Role of IRS-2 in insulin and cytokine signalling

Xiao Jian Sun, Ling-Mei Wang*, Yitao Zhang, Lynne Yenush, Martin G. Myers Jr, Erin Glasheen, William S. Lane†, Jacalyn H. Pierce* & Morris F. White‡

Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215, USA
* Laboratory of Cell and Molecular Biology, National Institutes of Health, Bethesda, Maryland 20892, USA
† Harvard Microchemistry Facility, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA
‡ To whom correspondence should be addressed

The protein IRS-1 acts as an interface between signalling proteins with Src-homology-2 domains (SH2 proteins) and the receptors for insulin, IGF-1, growth hormone, several interleukins (IL-4, IL-9, IL-13) and other cytokines[1–7]. It regulates gene expression and stimulates mitogenesis, and appears to mediate insulin/IGF-1-stimulated glucose transport[8]. Thus, survival of the IRS-1$^{-/-}$ mouse with only mild resistance to insulin was surprising[9,10]. This dilemma is provisionally resolved with our discovery of a second IRS-signalling protein. We purified and cloned a likely candidate called 4PS from myeloid progenitor cells and, because of its

a

Peptide	Sequence
p47	ALTDLVSEGR
p60	VAYNPYPEDYGDIEIgshk
p80	LPPASAATSQGP-a
p90	ETSVGFQNGLNYIAIDV
p96	LS-eGLQTLPSMQeYpL

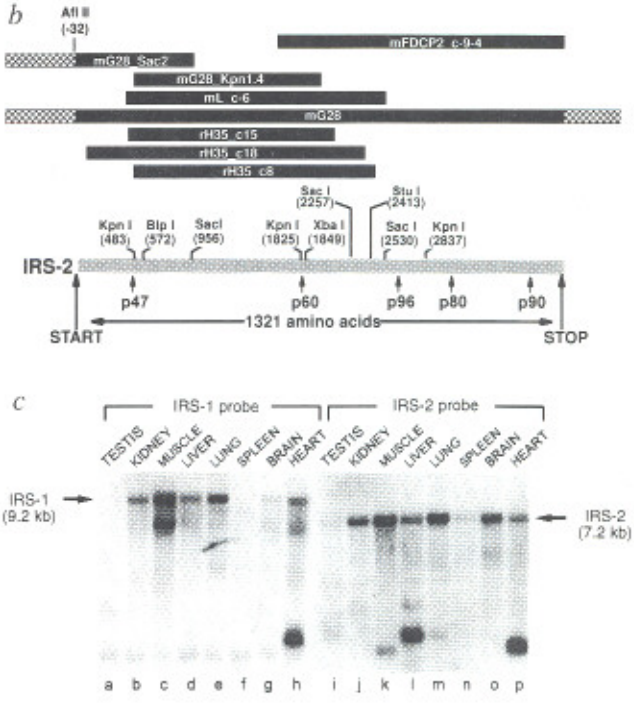


FIG. 1 The molecular cloning of IRS-2. *a*, The amino-acid sequences of five unique tryptic peptides obtained from 4PS are shown in upper case for full confidence, in lower case for less confidence. Dashes indicate positions that could not be assigned. *b*, Map of overlapping cDNA and genomic DNA clones encoding mouse and rat IRS-2. Mouse cDNA (mFDCP2c-9-4) was initially isolated from a cDNA library prepared with mRNA from FDC-P2 cells screened with an oligonucleotide based on the amino-acid sequence of p60. *c*, Northern blot of mouse tissue (Clontech) was hybridized at high stringency with an IRS-2 (2,987–3,325 bp) (lanes *i–p*) or IRS-1 cDNA probe (2,834–3,136 bp) (lanes *a–h*). The ^{32}P-labelled cDNA probes were prepared with Multiprime DNA Labeling System (Amersham)[14].

