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Cloning of a cDNA Encoding a 190-kDa Insulin Receptor Substrate-1-like Protein of Simian COS Cells [1]

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Major insulin signals such as stimulation of glucose uptake and DNA synthesis and modification of hexose metabolism are mediated by the tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1; pp180) in many species of cells.

We cloned cDNA encoding a 190-kDa IRS-1-like protein (pp190) in simian COS cells and which is slightly larger than IRS-1 (pp180) of human, rat, and mouse cells. The deduced amino acid sequence of COS pp190 consisted of 1251 amino acids and was 96.4%, 87.9% and 88.7% identical to human, mouse and rat IRS-1. The COS pp190 bound to SH2 (src-homology 2) domains of p85, Grb2/Ash, and SH-PTP2, as did IRS-1. In IRS-1-knockout mice, insulin signals are thought to be mediated by IRS-2 (pp190),which is an alternative signaling molecule and is slightly larger than IRS-1. However, the COS pp190 may be a simian homologue of IRS-1, but not of IRS-2. The results of Southern blotting suggested the possibility that Chinese hamster ovary (CHO) cells have not only the IRS-1 gene but also a gene related to the COS pp190.

Insulin exerts physiological effects including stimulation of glucose uptake by binding to receptors. The activated insulin receptor phosphorylates insulin receptor substrate-1 (IRS-1)[1] (1-5) and Shc (4-6). The tyrosine-phosphorylated IRS-1 (pp180) has specific motifs each of which binds to other molecules containing SH2 (src-homology 2) domains, such as p85 of PI 3-kinase (1-5, 7, 8), Grb2/Ash (9,10), SH-PTP2 (11), and Nck (12).

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number D64157.

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Abbreviations : IRS-1, insulin receptor substrate-1; SH2, src-homology 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Shc, src homologous and collagen; CHO, Chinese hamster ovary; HIR, human insulin receptor; GST, glutathione S-transferase; kp, kilobase; SSC, sodium chloride-sodium citrate; PCR, polymerase chain reaction.

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The PI 3-kinase activated by IRS-1 binding is suggested to transmit the insulin signal to glucose transporters and to increase glucose uptake in the cell (13-17). The IRS-1-bound Grb2/Ash is implicated in activation of p21 ras and of MAP kinase (4, 5).

IRS-1-knockout mice have been generated to examine the role of IRS-1 (18, 19). It was suggested that an alternative molecule, tentatively termed IRS-2 (pp190), which is slightly larger than IRS-1 (pp180), transmitted insulin signals in the absence of IRS-1 in these knockout mice (19-21). In the course of our experiments, we found that simian COS cells have a 190-kDa IRS-1 like protein (pp190) which is slightly larger than the IRS-1 (pp180) of human, rat, and mouse cells. To examine the relationship among the COS pp190, IRS-1 (pp180), and IRS-2 (pp190), we cloned a cDNA encoding the COS pp190 and characteristics of the protein were studied.

MATERIALS AND METHODS

Cells and materials — Cells used: COS-HIR cells, simian COS cells stably overexpressing human insulin receptors (HIR); CHO-HIR cells, Chinese hamster ovary (CHO) cells overexpressing HIR (22); NIH-HIR cells, NIH 3T3 cells overexpressing HIR (23); IM9 cells, a human lymphocyte cell line. An anti-phosphotyrosine (pTyr) antibody, MB6093 was purchased from BioMarker (Israel) and an anti-GST (glutathione S-transferase) antibody was prepared as described previously (24). All other reagents were of analytical grade.

Cloning and sequence of COS pp190 — Double strand cDNA was synthesized from mRNA of COS cells, and inserted into λgt10 according to the manufacturer's instruction (Gibco/BRL). Independent 6.4×10^5 plaques were screened with a rat IRS-1 fragment coding N-terminal 1.8-kb fragment (1). The three independent clones obtained have a Kozac initiation codon and a 2.5-kb open reading frame, but they do not contain an in-frame stop codon. Therefore, we used the biotin capture PCR (polymerase chain reaction) to extend further the cDNA in the 3' direction (25). The PCR fragments (1.5-kb) were subcloned, and the four independent clones were sequenced to avoid misincorporation during PCR. The 1.5-kb PCR fragment contained an in-frame stop codon, and a full-length 3.8-kb cDNA encoding COS pp190 was assembled using appropriate restriction enzyme sites .

