recombinant plasmids

The HSD-0.7 cDNA was inserted into the vector pcDNA6/V5-HisB-HA as a BamHI/XhoI fragment to construct a plasmid that is capable of expressing the HA-tagged fusion protein in eukaryotic cells. Simultaneously, the cDNA encoding the full-length human Gβ1 subunit was cloned into vector pcDNA6/V5-HisB-FLAG to express the FLAG-tagged Gβ1 in cells. The fragments of HSD-0.7 cDNA were inserted into the BamHI/XhoI sites of pcDNA6/V5–HisB-HA, resulting in the truncated plasmids designated as pcDNA6/V5-HisB-HA-HSD-0.7A and pcDNA6/V5-HisB-HA-HSD-0.7B.

3.6. Cell culture and treatment

HEK-293 cells or COS-7 cells, used for this study were grown as a monolayer culture and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37°C under 5% CO_2 atmosphere. Cells were plated in appropriate dishes and grown until attaining 50% to 70% confluency. Expression plasmids were transfected into the cells using the calcium phosphate precipitation technique (Molecular Cloning: A Laboratory Manual). Before collecting COS-7 cells for MAPK assay, the cells were serum starved with DMEM containing 1mg/mL bovine serum albumin (BSA) for at least 16 h. In experiments using the plasmids, pCMV-RasN17 or pCMV-Ras (Clontech Laboratories Inc.), which express the dominant negative $p21^{RasN17}$ or wild-type $p21^{Ras}$, the plasmids were co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 respectively. In experiments using the nontoxic PKC inhibitor bisindolylmaleimide GF109203X, the serum-starved cells were treated with GF109203X or equal volume of dimethyl sulfoxide (DMSO), the vehicle for GF109203X, for 2 h prior to the collection of cells.

3.7. Immunofluorescent staining in HEK-293 cells

HEK-293 cells grown on coverslips were co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and pcDNA6/V5-HisB-FLAG-Gβ1. 48h after transfection, cells on coverslips were washed with phosphate-buffer saline (PBS, pH 7.0) two times, fixed with 4% paraformaldehyde in PBS for 10~15min at room temperature. After each subsequent step, the samples were washed with PBS three times. The cells were permeablized with 0.5% Triton in PBS, blocked with 3% BSA in PBS for 30 min at 37°C, incubated with the primary antibody (rabbit anti-Gβ1-C144 antiserum at 1:100 dilution and mouse anti-HA antibody at 1:100 dilution, Sigma) for 30 min at 37°C. As controls, preimmune serum (rabbit) was substituted as the primary antibody at a 1:100 dilution. Cells were incubated with the secondary antibody (FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG, Santa Cruz Biotechnology) at 37°C for 30 min. After washing with PBS, the samples were washed with deionized water two times. Coverslips were mounted upside-down on the slides with 90% glycerol containing 2% triethylenediamine (DABCO, Sigma) and examined under a LEICA TCS NT laser confocal microscope.

3.8. Preparation of human sperm smears and immunofluorescent staining

Semen samples were obtained by masturbation following 3 to 5 days of sexual abstinence from a healthy donor. Motile sperms were isolated by overlaying the semen samples with an equal volume of warm BWW(13), supplemented with 0.3% Bovine Serum Albumin, incubating the mixture at 37° C for 60 min and then collecting the BWW medium sperm suspension. Smears of sperm were done by swabbing small amount of the sperm suspension onto microscope slides. Then the sperm on the slides were fixed with 4% paraformaldehyde in PBS for 15min at room temperature. Subsequent steps were same to that mentioned in previous section except that rabbit anti-Gβ1-C144 antiserum and mouse anti-HSD-0.7 antiserum were used as the primary antibody and TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) were used as the secondary antibody. Before coverslips were mounted, the sperm cells were incubated in Hoechst Stain solution (Sigma) for 5 min to stain the nuclei. The labeled sperm smears were examined under a LEICA TCS NT laser confocal microscope.

3.9. Co-immunoprecipitation

HEK-293 cells co-transfected with pcDNA6/V5-FLAG-Gβ1 and pcDNA6/V5-HA-HSD-0.7 were lysed in cold lysis buffer consisting of 20 mmol/L HEPES (pH 7.0), 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 20 mmol/L -glycerophosphate, 1 mmol/L Na3VO4, 1 mmol/L DTT, 2 mmol/L PMSF, 10μg/ml Aprotinin containing 500μmol/L GDP or 200μmol/L GTPγS. The lysate was clarified by centrifugation at 14000 rpm for 20 min. To 1 mL aliquot of the supernatant 40μL 50% protein A-agarose were added to absorb extraneous cellular proteins and the mixture was incubated for 3 h at 4°C. The samples were centrifuged at 12000 rpm for 20s, the supernatant collected, and 40μL rabbit anti-HSD-0.7 antiserum added. After incubating at 4°C for 1 h, 50μL 50% protein A-agarose were added to the mixture and incubated at 4°C for another 1 h. The agarose particles were sedimented by centrifugation, the pellet was collected and washed with lysis buffer three times and finally washed with lysis buffer without Na_3VO_4. The samples were mixed with SDS sample buffer and analyzed by Western blot. Anti-FLAG antibody (Sigma) was used as the primary antibody and the HRP-conjugated goat anti-mouse IgG as the secondary antibody. The blot was developed using ECL according to the Manufacturer instruction.

