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Short sequence-paper

Molecular cloning and expression of a novel human cDNA related to the diazepam binding inhibitor

Kyoungho Suk [a], Yun-Hee Kim [a], Dae-Yeon Hwang [a], Sung-Hee Ihm [b], Hyung Joon Yoo [b], Myung-Shik Lee [a,c,*]

[a] *Clinical Research Center, Samsung Biomedical Research Institute, Seoul, South Korea*
[b] *Department of Internal Medicine, Hallym University College of Medicine, Seoul, South Korea*
[c] *Department of Medicine, Samsung Medical Center, Sungkyunkwan University College of Medicine, 50 Ilwon-dong, Kangnam-ku, Seoul 135-710, South Korea*

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Abstract

In order to isolate the unidentified autoantigens in autoimmune diabetes, a human pancreatic islet cDNA library was constructed and screened with the sera from the diabetic patients. From the library screening, one clone (DRS-1) that strongly reacted with the sera was isolated. Subsequent sequence analysis revealed that the clone was a novel cDNA related to the diazepam binding inhibitor. DRS-1 was expressed in most tissues including liver, lung, tonsil, and thymus, in addition to pancreatic islets. DRS-1 was in vitro translated and the recombinant DRS-1 protein was expressed in *Escherichia coli* and purified. The size of the in vitro translated or bacterially expressed DRS-1 protein was in agreement with the conceptually translated polypeptide of DRS-1 cDNA. Further studies are required to test whether or not DRS-1 is a new autoantigen in autoimmune diabetes.

Keywords: Diazepam binding inhibitor; Pancreatic islet; cDNA library; Autoantigen; Autoimmune diabetes

In autoimmune diabetes, pancreatic islets are destroyed by the chronic autoimmune responses to islet cells and the autoantibodies to various islet antigens are detected in a large panel of diabetic sera [1,2]. The identified islet autoantigens include glutamic acid decarboxylase (GAD), IA-2, insulin, and carboxypeptidase-H. Although the pathological implications of the autoantibodies to these antigens are not well elucidated, the presence of the autoantibodies in the patients' sera has been used as a diagnostic marker for autoimmune diabetes. Characterization of the autoantigens responsible for the islet destruction will greatly enhance our understanding of the pathogenic mechanism of the autoimmune diabetes. Biochemical as well as molecular approaches have been used for the identification and characterization of some of these diabetic autoantigens [3–5]. Current work was initially undertaken to isolate the unidentified autoantigens in autoimmune diabetes using a molecular approach. We constructed a cDNA library of human pancreatic islets and screened the library with diabetic patient sera. From screening with pooled sera of 10 patients, we isolated three clones that strongly reacted with the patients' sera. Subsequent sequence determination revealed that two of these cDNA clones were derived from the same

* Corresponding author. Fax: +82 (2) 3410-3849; E-mail: mslee@smc.samsung.co.kr

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GATGGCGTACTTGGCTTGGAGACTGGCTTTGCGTTCGTGTCCGAGGTCACTAGTTTCCCG   60
GGAGTTCAGCTGCACATGAATAGAACAGCAATGAGAGCCAGTCAGAAGGACTTTGAAAAT  120
               MetAsnArgThrAlaMetArgAlaSerGlnLysAspPheGluAsn

TCAATGAATCAAGTGAAACTCTTGAAAAAGGATCCAGGAAACGAAGTGAAGCTAAAACTC  180
SerMetAsnGlnValLysLeuLeuLysLysAspProGlyAsnGluValLysLeuLysLeu

TACGCGCTATATAAGCAGGCCACTGAAGGACCTTGTAACATGCCCAAACCAGGTGTATTT  240
TyrAlaLeuTyrLysGlnAlaThrGluGlyProCysAsnMetProLysProGlyValPhe

GACTTGATCAACAAGGCCAAATGGGACGCATGGAATGCCCTTGGCAGCCTGCCCAAGGAA  300
AspLeuIleAsnLysAlaLysTrpAspAlaTrpAsnAlaLeuGlySerLeuProLysGlu