METHODS. Cell lysates from insulin-stimulated FDC-P2 cells (20×10^9) were clarified by centrifugation and passed over the Sepharose-immobilized GST-fusion protein containing the N-terminal SH2 domain from anti-p85 antibody, washed, and eluted with 100 mM Tris-HCl (pH 7.4) containing 40 mM glutathione, 40 mM DTT, 250 mM NaCl, 0.2 mM sodium orthovanadate and 0.4 mM PMSF. The concentrated eluate was resolved by 7.5% SDS–PAGE, transferred to PVDF membranes and stained with 0.5% Ponceau-S. The area corresponding to 4PS was excised and digested *in situ* with trypsin as described[31]. The eluted peptides were separated by narrow-bore high-performance liquid chromatography using a Vydac C18 reverse-phase column (2.1 mm × 150 mm). The sequences of five unique tryptic peptides (p47, p60, p80, p90, p96) were determined by automated Edman degradation as described[32,33]. The optimized oligonucleotide probe based on the sequence of p60 (*a*) was prepared for library screening with a pair of oligonucleotides containing a 10-nucleotide overlap (underlined sequence), as previously described: 5′-GTGGCCTACAACCCATACCCTGAGGAC-3′ and 5′-AATCTCAATGTCGCCATAGTCCTCAGGG-3′ (ref. 14). About 10^6 plaques were screened with the ^{32}P-labelled oligonucleotide probe (2.5×10^6 c.p.m. ml^{-1}) and one cDNA clone (mFDCP2c-9-4) was obtained from the mouse FDC-P2 cell poly(T)-primed cDNA library prepared in λExlox (Novagen), and one genomic clone (mG28) was isolated from a mouse genomic library in λFIX (Stratagene). Both strands of the 2.4-kb cDNA insert were sequenced with Sequenase (US Biochem.) using specific primers selected at convenient intervals. A mouse lung cDNA library was screened with the 5′ end of clone mFDCP2c-9-4 revealing a new cDNA (mLc-6) that partially overlapped with mFDCP2c-9-4. Two genomic fragments obtained by digestion of mG28 with *Kpn*I (mG28Kpn1.4) or *Sac*I (mG28Sac2) which hybridized with the 5′ end of mLc-6, were subcloned and sequenced. The sequences were aligned and the open reading frame of 3,963 nucleotides was identified using the EUGENE and SAM programs. Rat cDNA corresponding to the 5′ end of mouse IRS-2 was identified with mG28Sac2 in a rat H35 hepatoma cDNA library (rH35c15, rH35c18, and rH35c8). Most of the mouse genomic 5′ sequence was confirmed by alignment with rH35c18, although cDNA clones encoding the extreme 5′ end were never obtained. Northern blots were hybridized overnight at 42 °C with specific probes as described[14].

resemblance to IRS-1, we designate it IRS-2. Alignment of the sequences of IRS-2 and IRS-1 revealed a highly conserved amino terminus containing a pleckstrin-homology domain and a phosphotyrosine-binding domain, and a poorly conserved carboxy terminus containing several tyrosine phosphorylation motifs. IRS-2 is expressed in many cells, including tissues from IRS-1$^{-/-}$ mice[11], and may be essential for signalling by several receptor systems.

Using a Sepharose-coupled SH2 domain derived from p85, about 5 μg 4PS were partially purified from 20×10^9 insulin-stimulated FDC-P2 cells[4]. Five tryptic peptides derived from 4PS were subjected to automated Edman microsequencing: two (p60 and p90) were ~35% identical to IRS-1, whereas the other three (p47, p80 and p96) had no significant homology with any GeneBank entries (Fig. 1*a*).