3.10. MAPK assay

COS-7 cells were washed with cold phosphate-buffered saline (PBS). Whole cell protein extracts were

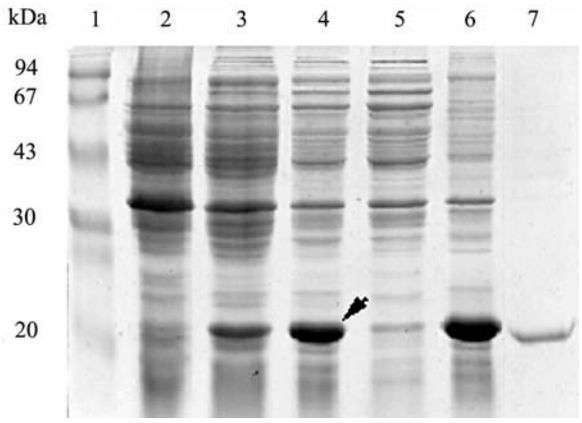

Figure 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of proteins obtained from the lysate of *E. coli* BL21 transformed with pET30a-Gβ1-C144. Lane 1, standard protein markers; lane 2, negative control, pET30a (+) cells induced with isopropyl-β-D-thiogalactoside (IPTG); lane 3, pET30a (+)-Gβ1-C144 transformed cells, incubated without IPTG; lane 4, pET30a (+)-Gβ1-C144 transformed cells, induced with IPTG; lane 5, supernatant of lysate of IPTG-induced pET30a (+)-Gβ1-C144 transformed cells; lane 6, residue fraction from lysate of IPTG-induced pET30a (+)-Gβ1-C144 transformed cells; lane 7, His_6-tagged-Gβ1-C144 purified by Ni^+-NTA chromatography. Protein bands were stained with Coomassie blue. A representative gel of three experiments is depicted.

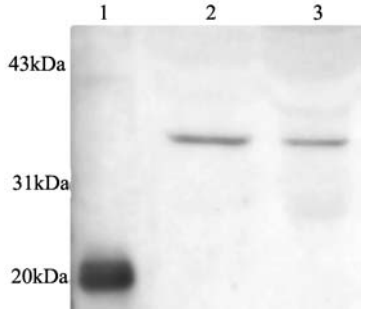

Figure 2. Anti-Gβ1-C144 antiserum detects Gβ1 in tissues extracts and His6-tagged-Gβ1-C144 expressed in *E.coli* BL21. Lane 1, pET30a (+)-Gβ1-C144 transformed cells, induced with IPTG, immunostained with polyclonal anti-Gβ1-C144 antiserum. Lane 2, human testis extract, proteins reacted with polyclonal anti-Gβ1-C144 antiserum. Lane 3, human ovaries extract, proteins reacted with polyclonal anti-Gβ1-C144 antiserum. Note stained ~20kDa band in lane 1 and higher molecular weight (~37kDa) stained bands in human testis and ovaries extracts (lanes 2 and 3).

prepared by lysing cells on ice in a lysis buffer containing 50 mmol/L HEPES (pH 7.0), 150 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L EDTA, 20 mmol/L -lycerophosphate, 1 mmol/L ortho-vanadate, 2 mmol/L $MgCl_2$, 10 mmol/L sodium pyrophosphate, 1% Nonidet P-40, 10% glycerol, 1 mmol/L PMSF, 1μg/ml Leupeptin, 10μg/ml Aprotinin. After centrifugation, the supernatant was loaded onto a 10% polyacrylamide gel. BCA assay was performed to normalize the protein concentration of each sample, with BSA as a standard. The phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 were detected by Western blot. The phosphorylated ERK1/2 was detected by utilizing phospho-p44/42 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology) as the primary antibody. Following incubation with HRP-conjugated anti-rabbit antibody (1:5000: Santa Cruz Biotechnology), signals were detected using ECL. After that, the anti-p-ERK1/2 antibody on the PVDF membrane was stripped using the strip buffer (60 mmol/L Tris-Cl, pH 6.7, 2%SDS, 0.8% 2-mercaptoethanol) for 60 min at 70°C, the PVDF membrane was blocked and incubated with the next antibody. Blots were initially incubated with anti-phospho-ERK1/2, followed by incubation with antibody against total ERK1/2 (Santa Cruz Biotechnology). Data are representative of at least three independent experiments. Densitometry of the blots were perform by using the software facility UVIsoft UVIband Windows Application V97.04, and further analyzed by Microsoft Excel 2000.

