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Absence of functional insulin receptor substrate-3 (*IRS-3*) gene in humans

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Abstract

Aim/hypothesis. Insulin receptor substrate (IRS) proteins play important roles in insulin action and pancreatic beta-cell function. At least four mammalian IRS molecules have been identified. Although genes and cDNAs encoding human IRS-1, IRS-2, and IRS-4 have been cloned, IRS-3 has been identified only in rodents. Thus, we have attempted to clone the human *IRS-3* gene.
Methods. Insulin-stimulated rat or human adipocytes were subjected to Western blot analysis to assess IRS-3 tyrosine phosphorylation. Human liver and adipose cDNA libraries were screened in an effort to clone IRS-3 cDNA. A PCR-based approach was designed to amplify IRS-3 cDNA. Reverse transcription PCR was carried out using mRNA from adipose tissue, liver, and skeletal muscle as templates in combination with an in silico screen using mouse *IRS-1*, *IRS-2* and *IRS-3* in a tblastn search of the draft public human genome.
Results. In human adipocytes we did not detect a M_r 60 000 phosphoprotein corresponding to IRS-3, whereas in rat adipocytes IRS-3 protein and insulin-stimulated tyrosine phosphorylation was readily observed. None of the molecular approaches provided evidence for a functional *IRS-3* gene in human tissue. Two deletions in human *IRS-3* gene were identified using bioinformatics. The human *IRS-3* gene product is predicted to lack a phosphotyrosine binding domain and also the sequence corresponding amino acid 353–407 of murine *IRS-3*. The contiguous sequence of genomic DNA between these two homologous regions does not have the coding information for human *IRS-3*.
Conclusion/interpretation. In silico screening of the human *IRS-3* genome region, combined with further biological and molecular validation, provides evidence against a functional IRS-3 in humans. [Diabetologia (2002) 45:1697–1702]

Keywords Adipocytes, bioinformatics, comparative genomics, diabetes, expressed sequence tag, genetics, insulin signalling, phosphatidylinositol 3-kinase, phosphotyrosine binding domain, pleckstrin homology domain.

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Abbreviations: IRS, Insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PTB, phosphotyrosine binding domain; PH, pleckstrin homology domain; SDS, sodium dodecyl sulphate; EST, expressed sequence tag; TBST, Tris-buffered saline with Tween-20.

Insulin receptor substrates (IRS) serve a crucial function in mediating the action of insulin, insulin-like growth factors, and various cytokines [1]. To date, four IRS proteins have been identified in various mammalian species [2, 3, 4, 5, 6]. IRS-1 plays a prominent role in insulin signalling to phosphatidylinositol (PI) 3-kinase [1] and defects have been noted in this target in skeletal muscle [7, 8] and adipocytes [9] from patients with Type II (non-insulin-dependent) diabetes mellitus. In adipose tissue [9], but not skeletal muscle [8, 10], IRS-2 seems to partially compen-

sate for impaired insulin action on IRS-1, as shown by normal IRS-2-associated PI 3-kinase. These correlative studies in humans suggest that *IRS* genes could functionally compensate for each other to maintain signal transduction.

Animal models have been useful tools to illustrate the importance of IRS molecules in disease pathogenesis. When the *IRS-1* gene was inactivated in knockout mice, this led to growth retardation and insulin resistance [11, 12]. Nevertheless, because of partial redundancy, other IRS proteins had the ability to compensate for the absence of IRS-1 [11, 12]. For example, in adipocytes of *IRS-1* knockout mice, IRS-3 was identified as the principal substrate for the insulin receptor tyrosine kinase [13, 14]. To date, the role of IRS-3 has not been evaluated in humans. Since aberrant insulin signal transduction in adipose tissue can contribute to metabolic disturbances and insulin resistance in Type II diabetes, we attempted to clone the human *IRS-3* gene.