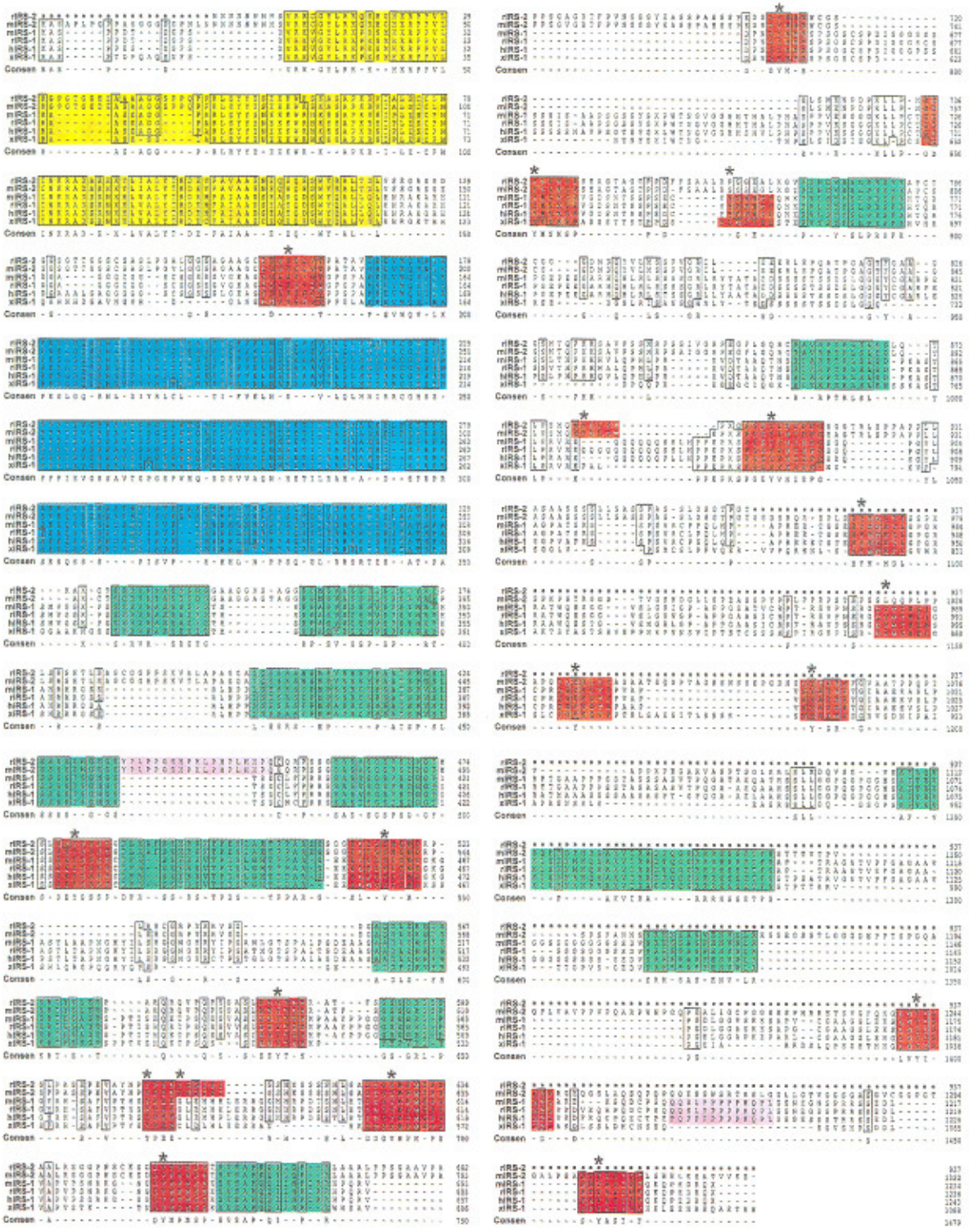


FIG. 2 Sequence alignment between IRS-1 and IRS-2. An optimized alignment of the complete mouse (m) and partial rat (r) amino-acid sequences of IRS-2 against the complete mouse (m), rat (r), human (h) and *Xenopus* (x) IRS-1 sequences was obtained using the PILEUP program (Genetic Computer Group, Madison, Wisconsin). Gaps are indicated by dashed lines, identities are boxed and the consensus sequence is shown. The IH-1^{PH} and the IH-2^{PTB} domains are shown in yellow and blue, respectively; conserved tyrosine-phosphorylation motifs are pink, and the putative phosphorylation site is labelled with an asterisk. Conserved regions of unknown function in the C terminus are shown in green and proline-rich motifs in purple. Incomplete rIRS-2 sequence is indicated by small square dots.

A complementary DNA library from the FDC-P2 cells was screened with an optimized nucleotide probe based on the amino-acid sequence of p60 (Fig. 1*a*). A single clone was isolated (mFDCP2c-9-4) which contained a 2.4-kilobase (kb) insert that was 41% identical to nucleotides 1,353–3,538 of mouse IRS-1 (Fig. 1*b*). The conceptual translation was 35% identical to amino-acid residues 451–1,234 in mouse IRS-1, and it contained the exact amino-acid sequences of p60, p80, p90 and p96. Based on these observations, we designated the putative protein 'IRS-2'.