4. RESULTS

4.1. Expression and purification of recombinant proteins

The recombinant construct pET30a (+)-Gβ1-C144 was transformed in *E.coli* BL21. The predicted molecular weight of the fusion protein of Gβ1-C144 expressed by the construct plasmid was ~20kDa (Figure 1, lane 4, the expressed fusion protein is indicated by arrow). The fusion protein was located mainly in the inclusion body fraction, which was purified by affinity chromatography on a Ni^+-NTA resin column. Figure 1, lane 7 shows the purified protein of His6-tagged-Gβ1-C144.

4.2. Anti-Gβ1-C144 antiserum detects Gβ1 in tissue extracts and His6-tagged-Gβ1-C144 expressed in E.coli BL21

ELISA was used to determine the antibody titer of the antiserum raised in rabbits immunized with the purified fusion protein expressed by pET30a (+)-Gβ1-C144. The titers were $\varepsilon 3.2\times10^4$. Western blot analysis showed that the antiserum specifically reacted with the expressed polypeptide obtained from cells transformed with recombinant pET30a (+)-Gβ1-C144 (Figure 2, lane1). The human Gβ1 subunit of higher molecular weight was detected in human testis and ovary extracts (Figure 2, lane 2 and 3).

4.3. Localization of the HA-tagged-HSD-0.7 and FLAG-tagged-Gβ1 are superimposable

HEK-293 cells co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and pcDNA6/V5-HisB-FLAG-Gβ1 were immunofluorescently stained and examined by laser confocal imaging (Figure 3). No fluorescence could be observed in controls (data not shown). Figure 3A shows the HA-tagged-HSD-0.7, which expressed in the cytoplasm of the HEK-293 cells (Green fluorescence). Figure 3B shows the localization of FLAG-tagged-Gβ1 (Red fluorescence in cytoplasm). The stained sites of the two proteins were superimposable (Figure 3C, yellow fluorescence).

4.4. HSD-3.8 and Gβ1 are colocalized in human sperm

Human sperm smears were prepared, and slides were immunofluorescently stained and examined by laser

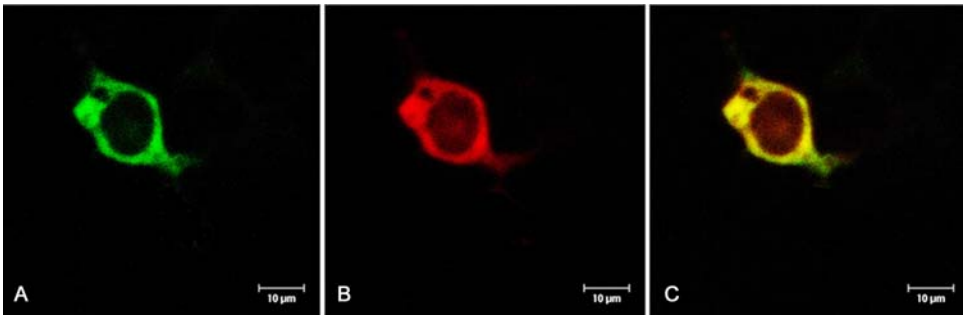

Figure 3. Co-localization of FLAG-tagged-Gβ1 and HA-tagged-HSD-0.7, determined by laser confocal immunofluoresent imaging. Transfected HEK-293 cells were fixed, permeabilized, and incubated with polyclonal anti-HSD-0.7 antiserum and monoclonal anti-FLAG antibodies as the primary antibodies, followed by treatment with the secondary antibodies, FITC-conjugated AffiniPure goat anti-rabbit IgG (green) and TRITC-conjugated goat anti-mouse IgG (red). Superimposed images (A and B) depicted in C as yellow colored spots of HA-tagged-HSD-0.7 (A, green fluorescence) and FLAG-tagged-Gβ1 (B, red fluorescence).

