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Single-Chain Antibody against Human Lipocalin-Type Prostaglandin D Synthase: Construction, Expression, Purification, and Activity Assay

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Abstract—An active form of single-chain antibody (ScFv) from murine monoclonal antibody 4A7, which is specific for lipocalin-type prostaglandin D synthase (L-PGDS), was produced in *Escherichia coli*. The complementary DNA fragments encoding the variable regions of heavy chain (VH) and light chain (VL), which amplified from hybridoma 4A7 producing a monoclonal antibody (IgG1) against L-PGDS, were connected by a $(Gly_4Ser)_3$ linker using an assembly polymerase chain reaction. The resultant ScFv were cloned into the vector pGEM and expressed in *E. coli* as inclusion bodies. The expressed ScFv fusion proteins were purified by Ni^{2+}-nitrilotriacetic acid chromatography. The purity and activity of purified ScFv were confirmed by SDS-PAGE and ELISA. The result revealed that 4A7 ScFv conserved the same characteristics of specific recognition and binding to sperm as the parental 4A7 monoclonal antibody.

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Key words: prostaglandin D synthase (L-PGDS), single-chain antibody (ScFv), expression

Prostaglandin D synthase (L-PGDS, EC 5.3.99.2) catalyzes the isomerization of the 9,11-endoperoxide group of prostaglandin H_2 (PGH_2), a common precursor of various prostanoids [1], to produce prostaglandin D_2 (PGD_2) with 9-hydroxy and 11-keto groups in the presence of sulfhydryl compounds [2]. PGD_2 is actively produced in a variety of tissues as a major prostanoid and is involved in numerous physiological and pathological functions. For example, PGD_2 is known as a potent endogenous somnogen [3], nociceptive modulator [4], anticoagulant, vasodilator, bronchoconstrictor, and also as an allergic and inflammatory mediator [5] released from mast cells. L-PGDS is localized in the central nervous system and male genital organs of various mammals and in the heart of human beings and is secreted into the cerebrospinal fluid, seminal plasma, and plasma, respectively [6]. The L-PGDS concentrations in these body fluids are useful for the diagnosis of several neurological disorders, dysfunction of sperm formation, and cardiovascular and renal diseases [7]. But the localization of L-PGDS in the cell is not clear [8]. The major function of L-PGDS in male fertility would be related to its capability of providing, beyond the blood–testis barrier, thyroid hormones and retinoids to the developing germ cells in the seminiferous tubules and the maturing spermatozoa in the epididymis [9], but the precise role of L-PGDS in male reproduction is still unclear [10].

Recombinant antibodies and their fragments in combination with new therapeutic approaches such as retargeting have become a promising resource for the design of high-affinity specific targeting drugs. Recent construction of engineered targeting molecules has demonstrated the potential for multivalent high affinity reagents built from small binding fragments (25 kD), which can potentially target a tumor more efficiently than large monoclonal antibodies (MAbs). Antibody fragment units have also been engineered for gene therapy, imaging, immunotherapy, radio immunotherapy, chemotherapy, and prodrug therapy [11]. ScFvs corresponding to the VH and VL immunoglobulin domains connected by a biologically inert flexible linker create the smallest antibody fragments that usually retain specific binding characteristics.

Abbreviations: L-PGDS) lipocalin-type prostaglandin D synthase; MAb) monoclonal antibodies.

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The purpose of the present study was to design and express the ScFv of L-PGDS, which could be used as a basic tool for gaining further insight into the potential function of L-PGDS in male reproduction and analyzing the localization of L-PGDS in cells.

MATERIALS AND METHODS

Mouse hybridoma cell lines. Hybridoma 4A7 producing a monoclonal antibody (IgG1) against L-PGDS was used in this study [12].

mRNA isolation. Cells of hybridoma 4A7 producing the anti-L-PGDS MAb were cultured in Iscove modified medium containing 20% (v/v) fetal bovine serum, 2% (v/v) glutamine (200 mM), and 1% (v/v) gentamicin (10 mg/ml) in a 5% CO_2 humidified incubator. RNAs were prepared from about 10^9 hybridoma cells by the guanidine isothiocyanate method. mRNAs were purified by affinity chromatography on an oligo(dT)-cellulose column (Pharmacia, Sweden) as specified by the manufacturer.

RT-PCR amplification of Ig variable regions. cDNA was synthesized from the mRNA template with a Reverse Transcription Kit (Clontech, USA). cDNAs encoding the antibody variable domains (VH and VL) were amplified by PCR under standard conditions using *Taq* polymerase (Promega, USA) in a MiniCycler thermocycler (MJ Research, Inc, USA). VH and VL were amplified with the following primers.