The complete coding region of IRS-2 (3,963 nucleotides) was assembled from overlapping mouse cDNA and genomic fragments, and confirmed with homologous rat clones (Fig. 1*b*). Northern analysis indicated that the principal messenger RNA transcript in mice for IRS-2 is 7.2 kb, whereas the transcript for IRS-1 is 9.2 kb (Fig. 1*c*). IRS-2 is not limited to haematopoietic cells, as it is expressed in skeletal muscle, lung, brain, liver, kidney, heart and spleen (Fig. 1*c*). Moreover, it was detected with a similar tissue distribution in IRS-1$^{-/-}$ mice, suggesting that it is the alternative insulin receptor substrate in that background[11].

The IRS-2 cDNA encodes a peptide of relative molecular mass 145K, which is 10K longer than IRS-1. Alignment of murine IRS-2 with IRS-1 from *Xenopus*[12], mouse[13], rat[14] and human[15] sources revealed two IRS-homology (IH-1 and IH-2) domains at the amino terminus (Fig. 2). The IH-1 domain contains 101 (IRS-1) to 112 (IRS-2) amino acids with 69% identity, whereas the IH-2 domain contains 156 (IRS-1) to 160 (IRS-2) amino acids with 75% identity (Fig. 2). By contrast, the C-terminal regions of IRS-1 and IRS-2 are 35% identical; however, approximately 20 common or unique tyrosine-phosphorylation motifs are located in relatively similar positions, suggesting that both IRS-1 and IRS-2 bind multiple SH-2 proteins, including p85, Grb-2 and SH-PTP2 (Fig. 2).

The expression and tyrosine phosphorylation of endogenous IRS-2 was investigated in ordinary FDC-P2 cells, and compared to recombinant IRS-1 or IRS-2 expressed in 32D cells which do not contain these endogenous proteins[5]. Cell extracts were immunoprecipitated with antibodies against PY, IRS-2 or IRS-1, and tyrosine phosphorylation was detected by immunoblotting with anti-PY antibody. During insulin or IL-4 stimulation, anti-PY and anti-IRS-2 immunoprecipitated tyrosine-phosphorylated IRS-2 from FDC-P2 cells and 32D^{IRS2} cells (Fig. 3*a*, *b*), whereas anti-IRS-1 did not (Fig. 3*c*). In contrast, anti-PY and anti-IRS-1 strongly immunoprecipitated IRS-1 from 32D^{IRS1} cells (Fig. 3*a*, *c*), whereas anti-IRS-2 did not crossreact