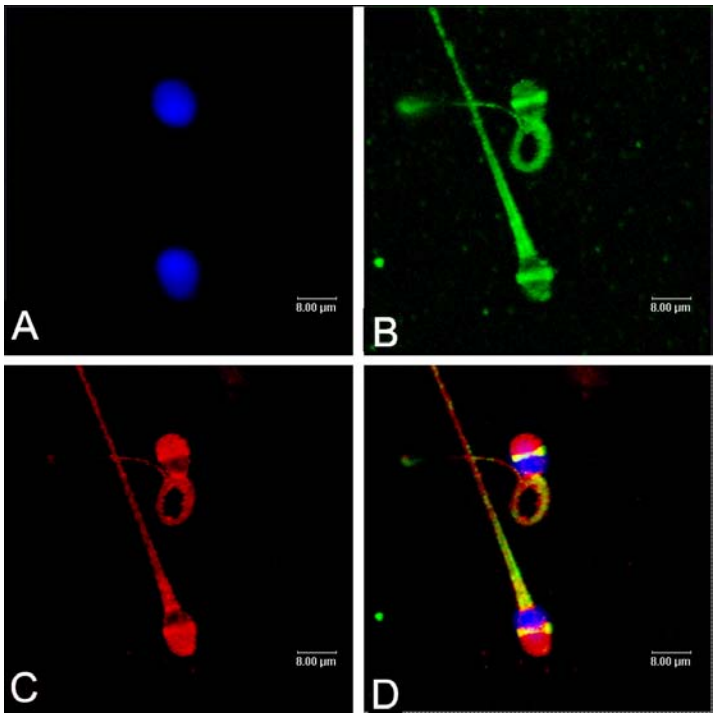

Figure 4. Co-localization of Gβ1 and HSD-3.8 in human sperm, determined by laser confocal immunofluoresent imaging. Human sperm smears were prepared. The sperm on the slides were fixed, permeabilized, and incubated with polyclonal anti-HSD-0.7 mouse antiserum and anti-Gβ1 rabbit antiserum as the primary antibodies, followed by treatment with the secondary antibodies, FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG. The nuclei were labeled by Hoechst. Figure 4A shows the staining of nuclei (Blue fluorescence). Figure 4B shows the staining of HSD-3.8 (Green fluorescence). Figure 4C shows the staining of Gβ1 (Red fluorescence). Figure 4D is the overlapped result. The two proteins were partial colocalized in human sperm (Figure 4D, yellow fluorescence). Figure 4D is the overlapped result. The two proteins were partial colocalized in human sperm (Figure 4D, yellow fluorescence).

confocal imaging (Figure 4). Figure 4A shows the staining of nuclei (Blue fluorescence). Figure 4B shows the staining of HSD-3.8 (Green fluorescence). Figure 4C shows the staining of Gβ1 (Red fluorescence). Figure 4D is the overlapped result. The two proteins were partial colocalized in human sperm (Figure 4D, yellow fluorescence).

4.5. HA-tagged-HSD-0.7 and FLAG-tagged-Gβ1 co-precipitate in the presence of GDP

HEK-293 cells were co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and pcDNA6/V5-HisB-FLAG-Gβ1. After 24 h the cells were collected and lysed. The effect of GTP or GDP on the co-precipitation of HA-

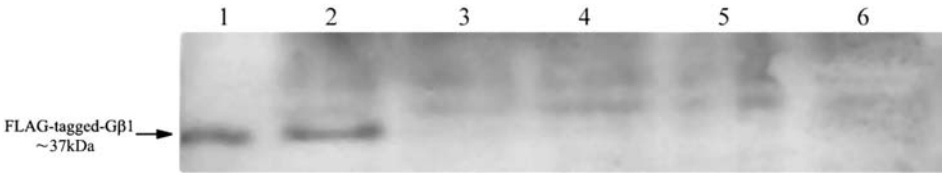

Figure 5. Co-immunoprecipitation of FLAG-tagged-Gβ1 subunit and HA-tagged-HSD-0.7. Lane 1, lysate of overexpressed FLAG-tagged-Gβ1 in HEK-293 cells, immunostained with monoclonal anti-FLAG antibodies; lane 2, lysate of HEK-293 cells co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and pcDNA6/V5-HisB-FLAG-Gβ1, immunoprecipitated with anti-HSD-0.7 antiserum in the presence of 500μmol/L GDP; lane 3, negative control, lysate of HEK-293 cells transfected with pcDNA6/V5-HisB-FLAG-Gβ1, immunoprecipitated with anti-HSD-0.7 antiserum; lane 4, negative control, lysate of HEK-293 cells co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and pcDNA6/V5-HisB-FLAG-Gβ1, immunoprecipitated with preimmune serum; lane 5, lysate of HEK-293 co-transfected with pcDNA6/V5-HisB-FLAG-Gβ1 and pcDNA6/V5-HisB-HA-HSD-0.7; immunoprecipitated with anti-HSD-0.7 antiserum in the presence of 200μmol/L GTPγS; lane 6, lysate of HEK-293 co-transfected with pcDNA6/V5-HisB-FLAG-Gβ1 and pcDNA6/V5-HisB-HA-HSD-0.7; immunoprecipitated with anti-HSD-0.7 antiserum. A representative gel of three experiments is shown.

agged-HSD-0.7 and FLAG-tagged-Gβ1 with rabbit anti-HSD-0.7 antiserum was determined. The addition of 500μmol/L GDP to the cell lysate promoted co-precipitation of HA-tagged-0.7 and FLAG-tagged-Gβ1 (Figure 5, lane 2). However, in the presence of 200 μmol/L GTPγS, co-precipitation did not occur (Figure 5, lane 5). This finding suggests that GDP is required for binding of these two components.