5' end primers for VL signal peptide:

LL1: GGGGATATCCACCATGGAGACAGACACACTCCTGCTAT,

LL2: GGGGATATCCACCATGGATTTTCAAGTGCAGATTTTCAG,

LL3: GGGGATATCCACCATGGAGWCACAKWCTCAGGTCTTTRTA,

LL4: GGGGATATCCACCATGKCCCCWRCTCAGYTYCTKGT,

LL5: GGGGATATCCACCATGAAGTTGCCTGTTAGGCTGTTG;

3' end primer for Vκ spanning Cκ and Jκ1:

MVK: GGATACAGTTGGTGCAGTCGACTTACGTTTKATTTCCARCT;

5' primers for VH signal peptide:

VHL1: GGGGATATCCACCATGGRATGSAGCTGKGTMATSCTCTT,

VHL2: GGGGATATCCACCATGRACTTCGGGYTGAGCTKGGTTTT,

VHL3: GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT,

VHL4: GGGGATATCCACCATGATRGTGTTRAGTCTTYTGTRCCTG;

3' end primer for VH spanning CH1 and JH:

MVH: GACHGATGGGGSTGTYGTGCTAGCTGMRGAGACDGTGA,

where R means A or G; Y means A or C; M means A or T; W means A or T; K means G or T; S means G or C.

The 5' end primers contain *EcoR*V restriction site, and 3' end primers contain *Sal*I and *Nhe*I restriction sites. PCR was done for 35 cycles (1 cycle is 0.5 min at 94°C, 0.5 min at 60°C, and 45 sec 72°C) in 50 μl of the following reaction mixture: 78 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.05% W-1 detergent (Gibco BRL, Great Britain), 0.2 mg/ml bovine serum albumin (BSA), 200 μM each dATP, dCTP, dGTP, and dTTP, 1 μM each primer, 10 ng of template, and 2.5 U of *Taq* DNA polymerase (Gibco BRL). The PCR products were analyzed on a 2% low-melting-point agarose gel in TAE buffer (Tris/acetate/EDTA) and visualized with ethidium bromide. PCR products of the expected size were excised from the gel and purified with a Geneclean II kit (Bio 101; Vista, USA) as specified by the manufacturer. The heavy and light chain fragments were sequenced in the Shengong Biocompany of Shanghai.

Construction of ScFv. ScFv was created by joining the VH and VL genes together by PCR splicing with overlap extension using oligonucleotides that encoded a 15-amino-acid linker $(Gly_4Ser)_3$ between the C-terminus of the *VH* and the N-terminus of the *VL* gene (Fig. 1). Each amplification was performed in a total volume of 100 μl that contained the following components in addition to those listed above: 200 μM of each dNTP (Pharmacia), 1 unit Taq DNA polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. The reaction mixture was subjected to 25 cycles of amplifica-

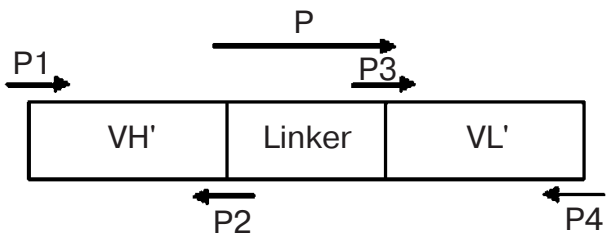

Fig. 1. Assembly of the gene for the soluble form of L-PGDS ScFv by splice overlap extension polymerase chain reaction (PCR).

tion; each thermal cycle consisted of 1 min at 94°C for denaturation followed by 1 min at 50°C for primer template annealing and 2 min at 72°C for extension; at the end of the amplification, the mixture was subjected to a final 10 min extension period (72°C). Amplification primers were as follows:

P1, 5'-CTGGCTAGCGATGTACAGCTTCAGGAG-TC-3' (contains site for *Nhe*I);

P2, 5'-CTGAACCGCCTCCACCTGAGGAGACAGT-GACCAGAG-3' (contains linker For);

P3, 5'-CTCTGGCGGTGGCGGATCGGATGTTGT-GATGACCCAGAC-3' (contains linker Rev);

P4, 5'-ATGCGTCGACCGACTTACGTTTGATTTCC-3' (contains *Sal*I restriction site);

P, 5'-CAGGTGGAGGCGGTTCAGGCGGAGGTG-GCTCTGGCGGTGGCGGATCGGA-3' (contains linker).

The modified genes were purified on agarose gels and 200 ng of each fragment was combined in a PCR reaction with *Pfu* DNA polymerase (Stratagene, USA). The resulting 0.7 kb PCR fragment was ligated into the pGEMT vector, cloned into *Escherichia coli*, and sequenced.