GCTGCCAGGCAGAACTATGTGGATTTGGTGTCCAGTTTGAGTCCTTCATTGGAATCCTCT  360
AlaAlaArgGlnAsnTyrValAspLeuValSerSerLeuSerProSerLeuGluSerSer

AGTCAGGTGGAGCCTGGAACAGACAGGAAATCAACTGGGTTTGAAACTCTGGTGGTGACC  420
SerGlnValGluProGlyThrAspArgLysSerThrGlyPheGluThrLeuValValThr

TCCGAAGATGGCATCACAAAGATCATGTTCAACCGGCCCAAAAAGAAAAATGCCATAAAC  480
SerGluAspGlyIleThrLysIleMetPheAsnArgProLysLysLysAsnAlaIleAsn

ACTGAGATGTATCATGAAATTATGCGTGCACTTAAAGCTGCCAGCAAGGATGACTCAATC  540
ThrGluMetTyrHisGluIleMetArgAlaLeuLysAlaAlaSerLysAspAspSerIle

ATCACTGTTTTAACAGGAAATGGTGACTGTTACAGTAGTGGGAATGATCTGACTAACTTC  600
IleThrValLeuThrGlyAsnGlyAspCysTyrSerSerGlyAsnAspLeuThrAsnPhe

ACTGATATTCCCCCTGGTGGAGTAGAGGAGAAAGCTAAAAATAATGCCGTTTTACTGAGG  660
ThrAspIleProProGlyGlyValGluGluLysAlaLysAsnAsnAlaValLeuLeuArg

GAATTTGTGGGCTGTTTTATAGATTTTCCTAAGCCTCTGATTGCAGTGGTCAATGGTCCA  720
GluPheValGlyCysPheIleAspPheProLysProLeuIleAlaValValAsnGlyPro

GCTGTGGGCATCTCCGTCACCCTCCTTGGGCTATTCGATGCCGTGTATGCATCTGACAGG  780
AlaValGlyIleSerValThrLeuLeuGlyLeuPheAspAlaValTyrAlaSerAspArg

GCAACATTTCATACACCATTTAGTCACCTAGGCCAAAGTCCGGAAGGATGCTCCTCTTAC  840
AlaThrPheHisThrProPheSerHisLeuGlyGlnSerProGluGlyCysSerSerTyr

ACTTTTCCGAAGATAATGAGCCCAGCCAAGGCAACAGAGATGCTTATTTTTGGAAAGAAG  900
ThrPheProLysIleMetSerProAlaLysAlaThrGluMetLeuIlePheGlyLysLys

TTAACAGCGGGAGAGGCATGTGCTCAAGGACTTGTTACTGAAGTTTTCCCTGATAGCACT  960
LeuThrAlaGlyGluAlaCysAlaGlnGlyLeuValThrGluValPheProAspSerThr

TTTCAGAAAGAAGTCTGGACCAGGCTGAAGGCATTTGCAAAGCTTCCCCCAAATGTCTTG 1020
PheGlnLysGluValTrpThrArgLeuLysAlaPheAlaLysLeuProProAsnValLeu

AGAATTTCAAAAGAGGTAATCAGGAAAAGAGAGAGAGAAAAACTACACGCTGTTAATGCT 1080
ArgIleSerLysGluValIleArgLysArgGluArgGluLysLeuHisAlaValAsnAla

GAAGAATGCAATGTCCTTCAGGGAAGATGGCTATCAGATGAATGCACAAATGCTGTGGTG 1140
GluGluCysAsnValLeuGlnGlyArgTrpLeuSerAspGluCysThrAsnAlaValVal

AACTTCTTATCCAGAAAATCAAAACTGTGATGACCACTACAGCAGAGTAAAGCATGTCCA 1200
AsnPheLeuSerArgLysSerLysLeu*