4.6 HSD-0.7 activates ERK1/2 in COS-7 cells

The effect of HSD-0.7 on G-protein-mediated signal transduction was studied in HEK-293 and COS-7 cells transfected with pcDNA6/V5-HisB-HA-HSD-0.7, by determining ERK1/2 activation. In COS-7 cells transfected with pcDNA6/V5-HisB-HA-HSD-0.7, ERK1/2 was markedly activated (Figure 6A and Figure 6B, lane 2), compared to cells transfected with vector (Figure 6A and Figure 6B, lane 3) ($p<0.01$), whereas with HEK-293 cells, the activation was slight (data not shown). Hence, all subsequent experiments were performed with COS-7 cells. Phospho-ERK1/2 (p-ERK1/2) in the cells was measured at 36 h, 48 h and 60 h after transfection (Figure 7A and Figure 7B, lane 2, 3, 4). In COS-7 cells, HSD-0.7 activated ERK1/2 over the time period of measurement. However, the p-ERK1/2 in control cells was much weaker (Figure 7A and Figure 7B, lane 4, 5, 6) ($p<0.01$).

4.7. The truncated fragments of HSD-0.7 lacked TPR or P-loop could not trigger the ERK1/2 activation

Schematic representation of the structure of HSD-0.7 and truncated fragments and encoded polypeptides is shown in Figure 8. Overexpression of HA-tagged-HSD-0.7A, which contains only the TPR region of HSD-0.7, did not activate ERK1/2 in COS-7 cells (Figure 9A and Figure 9B, lane 2). Neither ERK1/2 was activated in COS-7 cells transfected with pcDNA6/V5-HisB-HA-HSD-0.7B, while expressing the P-loop of HSD-0.7 (Figure 9A and Figure 9B, lane 3).

4.8. Activation of ERK1/2 with HSD-0.7 is PKC-dependent

To determine whether a PKC-dependent pathway might be involved in HSD-0.7-induced ERK1/2 activation, the effect of the PKC inhibitor, GF109203X, was studied. GF109203X previously has been established as a potent *in vivo* PKC inhibitor in COS-7 cells(14, 15). Serum-starved COS-7 cells transfected with pcDNA6V5-HisB-HA-HSD-0.7 were treated with 3.5μmol/L GF109203X or DMSO as control for 2 h. Cells were lysed and activation of ERK1/2 assayed by Western blot (Figure 10A and Figure 10B). GF109203X appeared to inhibit HSD-0.7-induced activation of ERK1/2; whereas the effect of DMSO was minimal ($p<0.01$).

4.9. Expression of dominant negative $p21^{Ras}$ does not affect HSD-0.7/ERK signaling

As reported previously, PKC-dependent activation of MAPK in COS-7 cells appeared involving a pathway independent of p21Ras (12, 13, 16-18). Further support for the existence of a p21Ras-independent fraction of HSD-0.7 participates ERK1/2 activation was obtained experimentally by showing that p21Ras function was impaired upon expressing the dominant negative mutant p21RasN17. As shown in Figure 11A and Figure 11B, expressing p21RasN17 did not influence the involvement of HSD-0.7 in ERK1/2 activation ($p>0.05$).

5. DISCUSSION

The HSD-3.8 gene encodes a testis-specific protein. Immunization with the HSD-0.7, a fragment of HSD-3.8 protein, caused infertility in female rats(1). To identify the proteins interacting with HSD-3.8 during fertilization, HSD-0.7 cDNA was used to screen the human ovary MATCHMAKER cDNA library by yeast two-hybrid system. A truncated cDNA was identified and found to encode a polypeptide composed of 144 amino acids, being the C-terminus of human G protein β1 subunit. HSD-0.7 contains three TPR motifs and a P-loop domain. The capacity of TPR-containing protein(s) to interact with other protein(s) varies with different TPR motifs and is influenced by the composition and presence of vicinal groups. The C-terminal 144 amino acids of human Gβ1 subunit identified as the interacting polypeptide contains three WD40 domains, the groups structurally participate in protein-protein interaction(19).