Subjects and methods

Subject characteristics. The study group consisted of 11 subjects (10 women, 1 man), ranging in age from 29 to 54 years with a BMI of 21.2 to 45.5 kg/m^2. None of the subjects were taking regular medication or had any known metabolic disorder (besides obesity). All patients had been on stable diets and exercise habits. None of the women were post-menopausal. The subjects gave their informed consent prior to participation in the study. The ethics committee of Huddinge University Hospital approved the study. Subjects were studied after an overnight fast. Seven healthy volunteers underwent a biopsy of subcutaneous paraumbilical fat under local anaesthesia as described [15]. Biopsies were taken from an additional four subjects at the time of abdominal surgery (elective cholecystectomy because of uncomplicated gallstone disease or gastric banding because of obesity). General anaesthesia was induced by a short-acting barbiturate and maintained by fentanyl and nitrous oxygen. Subjects received intravenous saline. Subcutaneous adipose tissue was obtained from an abdominal incision at the beginning of surgery.

Animals. Male Wistar rats (130 g) were purchased from B and K Universal (Sollentuna, Sweden) and housed at the animal facility at Karolinska Institute for 1 week before experimentation. Rats were maintained on a 12 h:12 h light-to-dark cycle and were provided free access to water and standard rodent chow. Animals were studied in the post-prandial state. Rats were killed by CO_2 inhalation and cervical dislocation. Whole epididymal fat pads were removed. Stockholm's North Animal Ethics Committee approved the animal experiments.

Isolation of adipocytes. Human or rat adipose tissue was immediately transported to the laboratory and isolated adipocytes were prepared by collagenase treatment [15, 16]. Adipocytes were incubated for 10 min at 37 °C in the absence or presence of 1000 nmol/l insulin. When samples were used for immunoprecipitation with anti-IRS-3 or anti-phosphotyrosine, cells were washed and then lysed in extraction buffer consisting of 1% Triton X-100, 50 mmol/l Tris, pH 7.4, 1 mmol/l pervanadate (50:1 molar mixture of orthovanadate: H_2O_2), 1 mmol/l PMSF, 25 mmol/l benzamidine and 0.15 mol/l NaCl. After vigorous mixing, fat was removed by centrifugation. Protein was assessed in the supernatant using a commercial kit (Pierce, Rockford, Ill., USA). When samples were used for phosphotyrosine analysis, adipocytes were isolated and incubated as described above, except that the extraction buffer was replaced by sodium dodecyl sulphate (SDS) sample buffer consisting of 2% SDS, 100 mmol/l TrisCl, 1 mmol/l EDTA, 10% glycerol, pH 6.8, 1 mmol/l Na_3VO_4, 20 mmol/l dithiothreitol, 10 µg/ml aprotinin, 10 µmol/l leupeptin, 1 µg/ml pepstatin and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF). The samples were heated (95 °C) for 5 min, frozen in liquid nitrogen and stored at –80 °C. Protein was assessed in the lysate by a precipitating Lowry assay [17].

IRS-3 tyrosine phosphorylation. Cell extracts (800 µg) were immunoprecipitated with antiserum raised against the pleckstrin homology (PH) domain of rat IRS-3 (JD312) or an anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, Ky., USA). Thereafter, the immune complex was washed as indicated [8]. Aliquots were resuspended in Laemmli buffer with β-mercaptoethanol (5%, v/v) and heated (95 °C) for 5 min. Proteins of the immunoprecipitates or SDS-lysates were separated by SDS-polyacrylamide gel electrophoresis (7.5% resolving gel), transferred to nitrocellulose membranes, and blocked in 5% milk in TBST. Membranes were incubated with anti-phosphotyrosine antibody. Phosphorylated proteins were visualized by enhanced chemiluminescence.