AGGAAGGATGTGCTGTTACCTCTGATTTCCAGTACTGGAACTAAATAAGCTTCATTGTGC 1260
CTTTTGTAGTGCTAGAATATCAATTACAATGATGATATTTCACTACAGCTCTGATGAATA 1320
AAAAGTTTTGTAAAACAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and predicted amino acid sequences of human DRS-1 cDNA. The box indicates the conserved acyl CoA binding motif. The asterisk indicates the presumed termination codon. Poly(A) signal is underlined. The nucleotide sequence shown here has been deposited in the GenBank with the accession number of AF069301.

mRNA. The deduced amino acid sequence of these cDNA clones was related to the diazepam binding inhibitor (DBI), thus the cDNA clone was named DRS-1 (for DBI-related sequence-1). Further studies will be necessary to determine whether or not DRS-1 is a new autoantigen in autoimmune diabetes.

To construct a human pancreatic islet cDNA library, total RNA was isolated from human pancre-

atic islets obtained from the brain-dead donors by guanidinium thiocyanate–acid phenol–chloroform extraction as previously described [6]. Poly(A)$^+$ RNA was purified by the selection with biotinylated oligo(dT) and streptavidin coupled with paramagnetic particles (Promega, Madison, WI). The cDNA library was constructed using a ZAP Express-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, the first strand cDNA was synthesized from 5 μg of poly(A)$^+$ RNA with Moloney-murine leukemia virus reverse transcriptase and *Xho*I-linked oligo(dT) primers in the presence of methyl nucleotides, followed by the second strand synthesis with RNase H and DNA polymerase I. After the double-stranded cDNA was blunt-ended with dNTP and T4 DNA polymerase, the cDNA was ligated to the *Eco*RI linkers, then digested with *Xho*I. The sephacryl column-purified cDNA fragments were ligated into the *Eco*RI and *Xho*I sites of the bacteriophage lambda vector arms (ZAP-Express). The ligated vectors were packaged using the Gigapack II Gold extracts, followed by an amplification of the library in *Escherichia coli* Sure strain to yield 5×10^9 plaque-forming units/ml.

To screen the human islet cDNA library, the phage library of the total 5×10^5 clones was plated on the LB agar and incubated at 42°C for 4 h, and then overlaid at 37°C for 3 h with 10 mM IPTG-impregnated nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Plaque lifts were initially screened with the pooled sera from 10 diabetic patients, which were diluted 1:200 in 20 mM Tris-HCl (pH 7.5)–150 mM NaCl (TBS) containing 1% BSA and repeatedly preabsorbed with *E. coli* lysate. After the incubation of the plaque lifts with the diluted sera at 25°C for 1 h, bound antibody was detected by sequential incubations at 25°C with alkaline phosphatase-conjugated goat anti-human IgG antibody (Dako, Carpenteria, CA), and with chromogenic substrates (NBT and BCIP; Gibco-BRL, Gaithersburg, MD). Membranes were washed three times with TBS containing 0.05% Tween-20 after each incubation. Three positive clones were finally isolated after the secondary and tertiary screening, which were carried out in the same manner as the initial screening.

For isolation and sequence analysis of the DRS-1 cDNA clone, three positive phage clones from the library screening were subjected to in vivo excision by ExAssist helper phage coinfection (Stratagene) according to the manufacturer's instructions. When the pBK-CMV phagemids containing cDNA inserts of three positive clones were sequenced with T3 primer using an automated sequencing system (ABI 377; PE Applied Biosystems, Foster City, CA), two