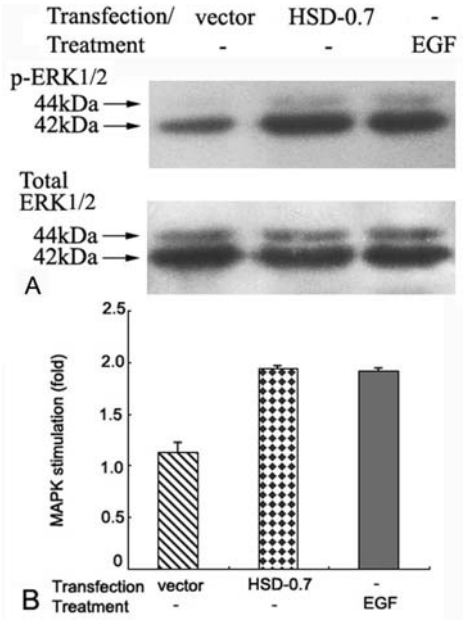

Figure 6. Activation of ERK1/2 in COS-7 cells transfected with pcDNA6/V5-HisB-HA-HSD-0.7. Transfection of COS-7 cells was performed using the calcium phosphate precipitation technique. Control cells were transfected with vector alone (the left lane). Cells were starved for at least 16 h, lysed and assayed for p-ERK1/2 and total ERK1/2, assayed by Western blot. As positive control, cells were starved and stimulated with 100 ng/mL human EGF for 15 min (the right lane). A, Western blot result. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, averaged results of three experiments performed with triplicate samples. Bars, averaged ERK activity expressed as fold stimulation respective to that obtained without stimulation or transfection.

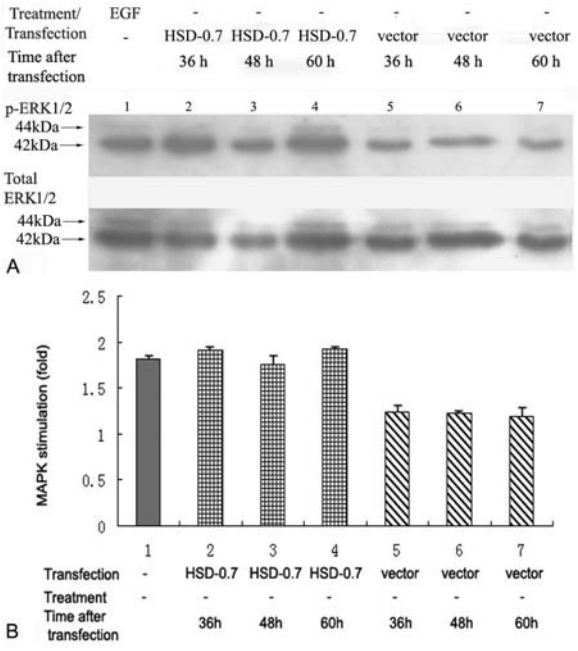

Figure 7. HSD-0.7 activates ERK1/2 over prolonged period of time. COS-7 cells were transfected, starved and lysed. The lysate proteins were analyzed by Western blot as described in Figure 5. Quantities of p-ERK1/2 and total ERK1/2 were measured at 36 h, 48 h, and 60 h after transfection (lanes 2, 3 and 4), respectively. As positive control, cells were starved and stimulated with 100 ng/mL human EGF for 15 min (lane 1). Negative control cells were transfected with the vector (lanes 5, 6 and 7). A, Western blot result. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, averaged results of three experiments performed with triplicate samples. Bars, averaged ERK activity expressed as fold stimulation respective to that obtained without stimulation or transfection.

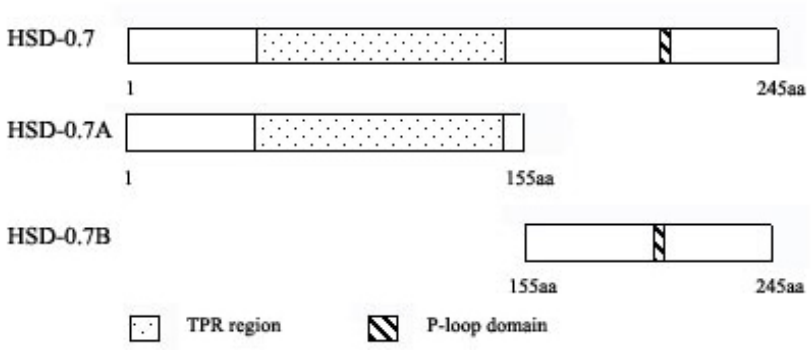

Figure 8. Schematics of the structures of HSD-0.7 and truncated fragments (HSD-0.7A, HSD-0.7B) encoded polypeptides.