Molecular approaches to detect human IRS-3. The mouse *IRS-3* gene contains two exons encoding the protein sequence. Because of the simple genomic structure, a search for human *IRS-3* was initiated by screening a PAC genomic library on filters (Genome Systems, St. Louis, Mo., USA). The filters were probed with ^{32}P-labelled mouse IRS-3 cDNA, with the hybridization temperature decreased to 45 °C to allow for the use of a heterologous probe. Two cDNA libraries were screened in an effort to clone hIRS-3 cDNA, a human liver cDNA library (Cat no. 937220; Stratagene; La Jolla, Calif., USA) and a human differentiated adipocyte cDNA library (Cat no. 937249; Stratagene). Reverse transcription was carried out using mRNA from adipose tissue, liver, and skeletal muscle as templates (Clontech Laboratory, Palo Alto, Calif., USA).

In silico screen of human IRS-3 genome region. The mouse *IRS-1*, *IRS-2* and *IRS-3* were used in a tblastn search (http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html) of the draft public human genome (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html). Two putative human *IRS-3* matches were found in chromosome 7 (entry NT_007751.8). The contiguous sequence, NT_007751.8, was studied further by searching for expressed sequence tags (EST's) in an effort to find support for potential *IRS-3* gene expression.

Materials and antibodies. All chemicals were purchased from Sigma (St. Louis, Mo., USA) or Merck (Rahway, NJ, USA). Phosphotyrosine antibodies were from Transduction Laboratories (polyclonal phosphotyrosine and RC20:HRP) or Affiniti Research Products Limited (PY20:HRP; Devon, UK). Dr. Martin G. Myers Jr. and Dr. Morris F. White (Joslin Diabetes Center, Boston, Mass., USA) generously provided the IRS-3 antibody (JD312).

Results

IRS-3 tyrosine phosphorylation. Human and rat adipocytes were incubated in the absence or presence of

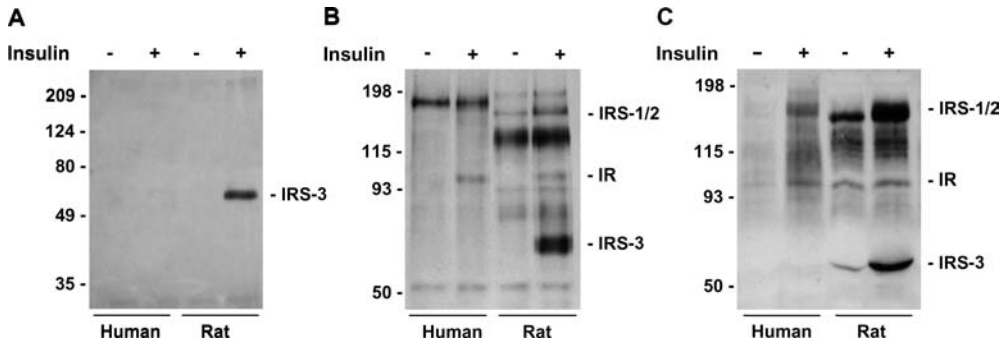

Fig. 1A–C. Insulin-stimulated IRS-3 tyrosine phosphorylation in adipocytes. Human or rat adipocytes lysates were immunoprecipitated (800 µg lysate) with anti-IRS-3 (rat) antibody (**A**) or with anti-phosphotyrosine antibody (**B**). **C** Human or rat adipocytes lysates were solubilized (75 µg lysate) in SDS sample buffer. Membranes were probed with an anti-phosphotyrosine antibody. Results are representative of four, two and seven subjects for (**A**), (**B**) and (**C**) respectively