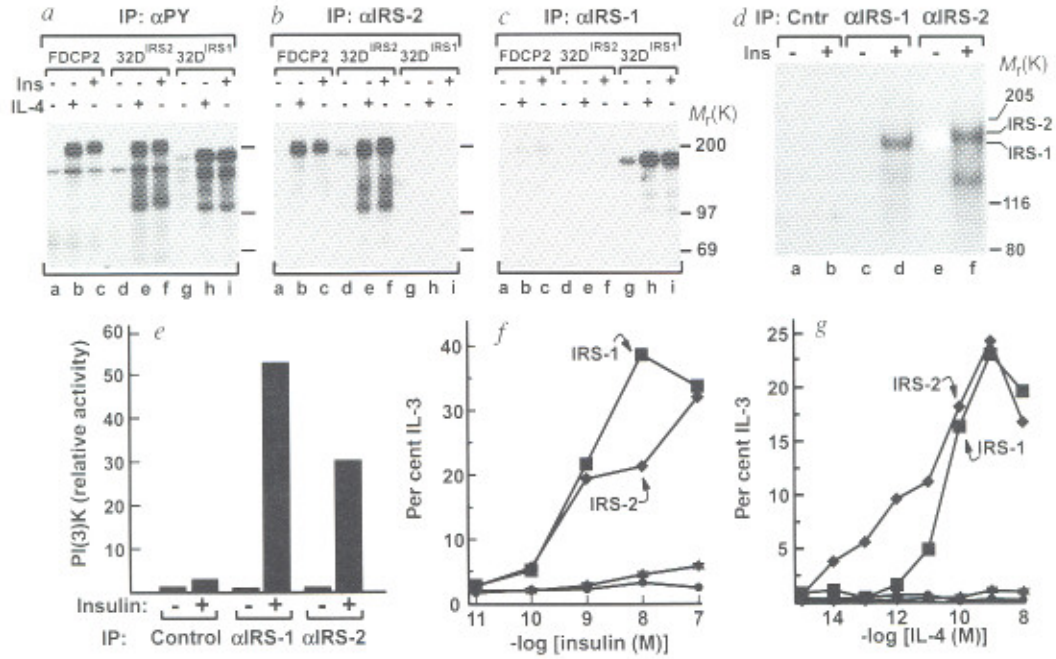
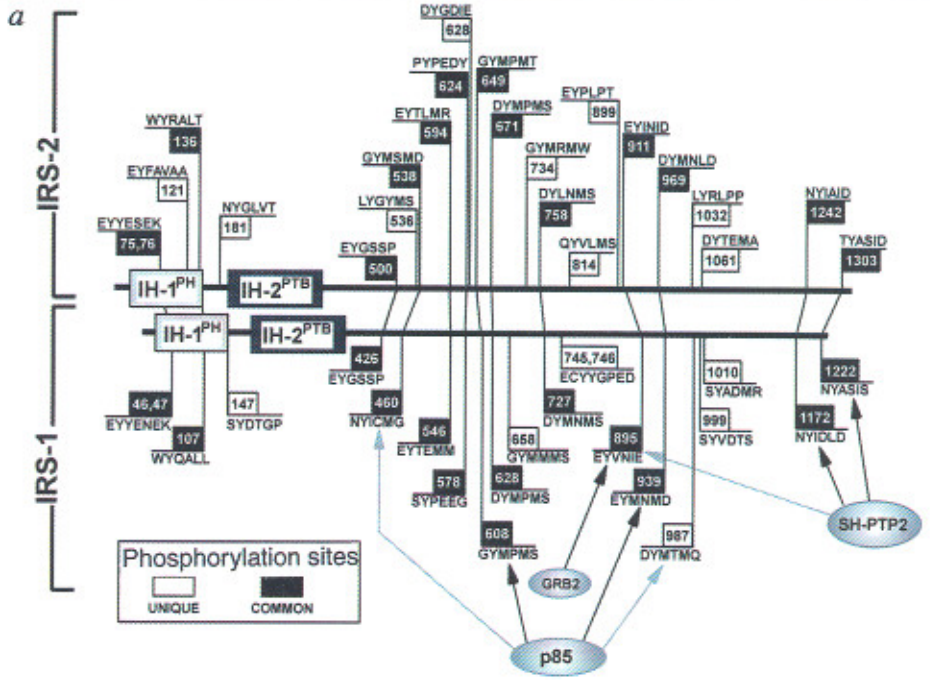


FIG. 3 The expression and function of IRS-2 in FDC-P2 cells, 32D cells and Fao hapatoma cells. *a–c*, Quiescent FDC-P2 cells, or 32D cells expressing the receptors for insulin and IL-4 (32D$^{IR/IL4R}$) and either IRS-1 or IRS-2 were incubated without or with 100 nM insulin or 10 nM IL-4 for 1 min. Cell lysates were immunoprecipitated with antibodies against PY, IRS-2 or IRS-1 (αPY, αIRS-2, αIRS-1), separated on 7.5% SDS–PAGE, and immunoblotted with αPY as described[34]. Quiescent rat Fao hepatoma cells (*d*) were incubated without or with 100 nM insulin for 1 min and immunoprecipitated with non-immune serum, αIRS-1 or αIRS-2, and immunoblotted with αPY; *e*, Phosphatidylinositol-3-OH kinase activity associated with the immuncomplexes was determined[34]. *f* and *g*, insulin and IL-4 stimulated [^{3}H]thymidine incorporation into 32D cells (circles) or 32D$^{IR/IL4R}$ cells before (stars) or after expression of IRS-1 or IRS-2 (as indicated).