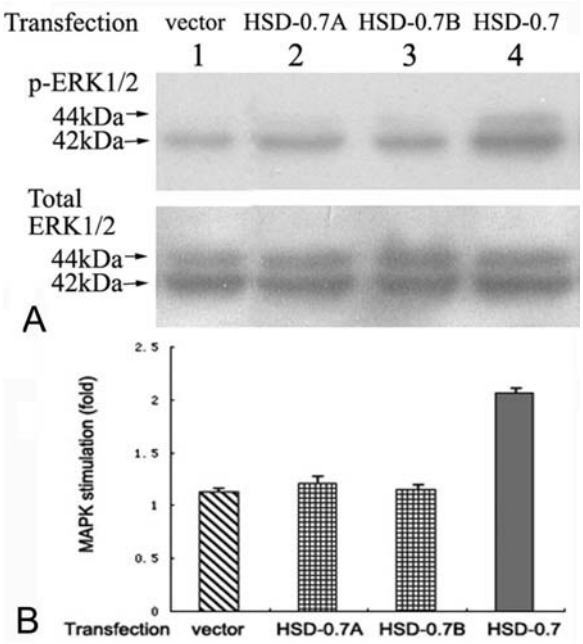

Figure 9. The truncated fragments of HSD-0.7, HSD-0.7A and HSD-0.7B could not trigger the ERK1/2 activation. COS-7 cells were transfected with pcDNA6/V5-HisB-HA-HSD-0.7A (lane 2) or pcDNA6/V5-HisB-HA-HSD-0.7B (lane 3), respectively. As positive control, cells were transfected with pcDNA6/V5-HisB-HA-HSD-0.7 (lane 4). Negative control cells were transfected with the vector (lanes 1). Transfected cells were starved and lysed. The p-ERK1/2 and total ERK1/2 in the lysate were assayed by Western blot. A, Western blot result. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, averaged results of three experiments performed with triplicate samples. Bars, averaged ERK activity expressed as fold stimulation respective to that obtained without stimulation or transfection.

Further experimentation is required to determine whether or not complexes of HSD-0.7 and human Gβ1 subunit occur *in vivo*. It is not feasible technically to study the effect of overexpression of FLAG-tagged-Gβ1-C144 *in vivo* since Gβ1 subunit lacking the N-terminal 42 amino acids degrades rapidly(20). To circumvent this problem, the FLAG-tagged-Gβ1 expression plasmid was constructed. The results of immunofluorescence staining in HEK293 cells showed that HA-tagged-HSD-0.7 and FLAG-tagged-Gβ1 are located in the cytoplasm and their sites are superimposable. In addition, the two proteins were also partial colocalized in human sperm. The present result suggests that the two proteins may form a complex *in vivo*. The co-immunoprecipitation experiments provide evidence that HSD-0.7 binds to Gβ1 *in vivo*, and that the binding depends on the presence of GDP.

The P-loop domain of HSD-0.7 is capable of binding GTP and possesses GTPase activity(1). This finding suggests that GTPase activity may be involved in the interaction of HSD-0.7 with human Gβ1 subunit. The association of HSD-0.7 and Gβ1, however, is mediated by GDP. It resembles the interaction of Gα subunit with Gβγ (21). G proteins play an important role in mouse egg activation; whereby the Gβγ subunit released at the time of fertilization may be involved in egg activation since microinjection with phosducin inhibits some events of sperm-induced egg activation, whereas microinjection with

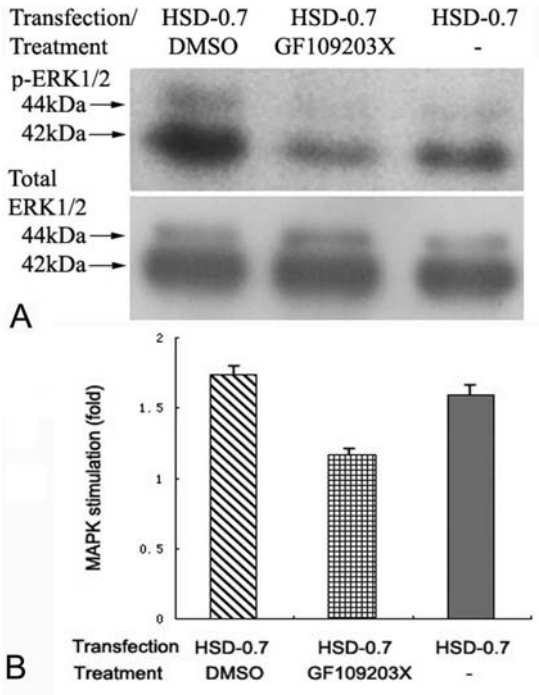

Figure 10. Effect of the PKC inhibitor GF109203X on HSD-0.7-induced MAPK activation. COS-7 cells were transfected with plasmid DNA encoding HA-tagged-HSD-0.7 and starved. Two hours before lysed, cells were incubated with 3.5 μmol/L GF109203X (middle lane) or equal volume of DMSO (left lane). COS-7 cells transfected with pcDNA6/V5-HisB-HA- HSD-0.7 and starved were used as control (right lane). A, Western blot result. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, averaged results of three experiments performed with triplicate samples. Bars, averaged ERK activity expressed as fold stimulation respective to that obtained without stimulation or transfection.