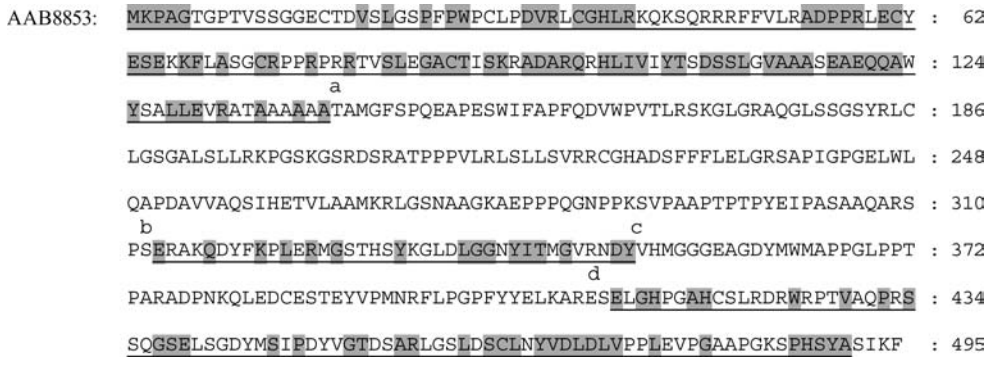

Fig. 2. Comparison between the *mIRS-3* gene and the *hIRS-3* psuedogene. The N- and C- terminal parts of mouse *IRS-3* (AAB8853) that match chromosome 7 in the human genome (NT_007751.8) are *underlined*. Residues of identity between the two species are *shadowed*. Regions corresponding to residues 141–312 and 351–410 (a–b and c–d respectively) of mouse *IRS-3* are missing in the human genome. The sections indicated between b–c were identified by manual examination of the human genome sequence

1000 nmol/l insulin for 10 min. Detergent extracts were subjected to immunoprecipitation with a rat IRS-3 or a phosphotyrosine antibody. Thereafter, immune complexes were analysed by SDS-PAGE, followed by immunoblot analysis using a phosphotyrosine antibody (Fig. 1A, B). Insulin markedly increased the phosphotyrosine content of a M_r 60 000 band corresponding to IRS-3 in rat adipocytes (Fig. 1A, B; lanes 3, 4). In contrast, the putative IRS-3 was not identified in human adipocytes studied under similar experimental conditions (Fig. 1A, B; lanes 1, 2). The antibodies to rat IRS-3 or phosphotyrosine did not recognize IRS-3 protein in human adipocytes. We then subjected SDS-lysates of human or rat adipocytes to immunoblot analysis with phosphotyrosine antibody (Fig. 1C). This experiment showed insulin-stimulated tyrosine phosphorylation of a M_r 180 000 band, presumably corresponding to IRS-1 and possibly IRS-2, in both rat and human adipocytes. Furthermore, a tyrosine phosphorylated M_r 60 000 protein, presumably IRS-3, was identified in insulin-stimulated rat adipocytes (Fig. 1C, lane 4). However, this M_r 60 000 protein was not detected in human adipocytes (Fig. 1C, lanes 1 and 2). The apparent absence of IRS-3 in human adipocytes can be explained as follows. First, our methods might lack the required sensitivity to detect this protein. Second, the pattern of expression of IRS-3 in human tissues might differ from that described in rodents. Thus, we did additional molecular analysis, as well as an in silico screen using bioinformatics applied to the human genome, to examine whether humans express the *IRS-3* gene.

Molecular approaches to detect human IRS-3. In the initial PAC genomic library screening, eight positive clones were obtained. PAC clones were obtained, and DNA was digested with three separate enzymes (Eco RI, Bam HI, and Hind III). Using Southern blot analysis, we identified DNA fragments that hybridised to the mouse IRS-3 cDNA probe. However, when these DNA fragments were sequenced, none of them proved to be human *IRS-3*.