A

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                                                                      60
DRS-1          MNRTAMRASQKDFENSMNQVKLLKKDPGNEVKLKLYALYKQATEGPCNMPKPGVFDLINK
Human DBI      -------MSQAEFEKAAEEVRHLKTKPSDEEMLFIYGHYKQATVGDINTERPGMLDFTGK
Porcine DBI    --------SQAEFEKAAEEVKNLKTKPADDEMLFIYSHYKQATVGDINTERPGILDLKGK
Bovine DBI     -------MSQAEFDKAAEEVKHLKTKPADEEMLFIYSHYKQATVGDINTERPGMLDFKGK
Mouse DBI      -------MSQAEFDKAAEEVKRLKTQPTDEEMLFIYSHFKQATVGDVNTDRPGLLDLKGK
Rat DBI        -------MSQADFDKAAEEVKRLKTQPTDEEMLFIYSHFKQATVGDVNTDRPGLLDLKGK
Chicken DBI    --------SEAAFQKAAEEVKELKSQPTDQEMLDVYSHYKQATVGDVNTDRPGMLDFKGK
Yeast DBI      -------MVSQLFEEKAKAVNELPTKPSTDELLELYALYKQATVGDNDKEKPGIFNMKDR
                             *     *  *   *     *  *   **** *      **
                                                                     120
DRS-1          AKWDAWNALGSLPKEAARQNYVDLVSSLSPSLESSSQVEPGTDRKSTGFETLVVTSEDGI
Human DBI      AKWDAWNELKGTSKEDAMKAYINKVEELKKKYGI--------------------------
Porcine DBI    AKWDAWNGLKGTSKEDAMKAYINKVEELKKKYGI--------------------------
Bovine DBI     AKWDAWNELKGTSKEDAMKAYIDKVEELKKKYGI--------------------------
Mouse DBI      AKWDSWNKLKGTSKESAMKTYVEKVDELKKKYGI--------------------------
Rat DBI        AKWDSWNKLKGTSKENAMKTYVEKVEELKKKYGI--------------------------
Chicken DBI    AKWDAWNALKGMSKEDAMKAYVAKVEELKGKYGI--------------------------
Yeast DBI      YKWEAWENLKGKSQEDAEKEYIALVDQLIAKYSS--------------------------
                ** *  *    * *   *   *  *
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B

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                                                                      60
DRS-1          MNRTAMRASQKDFENSMNQVKLLKKDPGNEVKLKLYALYKQATEGPCNMPKPGVFDLINK
ACBP/ECHM      -----MGASVEEFNAAKEKLGALKKDPGNEVKLKVYALFKQATQGPCNTPKPSMLDFVNK
                    * **   *          ************ *** **** **** ***   *  **
                                                                     120
DRS-1          AKWDAWNALGSLPKEAARQNYVDLVSSLSPSLESSSQVEPGTDRKSTGFETLVVTSEDGI
ACBP/ECHM      AKWDAWKSLGSVSQEEARQQYVDLISSLVGTEAPAVAAQPTG--STKGFQTLLVSTEDNI
               ******  ***   * *** **** ***          *       ** ** *  ** *
                                                                     180
DRS-1          TKIMFNRPKKKNAINTEMYHEIMRALKAASKDDSIITVLTGNGDCYSSGNDLTNFTDIPP
ACBP/ECHM      TTIRLNRPEKKNAITVEMYNELIEALDLAGKDSSVITVITGSGDYYCSGNDLNNFTKIPE
               * *  *** *****  *** *   **  * ** * *** ** ** * ***** *** **
                                                                     240
DRS-1          GGVEEKAKNNAVLLREFVGCFIDFPKPLIAVVNGPAVGISVTLLGLFDAVYASDRATFHT
ACBP/ECHM      VEYRRWLKMLGS-AEEYVKVYIDFPKPLIGVINGPAVGVSVTLLGLFDVVYATEKATFHT
                     *       * *   ******** * ****** ********* ***    *****
                                                                     300
DRS-1          PFSHLGQSPEGCSSYTFPKIMSPAKATEMLIFGKKLTAGEACAQGLVTEVFPDSTFQKEV
ACBP/ECHM      PFSQLGQSPEGCSSYLFPKIMGAAKASEMLLFNKKLTAAQACEVGLVTEVFPESSFQSEV
               *** *********** *****  *** *** * *****  **  ******** * ** **
                                                                     360
DRS-1          WTRLKAFAKLPPNVLRISKEVIRKREREKLHAVNAEECNVLQGRWLSDECTNAVVNFLSR
ACBP/ECHM      WTRLKAYAKLPKNSLVLSKQLIRAVEKEKLHAVNDAEVERLVERWLSDECMQAIMSFFQA
               ****** **** * *  **  **  * *******  *   *  *******  *   *

