

## Cloning and Increased Expression of an Insulin Receptor Substrate - 1-Like Gene in Human Hepatocellular Carcinoma

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Human insulin receptor substrate-1 (hIRS-1) cDNAs were cloned from a  $\lambda$ GT11 expression library using a monoclonal antibody (MAb) produced against a human hepatocellular carcinoma (HCC) cell line (FOCUS). The predicted amino acid sequence derived from both a genomic DNA fragment and the cDNAs showed a 90.5% identity to the previously reported rat IRS-1 cDNA [Sun, X.P. (1991) *Nature* 352, 73-77]. Multiple potential phosphorylation sites, that suggest an intrinsic function of this molecule in response to insulin action, were highly conserved between the two species. A c.a. 180 kDa hIRS-1 protein was immunoprecipitated and found to be phosphorylated on tyrosine residue(s) following insulin stimulation of HuH-7 HCC cells. Northern blot analysis demonstrated a single c.a. 5 kb transcript in HCC cell lines and tissues. Higher levels of hIRS-1 gene transcripts were observed in HCC tumors compared to adjacent non-involved normal liver. © 1992 Academic Press, Inc.

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A recent study has shown that the rat IRS-1 cDNA (1) encodes a pp 185 protein which is believed to be the major specific cellular substrate for insulin receptor tyrosine kinase (2). It is noteworthy that the structure of the rat IRS-1 revealed a unique molecule containing multiple phosphorylation sites including nine potential tyrosine phosphorylation YXXM and YXXM motifs (1). It has been suggested that these motifs following phosphorylation, bind to molecules containing the Src-homology domains 2 and 3 (SH2/SH3) (3). Thus, the IRS-1 molecule has been proposed to be a multi-site "docking" protein and therefore may be one of the main target molecules for insulin action within the cell and may play a role in intracellular signaling pathways (1). Here we show the molecular characteristics of the proposed human form of the protein and demonstrate after insulin stimulation of HCC cells tyrosine phosphorylation of a similar size protein to pp 185 found in rat liver (2).

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The abbreviations used are: IRS-1 (insulin receptor substrate-1), HCC (hepatocellular carcinoma), IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), SDS (sodium dodecyl sulfate), PMSF (phenylmethylsulfonyl fluoride) SH2/SH3 (Src homology 2/ Src homology 3), EGTA ([ethylenedis(oxyethylenenitrilo)] tetracetic acid, PI-3' kinase (phosphatidylinositol 3'-kinase).

## METHODS

**cDNA and Genomic Cloning.** A  $\lambda$ GT11 expression library constructed from the human HCC cell line (FOCUS) was screened with the FB-50 Mab selected from a library of MAbs produced against the FOCUS cell line as described previously (4). A cDNA designated FB-50.1 was initially isolated. Two additional cDNAs, namely FB-50.F9 and FB-50.B1 were isolated from FOCUS and pre-B cell (kindly provided by Dr. Rene Bernards, MGH Cancer Center) cDNA libraries, respectively. A c.a. 10 kb genomic DNA fragment was isolated from a human placental genomic library (Clontech, Palo Alto, CA) using the FB-50.F9 cDNA fragment as a probe. The relationship between the isolated hIRS-1 like cDNAs and genomic fragment and the previously reported rat IRS-1 cDNA are shown in Fig 1A. Nucleotide sequence analysis was performed as previously reported except T7 DNA polymerase (Pharmacia NJ) was used instead of the Klenow fragment (4).

**Northern blot analysis.** Total cellular RNA from FOCUS cells and HCC tissues were isolated and Northern blot was performed with the FB-50.F9 cDNA probe labeled with  $\alpha$ - $^{32}$ P-dCTP (New England Nuclear, Boston, MA) using the Multiprime labeling method (Amersham Corp., UK) as previously described (4).