Two additional approaches were used in an effort to clone human IRS-3 cDNA. We screened a human liver and a human differentiated adipocyte cDNA library in an effort to clone hIRS-3 cDNA. Despite screening 10^6 clones from each library, we did not obtain human

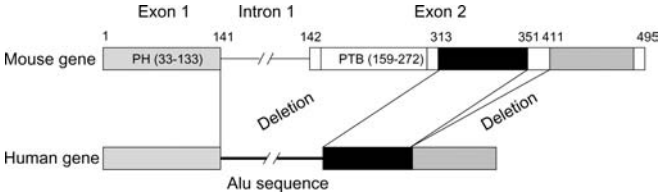

Fig. 3. Comparison of the structure of the mouse *IRS-3* gene and the human *IRS-3* psuedogene. The amino acid numbering system of *mIRS-3* was used to depict the structure of the human *IRS-3* psuedogene. There appear to be two deletions in the human psuedogene; Deletion A) downstream of exon 1, extending past the end of the phosphotyrosine binding domain (PTB) and Deletion B) the sequence corresponding to amino acid residues 353–407

IRS-3 cDNA. In addition, a PCR-based approach was designed to amplify IRS-3 cDNA. Degenerate oligonucleotide primers were synthesized based on regions of amino acid sequence identity between mouse and rat *IRS-3*. Reverse transcription was carried out using mRNA from adipose tissue, liver, and skeletal muscle as templates. Subsequently, the cDNA was amplified using either standard PCR with one set of primers, or nested PCR with two sets of primers. The amplified cDNA was analysed by electrophoresis followed by either ethidium bromide staining, or by Southern blots probed either with ^{32}P-labelled mouse IRS-3 cDNA or degenerate oligonucleotide probes. None of these approaches succeeded in detecting IRS-3 cDNA.

In silico screen of human IRS-3 genome region. Analysis of human genomic databases using mouse *IRS-3* identified a region in the human genome highly homologous to the N-terminus of mouse *IRS-3* (chromosome 7q21.3–7q22.1) encoding a protein sequence 60% identical to a region (amino acids 1–140) in the PH domain of mouse IRS-3 (Fig. 2). However, this human homologue has an in-frame stop codon immediately after the PH domain (Fig. 2). The stop codon was followed by an alu sequence and a poly A sequence. This observation possibly explains how the presumed gene rearrangement might have been introduced. Of interest, this region of the human genome is syntenic with the region of the mouse chromosome 5 containing the *IRS-3* gene [18]. The mouse *IRS-3* gene has been mapped within 1.6 cM (3200 kb) of the *erythropoetin* gene. The genetic sequence corresponding to a fragment of human *IRS-3* is located within 150 kb of the human *erythropoetin* gene [18]. The sequence of 650 kb of human chromosome 7q22 has been determined and annotated. In addition to the sequence corresponding to the PH domain of *IRS-3*, a second nucleotide sequence 732 base pairs downstream was identified to be 43% identical at the amino acid level to another region of mouse *IRS-3* (amino acids 313–491) (Fig. 3). This sequence seems to be composed of two sequences homologous to mouse *IRS-3*, interrupted by a gap. The contiguous sequence of genomic DNA between these two homologous regions lacks the coding information for a human *IRS-3* orthologue. Instead there are 732 bases of low complexity that are probably evolutionary vestiges of introns and exons of a human *IRS-3* gene. Further analysis of human EST databases also failed to identify any clones to support *IRS-3* gene expression. One putative human EST (accession no. AA777710) labelled as "Soares fetal liver spleen 1NFLS S1 homo sapiens cDNA clone 449296 3′ similar to "TR:O08724 insulin receptor substrate-3" was identified and determined to be a chimera between two mouse cDNA's, not a cDNA encoding human *IRS-3*. Thus, the in silico analysis of genomic and EST databases does not support the existence of a functional human *IRS-3* orthologue gene, suggesting that the *IRS-3* gene was lost subsequent to the evolutionary divergence between rodents and humans.