Soluble expression of ScFv. The constructed vector named pET-28a (+)/ScFv was cloned in *E. coli* BL21. The transformant was plated out on an NE plate (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China), incubated overnight at 26°C, and then stored at 4°C. We then inoculated 50 ml of super broth medium with a single bacterial colony at 26°C overnight with shaking at 250 rpm. We next inoculated 1 liter of super broth medium with this 50-ml pre-culture and shook the culture at 26°C at 200 rpm. To induce antibody expression, we grew the cultures to an OD_{600} of 0.5, added 0.02 mM IPTG, and continued shaking until the culture reached the stationary phase. Usually this was within 4 to 6 h after induction, when the final OD_{600} reached 3 to 6. We then harvested the cells by centrifugation (8000*g*, 10 min, 4°C). To prepare the whole-cell extract, we resuspended the cell pellet in 20 ml of Hepes extraction buffer (20 mM Hepes and 0.5 M NaCl adjusted to pH with NaOH), disrupted the cells in a French press, added DNase I to final concentration of 10 μg/ml, centrifuged the suspension at 27,000 rpm (4820*g*) in a 70.1 T1 rotor at 4°C for 30 min, and carefully collected the supernatant containing the single-chain antibody. The solution was filtered through a 0.22-μm-pore-size filter and stored at −70°C. To prepare the periplasmic extract, we resuspended the cells in 10 ml of ice-cold 1× TES (0.2 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose). To prepare 1/5× TES, we added 1 volume of 1× TES buffer to 4 volumes of distilled water. We added 15 ml of ice-cold 1/5× TES and vortexed it to resuspend the bacteria (this step induces a mild osmotic shock). We incubated the mixture on ice for 30 min and then centrifuged it at 10,000*g* for 10 min, carefully transferring the supernatant, which contains the soluble antibodies from the periplasm. The ScFv fragments were purified over a Ni^{2+} column (His trap kit, catalog No. 17-1880-01; Amersham Pharmacia Biotech AB, Sweden), and the concentration was measured by the Bradford method.

Gel electrophoresis and Western blot analysis. SDS-PAGE analysis was performed as a standard procedure using 12.5% acrylamide gels followed by staining with Coomassie Brilliant Blue (Serva, USA) or immunoblotting. For Western blot analysis, the proteins were transferred from the gels onto a PROTRAN nitrocellulose transfer membrane (Schleicher & Schuell, Germany) using a mini trans-blot system (Bio-Rad, USA) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3). The membranes were soaked for 1 h in PBS-T (20 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, pH 7.4) supplemented with 5% nonfat milk powder. This was followed by 1-h incubation with anti-His tag antibody conjugated to horseradish peroxidase 1/2000 (Sigma, USA). The antibody was diluted in the blocking solution (PBS-T with milk powder). The proteins on the membranes were revealed by the classical procedure of the ECL reagents (Amersham Biosciences).

ELISA. Enzyme-linked immunosorbent assays (ELISA) were performed at room temperature as follows: ovalbumin (10 μg/ml, Sigma) as a negative control was adsorbed for 2 h to ELISA plates (Maxisorb; Nunc) in 50 mM $NaHCO_3$ (pH 9.0). The excess antigen was washed out, and the plates were blocked for 2 h in B buffer (3% (w/v) skim milk and 1% (w/v) BSA in phosphate-buffered saline (PBS)). Primary antibodies (ScFvs or monoclonal antibodies (MAbs)), prepared in INC buffer (PBS, 1% (w/v) BSA, 1% (w/v) skim milk), were added to the wells at the concentrations indicated in each case and then incubated for 1 h. Unbound antibodies were removed by four 3-min washings of the wells with PBS. For detection of the bound E-tagged ScFvs, the anti-E-tag MAb–horseradish peroxidase (HRP) conjugate (1 μg/ml; Pharmacia) was added for 1 h in INC buffer. Bound MAb 6AC3 was revealed with a goat anti-mouse IgG–HRP conjugate in INC buffer (0.03 U/ml; Boehringer Mannheim, Germany). In every case, the ELISA was developed using *o*-phenylenediamine (Sigma) as a substrate of the peroxidase. The reaction was allowed to proceed for 10 min, stopped with 0.6 N HCl, and the OD_{492} of the plates was determined (Benchmark Microplate Reader; Bio-Rad).

Indirect binding assays by flow cytometry. Binding to antigen was assessed by indirect immunofluoroscence on human sperm. An aliquot of semen specimen (0.5 ml of samples with $(60\text{-}80)\cdot10^6$/ml spermatozoa or 1.5 ml for lower spermatozoa concentration) was added to 1.5 ml of

Hanks' balanced salt solution (HBSS; Sigma) containing 5% heat-inactivated fetal bovine serum (Sigma), 2 U/ml of potassium heparin, and 0.1% sodium azide and centrifuged at 350*g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 4-5 ml of the same medium to reach a final concentration of $(5\text{-}10)\cdot10^6$/ml spermatozoa. Direct immunofluorescence staining was performed in aliquots of 100 µl of sperm suspension, after vortex mixing to ensure monodispersion, using a fixed concentration (1 nM) of MAb, and increasing concentrations of ScFv (0.01-100 nM). It was incubated on ice for 30 min. The cells were washed three times in PBS/2% FCS, and then incubated for an additional 30 min with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs). After a final wash, the cells were resuspended in PBS/2% FCS and analyzed on a MoFlo flow cytometer (Cytomation, Fort Collins).