**Polyclonal antibody production.** Polyclonal antibodies were prepared for immunoprecipitation experiments against a recombinant hIRS-1 protein fragment derived from the FB-50.1 cDNA (Fig 1A, 1B). This cDNA was subcloned into the EcoRI site of pGEX-1 (Pharmacia, NJ). The prokaryotic expression vector was constructed to yield a fusion protein with glutathione S-transferase following the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). In these experiments, 1mM IPTG was used for induction of the fusion protein. Purification of the fusion protein was performed as described by Kaelin Jr., W.G., et al (5) using the glutathione-Sepharose (Sigma) as an affinity reagent. Approximately 0.1 to 0.2 mg of purified recombinant fusion protein emulsified with Freund's complete adjuvant (Difco) was intradermally injected into rabbits at multiple sites on the back. Booster injections were performed 3, 5, and 7 weeks later with c.a. 0.1-0.2 mg of purified fusion protein emulsified with Freund's incomplete adjuvant (Difco). Immune rabbit serum was obtained 7 days after the final injection for the immunoprecipitation experiments.

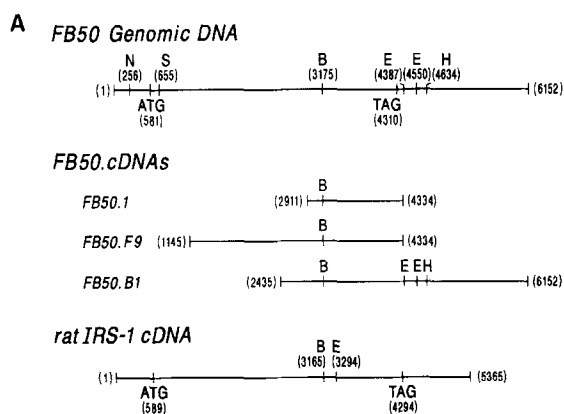
**Immunoprecipitation and Western Blot Analysis.** For the immunoprecipitation reactions, 1 ml of HuH-7 HCC cellular extracts in 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 50  $\mu\text{g}/\text{ng}$  PMSF, 0.2  $\mu\text{g}/\text{ml}$  leupeptin and 0.2  $\mu\text{g}/\text{ml}$  Aprotinin were immunoprecipitated with polyclonal antibody for 5 hours at 4°C (6) with and without a 5 minute exposure of cells grown to near confluency in 100 mm culture dishes to  $1 \times 10^{-7}$  M insulin at 37°C. After immunoprecipitation, the pellet was resuspended in the SDS sample buffer, resolved on SDS-7.5% polyacrylamide gels (7), transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-fusion protein antibodies followed by incubation with  $^{125}\text{I}$ -labeled goat anti-rabbit IgG F(ab') $_2$  (New England Nuclear, Boston, MA) as described previously (8). Parallel immunoprecipitation experiments were performed with an anti-phospho-tyrosine MAb (kindly supplied by Dr. Ed Harlow, MGH Cancer Center) followed by probing with  $^{125}\text{I}$ -protein A (New England Nuclear, Boston, MA). The blots were dried and autoradiographed.

## RESULTS AND DISCUSSION

The cloned genomic hIRS-1 DNA sequence from n.t. 1 to 6,152 corresponds to the transcribed region previously reported for the rat IRS-1 cDNA (1). As presented in Figures 1A and 1B, no intron sequences were found in this region. The sequence homology between human and rat IRS-1 cDNAs was found to be high. For example, there was a 76, 85, and 42% homology in the 5' non coding, protein coding and 3' non coding regions respectively. There are also 7 ATTTA sequences that have been previously shown to destabilize mRNA (9). In the hIRS-1 cDNAs, no poly-A tail was present. However, one polyadenylation signal AATAAA was found at

n.t. 6,130. In addition, there were two polyadenylation signals present in the genomic DNA fragment downstream of nucleotide 6,130 (data not shown).