METHODS. Antibodies against IRS-1 and IRS-2 were prepared in rabbits (HRP Corp.) as described[14]: αIRS-2 was obtained as a GST-fusion protein containing residues 619–746 of mouse IRS-2 (ref. 20), αIRS-1 (residues 1,221–1,234 of rat IRS-1) were made with synthetic peptides coupled to keyhole limpet haemocyanin[35]; αPY antibodies were rabbit polyclonal[36] or mouse monoclonal 4G10 (UBI). An IRS-2 expression vector was assembled in pCMV^{his} from two partial cDNA inserts (mFDCP2c-9-4 and mLc-6) and a genomic fragment (mG28Sac2) by using *Not*I, *Bip*I and *Afl*II restriction sites, and blunt-ended *Spe*I and *Hind*III subcloning sites. The correct orientation and reading frame was confirmed by DNA sequencing. 32D cells or cells expressing the IR and IL4R (32D$^{IR/IL4R}$) were transfected stably with IRS-1 or IRS-2 cDNA by electroporation as described[18]. Lysates from quiescent 32D cells incubated for 1 min in the absence or presence of 100 nM insulin or 10 nM IL-4 were immunoprecipitated with the indicated antibodies, separated by 7.5% SDS–PAGE and immunoblotted with αPY[34]. Fao cells were grown to confluence in RPMI-1640 medium and incubated with insulin (100 nM) for 10 min, and immune complexes were immunoblotted with αPY or assayed for PI(3)K activity as described[16]. Incorporation of [^{3}H]thymidine into 32D cells was used to quantify factor-induced proliferation. 32D cells (2×10^5 cells per ml) were incubated for 36 h in RPMI-1640 medium containing 10% FBS and the indicated growth factors or 5% WEHI-3-conditioned medium, incubated for 3 h with [^{3}H]thymidine and collected on glass filters (Whatman)[5]. Radioactivity was quantified by scintillation counting in ACS scintillation cocktail (Amersham). All determinations were carried out in triplicate, and data are reported as the percentage of incorporation of radioactivity into cells growing in medium supplemented with 5% WEHI-3-conditioned medium[18].



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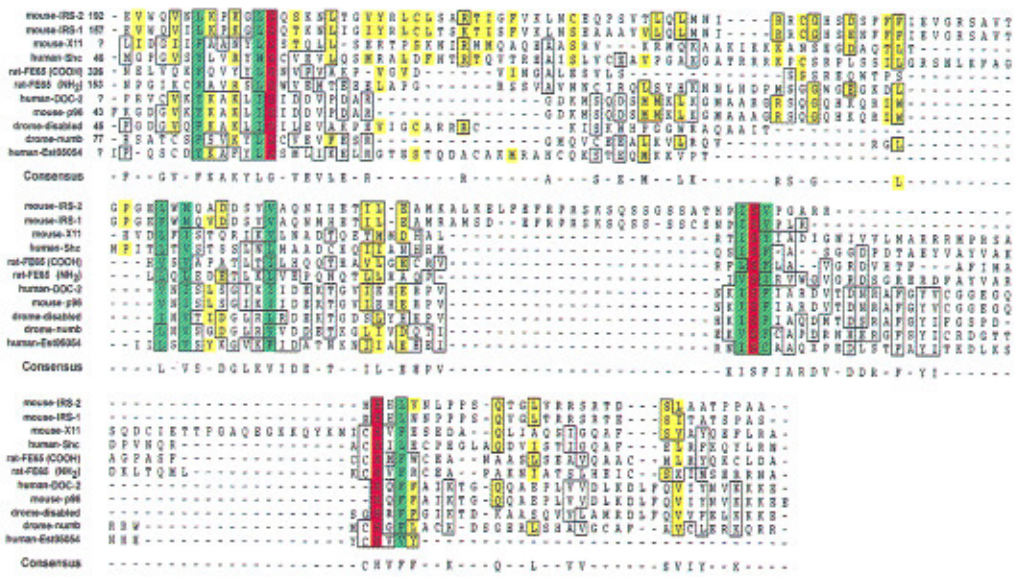

FIG. 4 The structural feature of IRS signalling proteins. *a*, The potential tyrosine-phosphorylation sites of IRS-1 and IRS-2 are presented in the context of surrounding amino-acid sequences. The conserved phosphorylation motifs are in solid boxes and the unique phosphorylation motifs are in open boxes. Binding sites in IRS-1 for GRB2, SH-PTP2 and PI(3)K are indicated; experimentally determined low-affinity sites are indicated by a grey arrow. Two IRS-homology domains, IH-1^{PH} and IH-2^{PTB} are indicated at the N terminus. *b*, The IH-2^{PTB} domain in IRS-1 and IRS-2 is aligned with the putative PTB domains identified in various proteins[37]. Gaps are indicated by dashed lines, similar residues are boxed; identical residues are indicated underneath (consensus). Three amino-acid residues diagnostic for the PTB domain (Gly, Ser and His) are indicated by red blocks[37]. Similar hydrophobic residues conserved in each sequence are highlighted in green. Residues in IRS-1 and IRS-2 with similarities in other PTB domains are highlighted in yellow.