Transient expression of COS pp190 and the binding to GST-fused SH2 domains — COS cells in 60-min dishes were transfected using Lipofection reagent (GIBCO/BRL) with plasmids containing pCXN2 promoter (3μg each transfection) (26). After 60-h incubation, the cells were treated with 10^{-7} M insulin for 1 min at 37°C, and lysed with a buffer containing 1% NP40, as described previously (24). To examine the binding activity to GST (glutathione S-transferase)-fused SH2 domains of PI 3-kinase p85, Grb2/Ash, and SH-PTP2, the cell lysates were incubated with the purified GST-fused SH2 domains from E. coli (24). The tyrosine-phosphorylated proteins binding to each GST-fused SH2 domain were precipitated with an anti-GST antibody and protein A-Sepharose (Pharmacia), electrophophoresed on 6% SDS-PAGE, and detected by Western blotting using an anti-pTyr antibody, as described previously (3).

Southern blotting — Genomic DNAs of COS cells and CHO cells were prepared as described (27). Each DNA (5μg) was digested with the appropriate restriction enzymes, electrophoresed on a 0.8% agarose gel, denatured *in situ* and transfered onto nitrocellulose membrane (Schleicher & Schuell). The membrane was hybridized with ^{32}P-labeled full length 3.8-kb cDNA coding COS pp190, or with ^{32}P-labeled full length 3.7-kb cDNA coding rat IRS-1 (pp180) in a hybridization solution containing 50% formamide at 42°C, and washed with 2 × SSC and 0.1% SDS three times for 10 min at room temperature and with 1 × SSC and 0.1% SDS for 30 min at 50°C (27).

RESULTS AND DISCUSSION

The tyrosine kinase activity of insulin receptors is crucial to transmit insulin signals (4, 5). We examined the insulin-stimulated tyrosine-phosphorylation in various cell lines overexpressing human insulin receptors (HIR), by Western blotting using an anti-phosphotyrosine (pTyr) antibody (Fig. 1). In response to insulin, the β-subunit of HIR was autophosphorylated in COS-HIR (lane2), CHO-HIR (lane4) and NIH-HIR (lane6) cells which are constitutively overexpressing exogenous large numbers of HIR. In IM9 cells which have a smaller number of endogeneous HIR than the other three lines, IRβ autophosphorylation is weak (lane8) in comparison with findings in the other three lines. Insulin stimulates tyrosine phosphorylation of IRS-1 (pp180) in CHO-HIR (lane4), NIH-HIR (lane6) and IM9 (lane8) cells. However, in the simian COS-HIR cells, a 190-kDa protein (COS pp190) which is slightly larger than IRS-1 (pp180) was tyrosine-phosphorylated as well as IRβ (lane2).

As the COS pp190 bound to PI 3-kinase and Grb2/Ash like IRS-1, the COS pp190 was expected to have some similarity to IRS-1 (Hayashi, H., *et al*, unpublished observation). We screened a cDNA library derived from COS cells with a ^{32}P-labelled rat IRS-1 fragment. The full length cDNA encoding COS pp190 was a 3756-bp open reading frame starting from a Kozac ATG to a TAG stop codon, and the deduced amino acid sequence consisted of 1251 amino acids, as shown in Fig. 2. The amino acid sequence of COS pp190 was 96.4%, 88.7%, and 87.9% identical to human (28, 29), rat (1), and mouse (30) IRS-1, respectively . The surrounding sequences of expected binding sites to Nck (Tyr^{151}), p85 (Tyr^{612} and Tyr^{950}), Grb2/Ash (Tyr^{905}), and SH-PTP2 (Tyr^{1188}) were highly-conserved. The additional 8~16 amino acids of COS pp190 compared to IRS-1 and the long Gln stretch (from Gln^{875} to Gln^{891}) (Fig.2) may contribute to the slower mobility of COS pp190 in SDS-PAGE (Fig.1). However, the total amino acid sequence of COS pp190 is not identical to the newly cloned IRS-2 (21).