Rate of immunofluorescence competitive inhibition was calculated as follows: (average fluorescence intensity in positive control – average fluorescence intensity in reaction mixture)/(average fluorescence intensity in positive control) × 100%.

Sperm agglutination assay. The standard slide agglutination assay was performed. Human semen samples, provided by healthy donors, were liquefied at room temperature. One part of semen diluted to $20\cdot10^6$ spermatozoa/ml in Ham's F-10 medium was gently mixed with one part of either 4A7 MAb or ScFv and diluted 1 : 5 in Ham's F-10. Anti-E-tag antibody alone served as negative controls. A sample of 20 µl of each mixture was placed on a plastic slide with a coverslip. After ~2 min, sperm agglutination and motility were observed and recorded with microscopy.

RESULTS

Amplification of VH and VL regions of IgG and construction of ScFv. Using universal antibody primers clones encoding heavy- and light-chain variable regions were successfully amplified from total RNA extracted from an anti-L-PGDS hybridoma. From nine primers, three genes were amplified (Fig. 2). These amplified DNAs were cloned and sequenced.

Both primer LL1 and LL5 amplified the *VL* gene. The *VL* gene amplified by primer LL5 and *VH* gene amplified by primer HL4 had four FW and three CDRs and were shown to correspond to the VH and VL regions of mouse immunoglobulins. Figures 3 and 4 showed the nucleotide and deduced amino acid sequences. These sequences were registered in the EMBL data bank (Accession Nos. DQ352183 and DQ352184), but in the *VL* gene amplified by primer LL1, the Cys residue at position 23 was converted to Tyr, and the second CDR connected with the J2 fragment directly with loss of four nucleotides, thus causing the reading frame to shift with

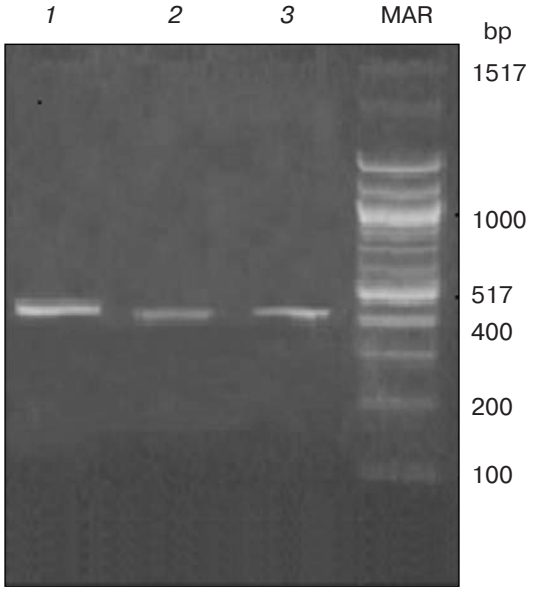

Fig. 2. Amplification of variable region genes of immunoglobulin light and heavy chains by RT-PCR: *1*) PCR product amplified by VHL4, 439 bp; *2*) PCR product amplified by LL5, 416 bp; *3*) PCR product amplified by LL1, 428 bp.

the appearance of the TAA terminator codon at amino acid residue 109 (data not shown). So, the *VL* gene amplified by primer LL1 was not functional.

To generate a ScFv fragment, the *VL* gene amplified by primer LL5 and *VH* gene amplified by primer HL4 were cloned from the cDNA and joined by a sequence encoding the 15-amino-acid-residue flexible $(Gly_4Ser)_3$ linker in a PCR amplification. Primers P, P1, P2, P3, and P4 were used to construct the *ScFv* gene. Figure 5 shows the nucleotide and deduced amino-acid sequences of the *ScFv* gene. These sequences were registered in the EMBL data bank (Accession No. 352185).

Expression of the *ScFv* gene in *E. coli*. The ScFv construct was cloned in pET-28a(+) and then transformed into *E. coli* BL21. The bacterial expression of the recombinant ScFv protein and extraction of soluble periplasmic protein are described in "Materials and Methods". The fusion protein was purified on a Ni^{2+} column. As demonstrated by SDS-PAGE, predominant fusion proteins were resolved at ~31 kD (Fig. 6).