DRS-1          KSKL
ACBP/ECHM      KSKL
               ****
```

Fig. 2. Alignment of amino acid sequences of DRS-1 and DBI of various species (A) or DRS-1 and carp ACBP/ECHM (B). The underlined sequence indicates the conserved acyl CoA binding motif. The asterisks denote consensus sequences. Gaps (−) are introduced to achieve maximum homology. DRS-1 showed a 46–54% local homology to DBI of various species. SWISS-PROT data base accession numbers of DBI sequences cited for comparison are as follows: human DBI, P07108; porcine DBI, P12026; bovine DBI, P07107; mouse DBI, P31786; rat DBI, P11030; chicken DBI, S63595; and yeast DBI, P31787.

clones were identical. The two identical clones were further characterized in this study. Restriction digestion of the isolated clone (DRS-1) revealed that the insert size was ~1.4 kb. Nucleotide sequence of the insert was determined to uncover that DRS-1 codes for polypeptide of 364 amino acid residues (Fig. 1). Data base sequence similarity search using the BLAST program (GCG, University of Wisconsin) revealed that the deduced amino acid sequence of DRS-1 is related to the human, porcine, bovine, mouse, rat, chicken, and yeast diazepam binding inhibitors (DBI) (Fig. 2A). DBI is a highly conserved protein that is widely distributed throughout phylogenesis [7]. DBI was first identified as a polypeptide that has binding activity and elicits behavioral effects expected for a ligand to the benzodiazepine site [7–9]. Although it was first thought that DBI has a specific distribution in brain nuclei and is highly concentrated in the hypothalamus and cerebellum [10–12], later studies demonstrated that the protein is expressed in a large number of different tissues [13]. DBI mediates various neurological actions, including the induction of anti-anxiety by binding to the benzodiazepine recognition sites associated with γ-aminobutyric acid ($GABA_A$) receptors [7]. Moreover, DBI turned out to be identical with the acyl CoA binding protein (ACBP), which was first purified from bovine liver and capable of binding and inducing synthesis of acyl CoA [14–17]. Sequence analysis of DRS-1 also uncovered that the protein possessed a conserved acyl CoA binding motif [18], suggesting a potential acyl CoA binding activity of DRS-1 protein (Figs. 1 and 2).

DRS-1 also showed a significant sequence homology with a cold-induced gene in the carp (ACBP/ECHM; GenBank accession number, AF006493, not published). ACBP/ECHM was cloned as a cold-induced gene in the carp liver by a differential display RT-PCR. ACBP/ECHM has N-terminal sequence similar to the acyl CoA binding protein and C-terminal sequence similar to the enoyl CoA hydratase. Although the sequence of ACBP/ECHM cDNA has been deposited in the GenBank, none of the functional or structural characterizations of the cDNA have been reported so far, except that the expression of ACBP/ECHM may be upregulated in the carp maintained at a low temperature. DRS-1 showed a 60% identity with ACBP/ECHM at an amino acid sequence level (Fig. 2B). Moreover, DRS-1 also has C-terminal sequence homologous to the enoyl CoA hydratase. This suggests that DRS-1 might be a human homolog of the carp ACBP/ECHM gene. The expression of DRS-1, however, was not affected by the cold shock in HeLa cells (data not shown).