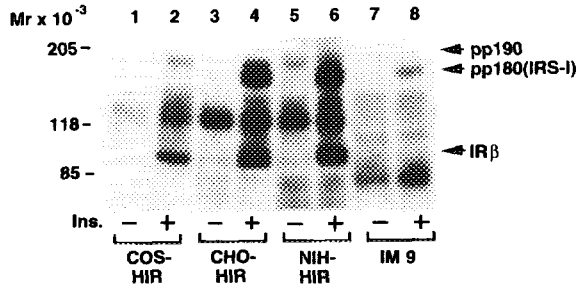

Fig. 1. Insulin-dependent phosphorylation of pp190 in COS cells and pp180 (IRS-1) in CHO cells, NIH cells and IM9 cells. Cells were treated with (+) or without (-) 10^{-7} M insulin for 1min, and the cell lysates were directly electrophoresed on 6% SDS-PAGE. After transfer onto nitrocellulose paper, the tyrosine phosphorylated proteins were detected with an anti-phosphotyrosine (pTyr) antibody. Cell lines ; COS-HIR, simian COS cells stably overexpressing human insulin receptors (HIR) ; CHO-HIR, hamster CHO cells stably overexpressing HIR ; NIH-HIR, mouse NIH3T3 cells stably overexpressing HIR ; IM9, human lymphocytes. Arrows indicate the pp190, pp180 (IRS-1), and insulin receptor β-subunit (IRβ).