ELISA. The binding of the different ScFv forms to L-PGDS was tested *in vitro* using ELISA. As shown in Fig. 7, the binding curves for L-PGDS of the ScFv 4A7, purified from the extracellular medium, were identical, clearly demonstrating that these two molecules have the same binding activity. The 4A7 MAb bound L-PGDS with a ~50-fold-higher apparent affinity than the *E. coli* recombinant antibodies. This value is within the range expected from the transformation from monovalent to bivalent mini-antibodies and probably reflects the sub-

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-57-ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG ATG TTC TGG ATT CCT GCT TCC
     M   K   L   P   V   R   L   L   V   L   M   F   W   I   P   A   S
-6- ATC ACT GAT GTT GTG ATG ACC CAG ACT CCA CTC TCC CTG CCT GTC GGT CTT
     I   T   D   V   V   M   T   Q   T   P   L   S   L   P   V   G   L
46- GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AAT
     G   D   Q   A   S   I   S   C   R   S   S   Q   S   L   V   H   N
97-AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG CCA GGC CAG TCT CCA
     N   G   N   T   Y   L   H   W   Y   L   Q   K   P   G   Q   S   P
148-AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA GAC AGG
     K   L   L   I   Y   K   V   S   N   R   F   S   G   V   P   D   R
199-TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG
     F   S   G   S   G   S   G   T   D   F   T   L   K   I   S   R   V
250-GAG GCT GAG GAT CTG GGA CTT TAT TTC TGC TCT CAA AGT ACA CAT ATT ACG
     E   A   E   D   L   G   L   Y   F   C   S   Q   S   T   H   I   T
301-TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGT AAG TCG
     W   T   F   G   G   G   T   K   L   E   I   K   R   K   S
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Fig. 3. The cDNA and amino acid sequences of the variable region gene of L-PGDS MAb light chain amplified by LL5.

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-54-ATG ATA GTG TTA AGT CTT CTG TAC CTG TTG ACA GCC ATT CCT GGT ATC CTG
     M   I   V   L   S   L   L   Y   L   L   T   A   I   P   G   I   L
-3- TCT GAT GTA CAG CTT CAG GAG TCA GGA CCT GGC CTC GTG AAA CCT TCT CAG
     S   D   V   Q   L   Q   E   S   G   P   G   L   V   K   P   S   Q
49- TCT CTG TCT CTC ACC TGC TCT GTC ACC GGC TAC TCC ATC ACC AGT GGT TAT
     S   L   S   L   T   C   S   V   T   G   Y   S   I   T   S   G   Y
100-TAC TGG AAC TGG ATC CGG CAG TTT CCA GAA AAC AGA CTG GAA TGG ATG GGC
     Y   W   N   W   I   R   Q   F   P   E   N   R   L   E   W   M   G
151-TAC ATA AGC TAC GAC GGT AGC GAT TAC TAC AAC CCA TCT CTC AAA AAT CGA
     Y   I   S   Y   D   G   S   D   Y   Y   N   P   S   L   K   N   R
202-ATC TCC ATC ACT CGT GAC ACA TCT AAG AAC CAG TTT CTC CTG AGG TTG AAT
     I   S   I   T   R   D   T   S   K   N   Q   F   L   L   R   L   N
253-TCT GTG AGT ACT GAG GAC ACA GCT ACA TAT TAC TGT GCA AAC TAC GGT AAT
     S   V   S   T   E   D   T   A   T   Y   Y   C   A   N   Y   G   N
304-AGC TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCC TCA
     S   F   A   Y   W   G   Q   G   T   L   V   T   V   S   S
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Fig. 4. The cDNA and amino acid sequences of the variable region gene of L-PGDS MAb heavy chain amplified by HL4.

stantial decrease in avidity of the 4A7 MAb after becoming a monovalent ScFv fragment. No L-PGDS-binding was detected using ovalbumin.

Ovalbumin was used as negative control in ELISA. Maximal binding was considered when the OD_{492} reached 2. The values shown are the average of at least two independent experiments in which binding to L-PGDS was determined in triplicate. No detectable signals (OD_{492} of ≤ 0.02) were observed in parallel ELISA tests using ovalbumin as a specificity control antigen.

Indirect binding assays by flow cytometry. After being measured by flow cytometry, purified L-PGDS ScFv