To investigate the expression of DRS-1, Northern

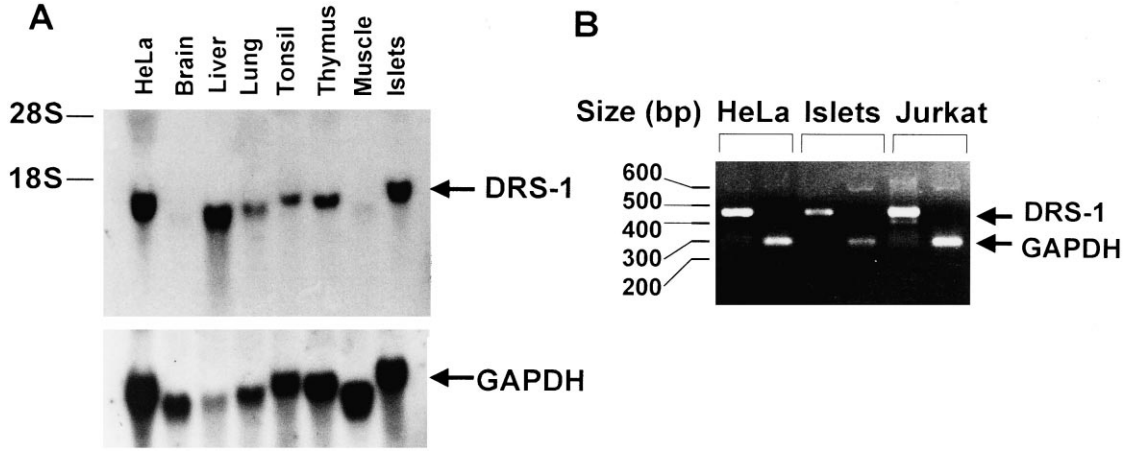

Fig. 3. Assessment of DRS-1 expression in different human tissues and cell lines. (A) Tissue distribution of DRS-1 was evaluated by the Northern blot analysis. Total RNA isolated from different tissues was blotted onto the membrane, which was then probed with a DRS-1 cDNA or human GAPDH cDNA. (B) Isolated RNA was subjected to RT-PCR with a primer set specific for either DRS-1 or human GAPDH. The PCR products were run on a 1% agarose gel, then stained with ethidium bromide. Specifically amplified products were not obtained in the RT-PCR without reverse transcriptase (data not shown).

blot analysis of RNA from different human tissues was carried out (Fig. 3A). For the Northern blot analysis, isolated total RNA (20 μg per sample) was electrophoresed in a 1.0% agarose gel containing 0.7% formaldehyde, 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate, and 1 mM EDTA. RNA was transferred to the Nytran membrane (Schleicher and Schuell, Keene, NH). After a UV crosslinking, the membranes were hybridized with a cDNA probe (10^6 cpm/ml) prepared by digesting a pBK-CMV phagemid harboring the human DRS-1 cDNA with *Eco*RI and *Xho*I to liberate a 1.4-kb insert. The membranes were then washed at 65°C, dried, and exposed to X-ray films. Human DRS-1 cDNA probe specifically hybridized with ~1.4-kb band in most tissues, including liver, lung, tonsil, thymus, and pancreatic islets, which suggests a ubiquitous expression of the DRS-1 gene (Fig. 3A). DRS-1 was most strongly expressed in liver. In brain and muscle, however, a very low level of expression was detected upon a prolonged exposure (data not shown). The DRS-1 gene was transcribed in most tissues as a single major mRNA species with a size that correlated with our cloned cDNA. Differences in the mobility of DRS-1 bands among different tissues were due to smiling in the gel during electrophoresis as demonstrated by the reprobing with GAPDH, in which the same mobility pattern was observed. The expression of DRS-1 in human cell lines (HeLa and Jurkat) and pancreatic islets was also assessed by RT-PCR (Fig. 3B). A specifically amplified product (480 bp) was detected in the HeLa and Jurkat cell lines as well as independently isolated pancreatic islets. This supported the ubiquitous expression of the DRS-1 gene. For RT-PCR, isolated RNA was subjected to the first strand synthesis reaction using a Superscript reverse transcriptase and oligo(dT) primers (Gibco-BRL). An aliquot of this reaction was amplified in the presence of a primer set specific for DRS-1, dNTP, and Taq DNA polymerase (Promega, Madison, WI). PCR was done for 25 cycles at 60°C annealing temperature. Nucleotide sequences of the forward and reverse primers used in PCR were 5′-CAA AGA TCA TGT TCA ACC GGC-3′ and 5′-CCT CTC CCG CTG TTA ACT TC-3′, respectively.