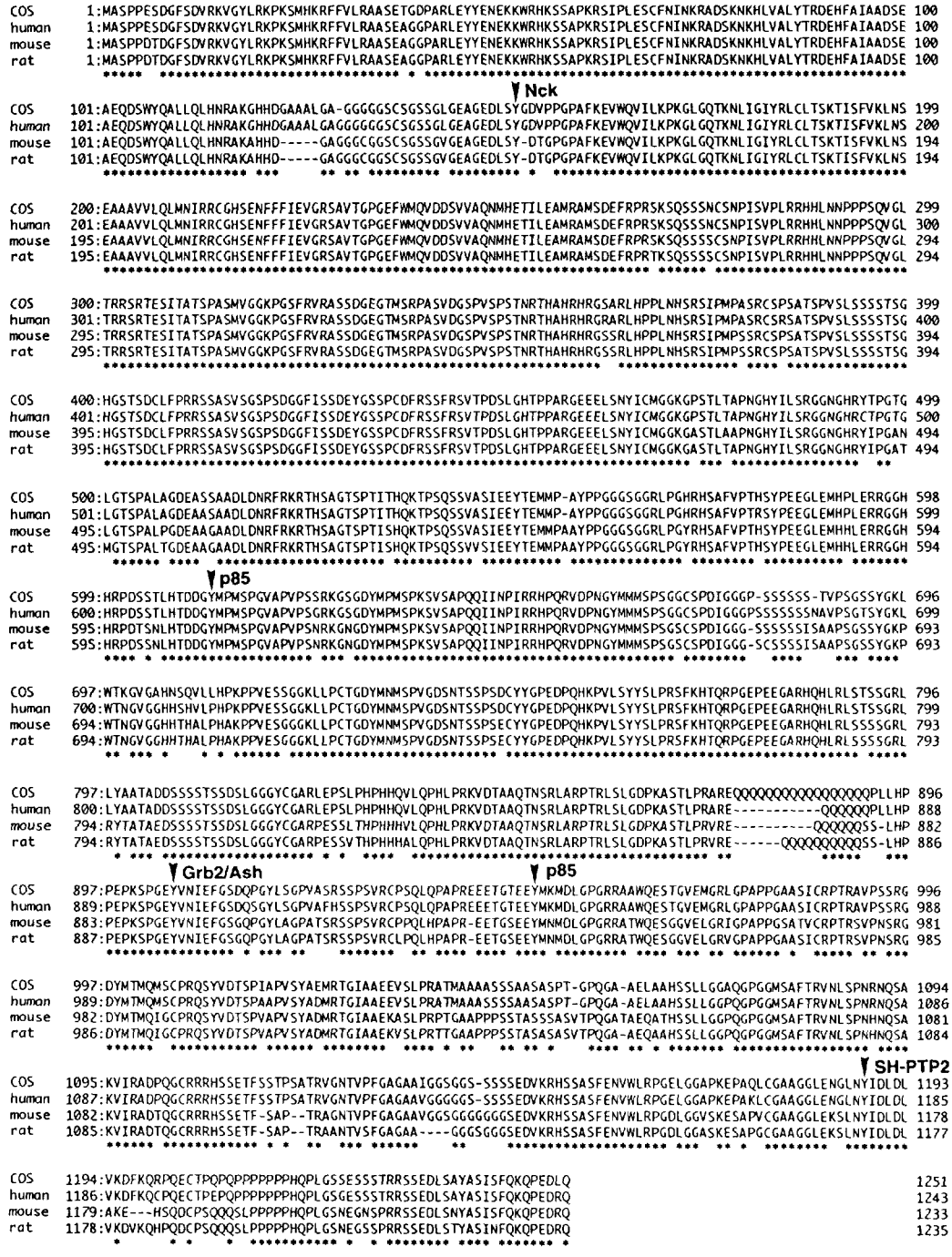

Fig. 2. Comparison of COS pp190 amino acid sequence with IRS-1 (pp180) sequences human, rat, and mouse. The deduced COS pp190 sequence is shown at the top. Reported IRS-1 sequences of human muscle (29), rat liver (1), and mouse genome (30) are shown in the second, third, and fourth lines, respectively. Bars (-) represent the gap. Identical amino acids are indicated by asterisks (*) at the bottom. Expected tyrosine residues to bind SH2 domains of Nck, p85 of PI 3-kinase, Grb2/Ash, and SH-PTP2 are indicated by arrowheads.

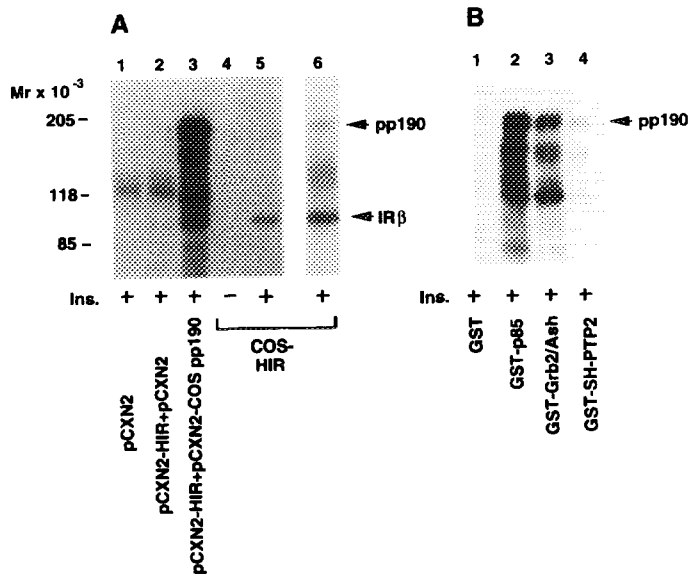

Fig. 3. Overexpression of pp190 in COS cells (A) and the binding to GST-fused SH2 domains *in vitro* (B). (**A**) The cDNA encoding pp190 was subcloned into a mammalian expression vector pCXN2, and overexpressed transiently in COS cells by transfection using Lipofection reagent and treated with 10^{-7} M insulin, as described under "MATERIALS AND METHODS". The transiently expressed proteins were electrophoresed on 6% SDS-PAGE and blotted with the anti-pTyr antibody. The transfected plasmids are ; lane 1, pCXN2 (3μg) ; lane 2, pCXN2 (1.5μg) and pCXN2-HIR (1.5μg) ; lane 3, pCXN2-HIR (1.5μg) and pCXN2-pp190 (1.5μg). To compare the endogenous pp190 of COS-HIR cells, the COS-HIR cell lysates treated with (+) or without (-) 10^{-7} M insulin were applied as control (lanes 4 and 5). Longer exposed autoradiography of lane 5 is also shown as lane 6. (**B**) The purified glutathione S-transferase (GST)-fused SH2 domains of PI 3-kinase (lane 2), Grb/Ash (lane 3), and SH-PTP2 (lane 4) were incubated with transiently overexpressed COS pp190 and insulin receptors in the presence of 10^{-7} M insulin, and the specifically-bound COS pp190 to each GST-SH2 domain was precipitated with an anti-GST antibody and Protein A-Sepharose. The bound tyrosine-phosphorylated proteins were electrophoresed on 6% SDS-PAGE, and blotted with an anti-pTyr antibody. The COS pp190 was also incubated with GST (lane 1) as control.

Next, we transiently-expressed the cDNA of COS pp190 in COS cells, as shown in Fig. 3A. An apparent tyrosine-phosphorylated 190-kDa protein was detected by Western blotting using the anti-pTyr, when the cDNA of COS pp190 was transfected with an insulin receptor cDNA (lane3). This means that the transiently-expressed 190-kDa protein was phosphorylated at tyrosine residues with the insulin receptor, then migrated at the same position as the endogenous pp190 of COS-HIR cells (lanes 3 and 6 which is a longer-exposed autoradiography of the lane5). In addition, Fig. 3B shows that the transiently-expressed COS pp190 specifically bound to the glutathione S-transferase (GST)-fused SH2 domains of PI 3-kinase p85 (lane2), Grb2/Ash (lane3), and SH-PTP2 (lane4), but the COS pp190 did not bind to GST (lane1). These characteristics of COS pp190 are the same as IRS-1 and IRS-2.