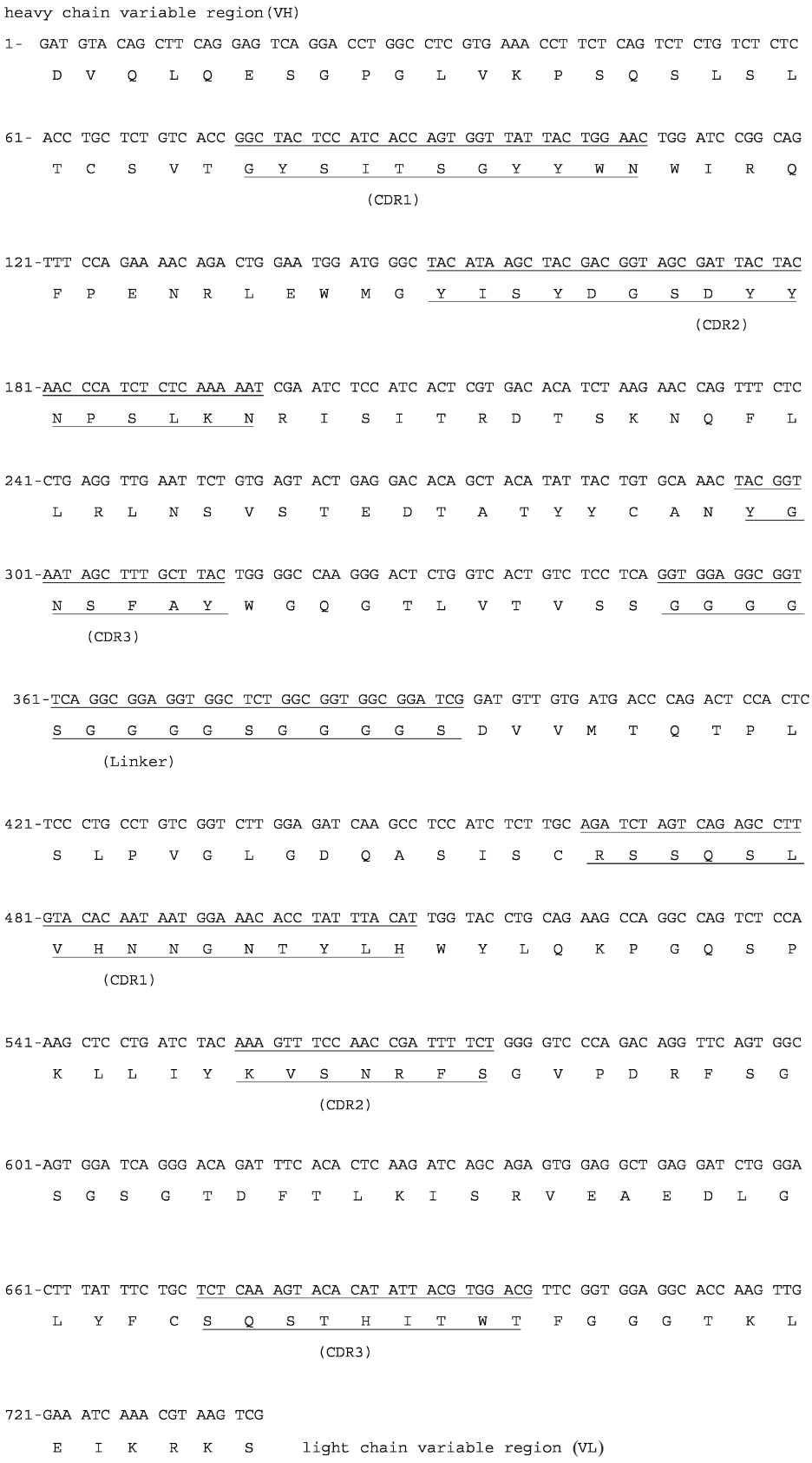

Fig. 5. The cDNA and amino acid sequences of L-PGDS ScFv. The amino acid sequence includes the heavy chain, linker (amino acid residues 136-251), and light chain sequences.

competitively bound to the L-PGDS antigen on sperm membrane, which caused the average fluorescence intensity to weaken significantly (table). In the presence of 10 μM L-PGDS MAb, the average fluorescence intensity of sperm can reach 85.65. However, on mixing 10 μM L-PGDS MAb and 20 μM ScFv the average fluorescence intensity of sperm dropped to 36.04. So the L-PGDS ScFv and MAb had similar antigen binding specificity.

Sperm agglutination assay. Agglutination of human spermatozoa with ScFv was investigated using the *in vitro* slide agglutination assay. Viable human spermatozoa were mixed with ScFv and observed by microscopy. Native 4A7 MAb and anti-E-tag antibody were included as positive and negative controls, respectively. The native 4A7 MAb and ScFv agglutinated spermatozoa in a tangled pattern, i.e. head-to-head, tail-to-tail, and head-to-tail (Fig. 8).