Discussion

In addition to IRS-1 and IRS-2, IRS-3 also mediates insulin action in adipocytes [13, 14]. IRS-3 undergoes tyrosine phosphorylation in rodent adipocytes, leading to activation of phosphatidylinositol 3-kinase, among other possible downstream signalling pathways [4, 13, 14, 19, 20, 21]. The human and mouse *IRS-1* and *IRS-2* proteins are 89% and 83% identical respectively [22]. Thus, homology between *IRS-3* proteins from these different species was expected to be high. To date, the human *IRS-3* orthologue gene has not been cloned and thus, the extent to which this gene is involved in insulin action in human adipocytes is not known. Using both an in silico screen and a biochemical approach, we failed to provide evidence for a functional *IRS-3* gene or protein in human adipocytes. Our in silico screen shows that humans have a degenerate *IRS-3* gene that lacks the information to code for either the phosphotyrosine binding domain or the sequence corresponding to amino acids 351–410 of murine *IRS-3*. Failure to detect insulin-stimulated tyrosine phosphorylation of a M_r 60 000 protein in human adipocytes in this study and in an earlier report [23] strongly supports the hypothesis that humans lack a functional *IRS-3* orthologue gene. However, we cannot exclude the possibility that some humans could express a functional *IRS-3* gene, but this seems unlikely based on the information in the EST data base and the size of the available genomic information. Our study is consistent with the hypothesis that in humans, the *IRS-3* orthologue gene has somehow become redundant, and degenerated through evolutionary processes. The bioinformatic screen showed that the majority of the *IRS-3* region is missing from the human genome. The human *IRS-3* gene product is predicted to lack a phosphotyrosine binding domain and also the sequence corresponding amino acid 351–410 of murine *IRS-3*.

The biological results presented in the present and previous work [23], strongly support the hypothesis that humans lack a functional *IRS-3* gene.

IRS-3 is not directly required to maintain glucose homeostasis, since inactivation of the murine *IRS-3* gene does not lead to a diabetic or insulin resistant phenotype [24]. However, the role of IRS-3 in mediating insulin action becomes more apparent when IRS-1 is lacking. IRS-3 is the major substrate for insulin-stimulated tyrosine phosphorylation in adipocytes from *IRS-1* knockout mice [13, 14]. Even more striking, double *IRS-1* and *IRS-3* knockout mice have severe glucose intolerance and insulin resistance compared to *IRS-1* knockout mice, highlighting the important compensatory role of IRS-3 in maintaining glucose homeostasis (Unpublished observation; P.G. Laustsen, M.D. Michael, B.E. Crute, S.E. Curtis, R.N. Kulkarni, S.R. Keller, G.E. Lienhard, and C.R. Kahn). Since humans do not have a functional *IRS-3* orthologue gene, an additional level of compensation in the insulin signalling pathway appears to be lost. In adipocytes from Type II diabetic or insulin resistant obese humans, or from people with a predominant genetic predisposition for Type II diabetes, there is a selective decrease in IRS-1 protein content [9, 25, 26]. In these subjects, IRS-2 does not seem to fully compensate for depressed expression of IRS-1, since insulin-stimulated tyrosine-associated PI 3-kinase activity, PKB phosphorylation [26] and glucose transport [9, 25, 26] is also impaired. This is consistent with double *IRS-1*/*IRS-3* knockout mice (Unpublished observation; P.G. Laustsen, M.D. Michael, B.E. Crute, S.E. Curtis, R.N. Kulkarni, S.R. Keller, G.E. Lienhard, and C.R. Kahn), whereby reduced IRS-1 protein expression in adipocytes has a deleterious effect on insulin action when IRS-3 is lacking. Thus, results from studies of insulin signalling in adipocytes from *IRS-3* null mice can offer a more physiologically relevant translational application to the delineation of molecular mechanisms contributing to insulin resistance in humans.

In summary, an in silico screen and biological experiments provide evidence against a functional *IRS-3* gene in humans. The lack of IRS-3 in humans arose from rearrangements within the *IRS-3* gene at some time subsequent to the evolutionary divergence between rodents and humans. Thus, IRS-1 and IRS-2, rather than IRS-3, appear to be the important mediators of insulin signalling in human adipocytes.

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