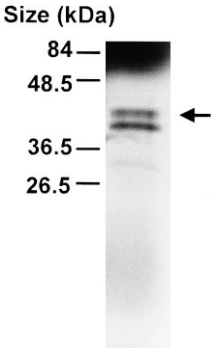

Fig. 4. In vitro translation of DRS-1. DRS-1 was in vitro translated and analyzed by 12% SDS-PAGE followed by autoradiography. The arrow indicates the presumed DRS-1 protein of ~40 kDa.

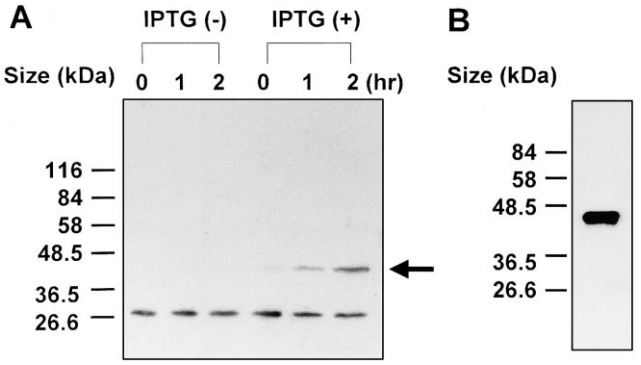

Fig. 5. Expression and purification of recombinant DRS-1 protein. (A) BL21(DE3) was transformed with pET28 expression vector harboring DRS-1 cDNA, followed by IPTG induction. IPTG (+) and IPTG (−) indicate transformed *E. coli* cells cultured with or without IPTG for 0–2 h, respectively. The expression of recombinant DRS-1 protein was evaluated by Western blot with anti-T7·Tag antibody, followed by chemiluminescent detection. The arrow indicates a recombinant DRS-1 protein of ~47 kDa tagged with T7 epitopes. Lower bands appear to be a protein that crossreacts with the antibody used. (B) Recombinant DRS-1 protein was purified using the Ni-NTA column. Purified protein was analyzed by a 10% SDS-PAGE and Coomassie blue staining.

In order to carry out in vitro translation of DRS-1, in vivo excised pBK-CMV phagemids harboring DRS-1 cDNA were linearized by digesting with *Xho*I. Linearized cDNA was gel-purified and used as a template in the coupled transcription and translation reaction using T3 RNA polymerase, [^{35}S]methionine, and TNT Coupled Reticulocyte Lysate Systems (Promega). When the translated proteins were analyzed by the SDS-PAGE and autoradiography, two major bands of ~36 kDa and ~40 kDa were detected (Fig. 4). The size of the higher molecular weight band (~40 kDa) correlated with

that of the conceptually translated polypeptide of DRS-1 cDNA. A lower molecular weight band (~36 kDa) appears to be a translated product whose translation was initiated from an internal ATG codon.

Recombinant DRS-1 protein was produced by using a prokaryotic expression vector system. A full-length DRS-1 cDNA was cloned into pET28 expression vectors with a compatible reading frame (Novagen, Madison, WI). Automatic nucleotide sequencing using a commercial kit (ABI 377, PE Applied Biosystems) was carried out to confirm the correct reading frame of the ligated plasmid. BL21(DE3), an *E. coli* lysogen expressing T7 polymerase, was transformed with the resulting plasmid. The expression of DRS-1 protein was evaluated by Western blot analysis using the horseradish peroxidase-conjugated anti-T7·Tag antibody (Novagen) (Fig. 5A). Expressed recombinant DRS-1 protein was purified using the Ni-NTA column (InVitrogen, San Diego, CA) (Fig. 5B).

In the present work, we report the isolation of a novel cDNA related to DBI from human pancreatic islets. DBI has been previously shown to inhibit glucose-stimulated insulin release from pancreatic islet β cells [19,20]. Since the novel clone, DRS-1, shows a significant sequence homology to DBI, two proteins may exert similar effects on β cells. Thus, if DRS-1 indeed affects β cell insulin secretion, the expression of DRS-1 in pancreatic islets may have an implication in the pathogenesis of autoimmune diabetes.

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