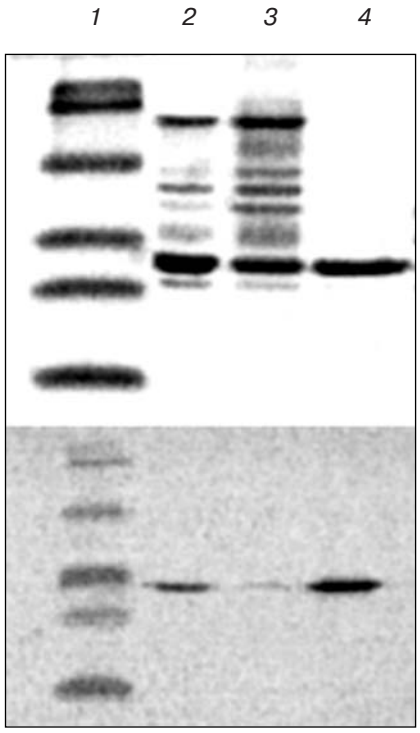

Fig. 6. Analysis of recombinant protein purification SDS-PAGE stained with Coomassie brilliant blue (upper panel). Western blot analysis with anti-His-tag antibodies (lower panel): *1*) protein molecular weight markers; *2*) induced; *3*) non-induced; *4*) purified ScFv protein.

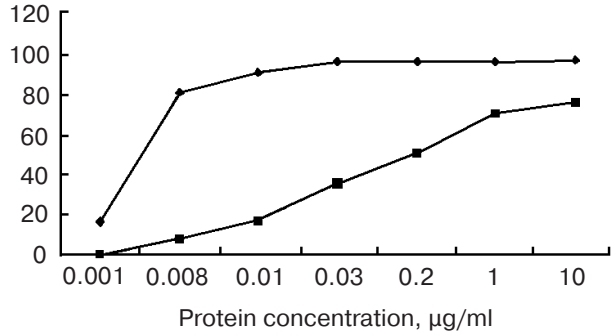

Fig. 7. Binding activity of secreted ScFv hybrid in ELISA. Relative binding to L-PGDS as a function of the concentration of antibodies (MAb 4A7 and ScFv 4A7).

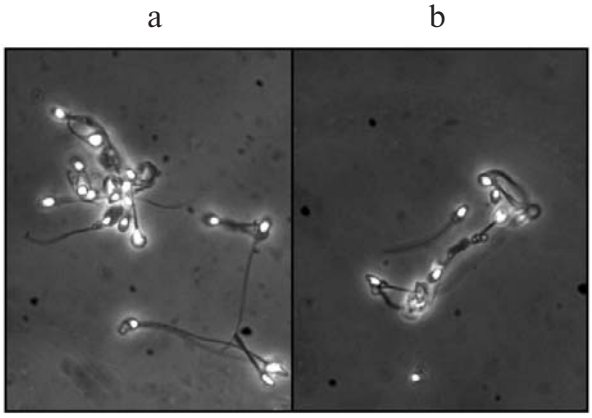

Fig. 8. Agglutination of human spermatozoa with ScFv. ScFv or 4A7 MAb was mixed with human spermatozoa at a concentration of $20 \cdot 10^6$ spermatozoa/ml and 1 : 10 dilution of antibody. After 2 min, sperm motility was observed and recorded under microscopy. a) ScFv; b) 4A7 MAb.

DISCUSSION

In this work, we constructed the *4A7 ScFv* gene, which was constituted as VH-$((Gly)_4Ser)_3$-VL, and expressed it in *E. coli*. The result from competitive immunofluoroscence on human sperm showed that the expressed ScFv retained the characteristic of specific recognition and binding to L-PGDS like 4A7 MAb.

In general, a hybridized cell can only produce one heavy chain gene and one light chain gene [13]. While we amplified two light chain genes and a heavy chain gene from the hybridized cells secreting anti-L-PGDS MAb. This phenomena was also reported by others researchers. Knight acquired three light chain genes from one mouse hybridized cell, and Luo obtained two *V*κ and two *VH* genes from mouse hybridized cell secreting anti-TNF-α, only one *V*κ and one *VH* gene were functional. So when cloning the variable gene from a hybridized cell, the appearing polygene and monofunctional gene was a natural phenomenon.

Western blot, flow cytometry, and agglutination analyses confirmed that ScFv recognizes the L-PGDS epitope on the human sperm surface. Western blots provided the initial confirmation of ScFv activity. Indirect binding assays by flow cytometry demonstrated that the L-PGDS ScFv and MAb had similar antigen binding specificity. ScFv was able to agglutinate human spermato-

Competitive inhibition of immunofluorescence

L-PGDS MAb (μM)	L-PGDS ScFv (μM)	Average fluorescence intensity	Competitive inhibition of immunofluorescence
0	40	3.96 ± 0.81	
Negative control			
10	20	36.04 ± 0.35	57.92%
10	10	50.71 ± 0.32	40.79%
10	5	61.12 ± 0.57	28.63%
10	0	85.65 ± 0.62	
Positive control			

zoa, indicating that ScFv can recognize L-PGDS on live spermatozoa. Furthermore, since ScFv demonstrates agglutination, we predict that it will block other aspects of fertilization.