Comparison of the predicted amino acid sequence between human (1,243 a.a.) and rat IRS-1 (1,235 a.a.) proteins are shown in Figure 2. There is a 90.5% identity at the amino acid level between the two proteins (1). More importantly, the multiple potential phosphorylation sites are highly conserved between the two species. For example, there are four cyclic AMP-dependent protein kinase (10) (R/K-R/K-X-S/T: a.a. 78, a.a. 528, a.a. 1,101 and a.a. 1,224 in Fig. 2), 13 protein kinase C (11) (S/T-X-R/K: a.a. 190, a.a. 301, a.a. 324, a.a. 352, a.a. 442, a.a. 625, a.a. 637, a.a. 775, a.a. 796, a.a. 921, a.a. 985, a.a. 1,085 and a.a. 1,219 in Fig. 2) and 10 tyrosine kinase (3) (E-X-Y-X-E: a.a. 552, Y-M-X-M: a.a. 613, a.a. 633, a.a. 663, a.a. 733, a.a. 942 and a.a. 990, Y-X-X-M: a.a. 466, a.a. 552 and a.a. 1,013, E-Y-Y-E: a.a. 46 in Fig. 2) sites. There is a highly conserved potential ATP-binding site at lysine 162 (A-X-K<sub>162</sub>-X-I/V/L) 15 residues downstream from the ATP consensus recognition sequence (G-X-G-X-X-G). A previous study has suggested that rat pp 185 protein encoded by the IRS-1 gene was phosphorylated not only at tyrosine but also at serine and threonine residues following insulin stimulation of FaO rat hepatoma cells (2). The presence multiple potential phosphorylation sites suggest an important intrinsic role of the molecule for signal transduction. Thus, the pp 185 may be a unique multi-site "docking" protein that interacts with other SH2/SH3 containing proteins following phosphorylation of tyrosine as well as for other tyrosine, serine and threonine kinases (1,3). Indeed, recent evidence suggests that phosphatidylinositol 3'-kinase (PI-3' kinase) was immunoprecipitated with anti-IRS-1 and this observation supports the concept that rat IRS-1 binds signal transduction molecules (1).



**Fig. 1.**

**A:** Physical map and relationship of the hIRS-1 genomic fragment (upper lane) and cDNAs (middle three lanes) compared to the previously reported rat IRS-1 cDNA (bottom lane). The nucleotide numbering sequence in rat cDNA are the same as previously described (1). Restriction enzyme cleavage sites are indicated as follows: N: Nsi I, S: Sph I, B: Bam HI, E: Eco RI, H: Hind III. **B:** Nucleic acid sequence of the hIRS-1 genomic DNA fragment. The predicted translation start codon (ATG), stop codon (TAG), a consensus sequence for mRNA destabilization (ATTTA) and a putative polyadenylation signal (AATAAA) are in a bold type. The protein coding region is underlined.

**B**

1 TGGTATTGTC GCGGCTGGTG GCGGCGGGGA CTGTTGACGG GTGGGAGGAC  
 101 TCCCCCTGCC CAAGGATATT TAATTTCGCT CGGGAATCGC TGCTTCCAGA  
 201 GACCCCGCAG TGTCGCCCTCC CTGTGCCGGA CTCACGCCGG GGCACACGAGA  
 301 GCTCTCGAGG GATCGGGGCT GCGCTCAGCC CGGACGCACT GCTTCCCGCG  
 401 AGCTGCGTCC TCCTTCAGCT GCGCTCAGCC GCGCGGGGCG GCGCGTGGAT  
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 601 CGATGGCTTC TCGAGCTGCG GCAAGGTGGG CTACCTGCGC AAACCTAAGA  
 701 CCGGCGCGCC TCGAGTACTA CGAGACACAG AAGAAGTGGC GGCACAAATC  
 801 AGCGGCGTGA CTCAAGAAC AGGACCTGCG TGGCTCTCTA CAGCCGCGAC  
 901 CGAGGCTCTC CTACAGCTGC ACAACCGTGC TAAGGCCAC CAGCAGCGAG  
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 2301 ACTCGGCTTT CGTGCCGACC CGTCTTACC CAGAGGAGGG TCTGGAATG  
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Fig. 1 - Continued