We next examined the gene(s) of COS pp190 by Southern blotting. As shown in Fig. 4A, several weak bands were detected in addition to a strong band for each restriction enzyme

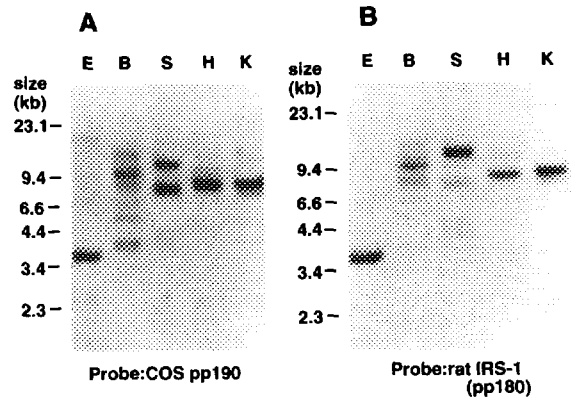

Fig. 4. Southern blot analysis of COS pp190 gene. Genomic DNA (each 5μg) of COS cells was prepared and digested with the restriction enzymes, EcoRI (E), BamHI (B), SmaI (S), HindIII (H), and KpnI (K). After being electrophoresed on a 0.8 % agarose gel, the digested DNAs were transferred onto nitrocellulose paper and probed with ^{32}P-labeled full-length 3.8-kb cDNA coding COS pp190 (**A**) and ^{32}P-labeled full-length 3.7-kbp cDNA coding rat IRS-1 (pp180) (**B**).

digestion of genomic DNA derived from COS cells, by hybridizing with the entire cDNA (3.8-kb) of COS pp190. Almost the same bands were detected by hybridizing with the entire rat IRS-1 cDNA (3.7-kbp) (Fig. 4B). These data suggest that COS cells have only one IRS-1-related gene, and that the pp190 may be a simian homologue of IRS-1 (pp180) of COS cells.

However, in CHO cells, sizes of the bands detected by the probe of the COS pp190 cDNA (3.8-kb) differed from those detected by the probe of the rat IRS-1 cDNA (3.7-kb), on

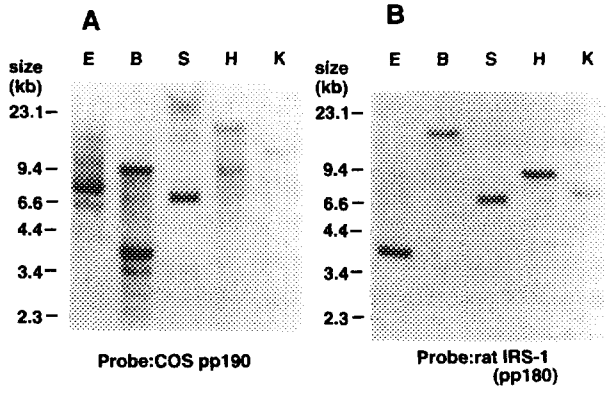

Fig. 5. Southern blot analysis of CHO genome using the COS pp190 cDNA (A) and rat IRS-1 (pp180) cDNA (B) as probes. Genomic DNA (5μg each) of CHO cells was prepared, and digested with the restriction enzymes, EcoRI (E), BamHI (B), SmaI (S), HindIII (H), and KpnI (K). The digested DNAs were electrophoresed, transferred onto nitrocellulose paper, and probed with the ^{32}P-labeled 3.8-kb COS pp190 cDNA (**A**) and ^{32}P-labeled 3.7-kb rat IRS-1 (pp180) cDNA (**B**), as in Fig. 4.

the Southern blotting after digestion of CHO genomic DNA by EcoRI (E), BamHI (B) and KpnI (K) (Fig. 5-A and -B).

These results suggest the possibility that CHO cells may carry not only the IRS-1 gene but also another gene related to COS pp190. Structural and functional relationships between IRS-1 (pp180), IRS-2 (pp190) and COS pp190 will provide new insight into the diverse IRS signaling system.

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