Over the past decade, the use of monoclonal antibodies has been expanded to a variety of clinical applications [14]. However, adverse effects such as binding interference, caused by using whole murine antibodies in humans, and the expense of tissue culture have presented obstacles to their utilization [15]. Recombinant antibody technology has presented a solution to both of these impediments because the generation of recombinant antibody fragments, such as ScFv, not only removes the majority of the immunogenic murine sequence, but may also be more economical. The ScFv construct can serve as a prototype for recombinant anti-sperm antibodies and for their use as contraceptive agents. Contraceptive efficacy in the rabbit model has been demonstrated with a variety of individual monoclonal sperm-agglutinating antibodies [16]. The conjunction of ScFv with additional sperm-agglutinating antibodies generated against sperm surface proteins such as PH-20, localized to the acrosome and equatorial segment, or LDH-C_4 on the midpiece, may serve as a topical agglutinin. Bispecific antibodies could also be generated to attach such sperm-reactive antibodies to one another [17]. These antibodies could be combined to create a contraceptive with an additive effect. In addition, generation of an active mini-antibody to the sperm surface opens the path to engineering chimeric fusion proteins or complement-recruiting diabodies with specialized cytotoxic properties using RASA (recombinant single chain antibodies against human spermatozoid surface) as a targeting domain [18]. Adequate large-scale expression, purification, and delivery strategies must be developed and *in vivo* feasibility trials performed as a prerequisite to commercialization.

Concerning L-PGDS, many questions remain unclear. First, the role of L-PGDS in male reproduction is still unclear. As early as 1975, Olsson identified β-trace in SF, observing that the protein, normally present at very high concentration, dramatically decreased in the semen of oligozoospermic men [19]. Surprisingly, this observation has been neglected for more than 20 years and only recently the phenomenon was partially explained in relationship with post-testicular obstructions [20]. Second, the origin of the protein in the male genital tract has not been unequivocally identified. In the human, the protein seems to be secreted mostly by Sertoli cells even if immunohistochemical studies showed that it is also localized in Leydig cells and in epithelial cells of the epididymides [21]. In other species the distribution of L-PGDS would be different, and besides the Sertoli cells, a significant synthesis would take place (i) in the epididymis of the mouse [22], stallion [23], and rat [22], (ii) in the Leydig cells of the mouse [24] but not in other species, (iii) in the germ cells of the bull [25]. Third, the production of L-PGDS by Leydig cells would suggest a possible involvement of this protein in steroidogenesis. However, another lipocalin, that is, α1-acid glycoprotein, but not L-PGDS, is known to bind testosterone and other steroids. Finally, different L-PGDS glycoforms are known to have the same enzymatic activity [26], but nothing is known about their transport activity. Moreover, whether different glycosylation patterns would affect the distribution of L-PGDS in the male reproductive tract is completely unknown [24, 27-29].

One of the reasons that so many questions about the L-PGDS are unclear is the function and character of the antibody. ScFv will partially resolve these questions. The use of single-chain antibodies enables the use of smaller molecules than regular antibodies, which are simpler to introduce into cells. The intracellular antibody strategy exploits the ectopic expression of recombinant antibodies to inhibit the *in vivo* function of selected molecules. Folding stability, cellular half-life, and the solubility properties of an antibody fragment depend on the intracellular compartment that it is located in. In the case of expression in the cell cytoplasm, the solubility of antibodies is very heterogeneous and formation of intracellular aggregates is a rather frequent event. This may occur

because the reducing environment of this compartment hinders the formation of intra-chain disulfide bonds of the variable domains of the heavy and light chains [30]. Two mRNA degradation strategies, RNAi and antisense RNA, are now available for the study of the function of specific genes. However, these two approaches are useful especially against short-lived proteins and therefore are not 100% effective for other molecules. The advantage of using single chains is their ability to adopt three-dimensional conformations and to homodimerize as whole antibodies, in this way mimicking the immune system. This feature makes single-chain antibodies attractive not only for research, but also for gene therapy. One approach is loading cells with dominant-negative proteins, so the native protein is in competition with its dominant-negative form and the cell is under stress. When using single-chain antibodies, the cell is able to continue its cycle until a definite point since the recombinant antibody neutralizes the native protein successively and noncompetitively. The use of proteins has a further advantage that in the future genetic engineering could be employed for intracellular expression of the antibody, thus allowing homing to specific cellular compartments without the need for conjugation of peptides to oligonucleotides or specific drugs.

In summary, we constructed and expressed the *4A7 ScFv* gene successfully. The result from competitive immunofluoroscence on human sperm showed that the expressed ScFv retained the characteristic of specific recognition and binding to L-PGDS as 4A7 MAb, and it could be used as a basic tool for gaining further insight into the potential function of L-PGDS in male reproduction and analyzing the localization of L-PGDS in cells.

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