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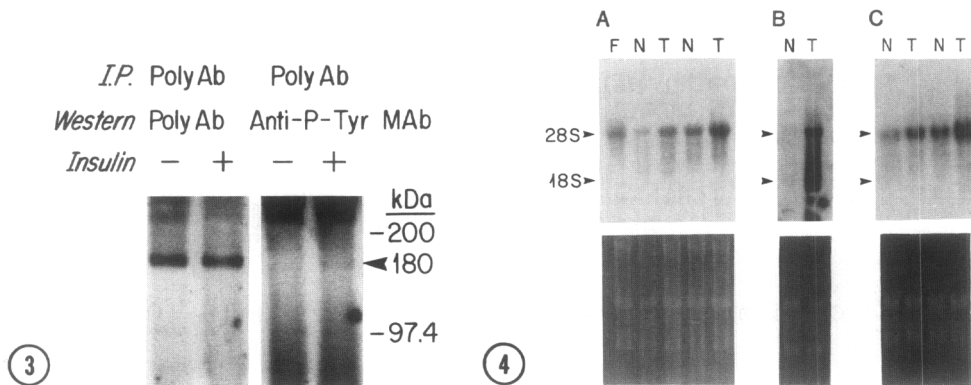
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1187 DCPSQQQLPPPPPHQLGSGESSSTRSSSEDLSAYASINQKQPEDRQ*

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**Fig. 2.** Comparison of the predicted amino acid sequence of hIRS-1 like gene to rat (r)IRS-1. Upper line shows hIRS-1 at the bottom line depicts rIRS-1 amino acid sequences. The consensus sequences for potential tyrosine phosphorylation sites are indicated by bold type.

We confirmed that the FB-50 Mab recognized the hIRS-1 protein, by preparing a polyclonal antibody against a recombinant fusion protein using the FB-50.1 cDNA as shown in Figure 1. Both antibody preparations immunoprecipitated a c.a. 180 kDa protein in HuH-7 cells and  $^{125}$ I labeled FB-50 Mab detected the same protein after immunoprecipitation with polyclonal antibody and Western blot analysis (data not shown). Finally, insulin stimulation studies of serum starved HuH-7 HCC cells revealed a 180 kDa phosphoprotein when immunoprecipitated with the polyclonal antibody, followed by Western blot analysis with an anti-phospho-tyrosine Mab (Figure 3). These results demonstrate that the cloned hIRS-1 cDNA encodes a c.a. 180 kDa peptide that represents the human analogue of the rat pp 185.

Since the FB-50 Mab produced against the FOCUS HCC cell line was used to isolate the hIRS-1 cDNA, we performed a Northern blot analysis on FOCUS cells, normal human liver and HCC tumors in order to determine if there was increased expression at the RNA level. As shown in Figure 4, a single c.a. 5 kb transcript was detected in FOCUS cells and all HCC tumors as well as normal liver. However, there was higher expression in HCC compared to the adjacent non-



**Fig. 3.** Demonstration that the polyclonal antibodies (poly Ab) prepared against a fusion protein encoded by the FB-50.1 cDNA immunoprecipitates a c.a. 180 kDa protein from HuH-7 HCC cells. This protein is phosphorylated on tyrosine residues (anti-P-Tyr MAb) following insulin stimulation of HuH-7 cells.

**Fig. 4.** Comparison of human IRS-1 like gene expression in human HCC vs. non-involved adjacent normal liver by Northern blot analysis. Top: 5 pairs of HCC tissues (T) and adjacent normal liver tissues (N) are depicted along with FOCUS HCC cells (F). The positions of 28 and 18 S rRNA are shown. Each lane contains equal amounts c.a. 20 µg of total RNA. Bottom: ethidium bromide-stained gel before transfer. Note that only a single c.a. 5 kb transcript was observed in HCC and normal liver.

involved normal liver. Since insulin is a potent growth factor for hepatocytes, over-expression of hIRS-1 gene in HCC suggests that it is upregulated during hepatocarcinogenesis.

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