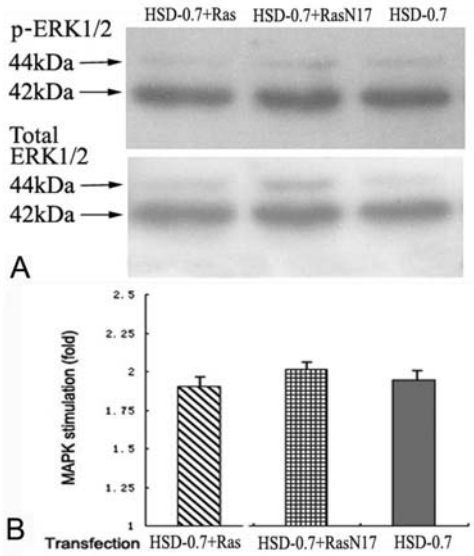

Figure 11. $p21^{RasN17}$ has no inhibitory effect on HSD-0.7-induced MAPK activation. COS-7 cells were co-transfected with pcDNA6/V5-HisB-HA-HSD-0.7 and plasmids encoding $p21^{RasN17}$ or $p21^{Ras}$, respectively. Cells transfected with pcDNA6/V5-HisB-HA-HSD-0.7 alone was performed as control. A, Western blot result. Only the representative result of a single reaction from triplicate samples is shown for every individual treatment. B, Averaged results of three experiments performed with triplicate samples. Bars, averaged ERK activity expressed as fold stimulation respective to that obtained without stimulation or transfection.

$\beta\gamma_t$ subunit neither activates mouse metaphase II–arrested eggs in the absence of sperm nor inhibits sperm-induced egg activation(9).

Overexpression of HA-tagged-HSD-0.7 activates ERK1/2 in COS-7 cells. The long-term activation of ERK1/2 caused by HSD-0.7 is resistant to the inhibitory action of the dominant negative Ras, $p21^{RasN17}$; whereas a potent *in vivo* PKC inhibitor, GF109203X appeared to inhibit HSD-0.7-induced activation of ERK1/2. These findings suggest that the ERK1/2-activated pathway initiated by HSD-0.7 is not $p21^{Ras}$ mediated, but rather PKC regulated. The present result indicates that HSD-0.7 activates ERK1/2 through a PKC dependant pathway and is similar to that observed in the study of microinjecting a dominant negative form of Ras (RasT) into an egg that did not influence the events associated with sperm-induced egg activation(9). Also the constitutively active form of Ras, (RasV12) does not induce egg activation in the absence of sperm(9). Thus a probable function of HSD-3.8 is to act as a signal-transducing G-protein or be coupled to a G protein, leading to the ERK1/2 activation. The role of the ERK pathway in sperm function is probably complex. The expression and activation of ERK1/2 varies during spermatogenesis in mice and it is suggested that these kinases could contribute to the mitotic proliferation of primary spermatogonia(22). The MAPK pathway functions during the process of fertilization and perhaps even earlier in the maturation of oocytes and germ cell differentiation(23).

In summary, the testis-specific protein HSD-3.8 functions as a G-protein-associated component by binding with Gβ1 in the presence of GDP. The HSD-3.8 protein activates ERK1/2 through a PKC-dependent G protein signal pathway, thereby influencing proliferation and differentiation of germ cells. If is further hypothesized that the HSD-3.8 may enter the oocyte following fusion of the membranes of sperm and oocyte, and promote sperm-induced egg activation. In these processes, the GTP/GDP conversion might play a crucial role. Hence, the basic action of HSD-3.8 is to activate MAPK pathway.

The present findings have wide implications based on the existence of a relationship in the expression of genes encoding sperm proteins during gametogenesis and carcinogenesis(24, 25). A group of cancer/testis genes has been identified and is under intense study as possible factors in the diagnosis and treatment of cancer. We have identified several genes encoding human sperm antigens(26). One of the components, YWK-II, can be considered to be a cancer/testis gene based on the report that the administration of limonene, a monoterpene, to rats bearing mammary carcinoma induced apoptosis and differentiation of the cancer cells, concurrently, with a marked expression of the YWK-II gene(27). Hence, the genes encoding sperm antigens including the HSD-3.8/SPAG1 identified in our studies may be involved in carcinogenesis. It is imperative that their expression in human cancer cells be investigated.

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Key Words HSD-0.7, G-protein, extracellular signal-regulated kinases (ERK), PKC, $p21^{Ras}$, tetratricopeptide repeat (TPR), phosphate binding-loop (P-loop)

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