(Fig. 3*b*). The co-migration during SDS PAGE of endogenous and recombinant IRS-2 slightly above IRS-1 suggested that we had the full-length cDNA encoding IRS-2.

The common ability of tyrosine-phosphorylated IRS-1 and IRS-2 to engage phosphatidylinositol-3-OH kinase (PI(3)K) was investigated in rat Fao hepatoma cells, which contain IRS-1 and a slower-migrating protein called pp185^{HMW} (ref. 16). Both IRS-1 and IRS-2 were immunoprecipitated from Fao hepatoma cells and immunoblotted with anti-PY (Fig. 3*d*); pp185^{HMW} appears to be identical to IRS-2. Insulin stimulated tyrosine phosphorylation of both IRS-1 and IRS-2 in Fao cells, and as expected, IRS-2 migrated slightly above IRS-1 (Fig. 3*d*). Interestingly, anti-IRS-2 coprecipitated a 125K tyrosine-phosphorylated protein which was not found in the anti-IRS-1 immunocomplex (Fig. 3*d*, lane f). Phosphatidylinositol-3-OH kinase associated with both IRS-1 and IRS-2 following insulin stimulation (Fig. 3*e*), suggesting that both IRS-proteins serve as interfaces between the insulin receptor and PI(3)K in the same cell background.

As untransfected 32D cells do not contain IRS-1 or IRS-2, many biological responses, including the activation of PI(3)K and p70^{s6k} and mitogenesis, are insensitive to insulin/IGF-1, IL-4, IL-13 and other cytokines[5,7,17,19]. Moreover, overexpression of the insulin receptor or the IL-4 receptor in 32D cells does not rescue the mitogenic response (Fig. 3*f*, *g*). However, expression of either IRS-2 or IRS-1 together with these receptors rescued insulin and IL-4 stimulated DNA synthesis (Fig. 3*f*, *g*). Thus IRS-1 and IRS-2 are interchangeable as mediators of insulin- and IL-4-stimulated mitogenesis in 32D cells. Interestingly, IRS-2 coupled more sensitively to the IL-4 receptor system than IRS-1.

Among the eight identified tyrosine-phosphorylation motifs in IRS-1, six are well conserved in IRS-2 (tyrosines at positions 649, 671, 911, 969, 1,242 and 1,303). The SH2 domains in p85 bind strongly to Y_{608}MPM and Y_{939}MNM in IRS-1 (ref. 20), which correspond to Y_{649}MPM and Y_{969}MNL in IRS-2 (Fig. 4*a*); however, the substitution of Met 972 by Leu may alter the interaction. Nevertheless, there are eight other YXXM motifs in

IRS-2 which may bind p85 after tyrosine phosphorylation (Fig. 4*a*). The SH2 domain of Grb-2 binds to a Y_{895}VNI motif in IRS-1 (refs 17, 20), which corresponds to Y_{911}INI in IRS-2 (Fig. 4*a*). IRS-1 also binds to SH-PTP2 at the Y_{1172}IDL and the Y_{1222}ASI motifs[20–22], which corresponds to the Y_{1242}IAI and Y_{1303}ASI motifs in IRS-2; however, the spacing between these motifs is 10 residues longer in IRS-2, which may alter the regulation of SH-PTP2 (Fig. 4*a*). Several other potential tyrosine-phosphorylation motifs unique to IRS-1 or IRS-2 may play distinct roles in signalling (Fig. 4*a*). Moreover, the C terminus possesses several other conserved motifs in an otherwise dissimilar peptide backbone, which could mediate protein–protein interactions (Fig. 2).

The IH-1 domain in IRS-1 was previously recognized as a pleckstrin-homology (PH) domain[23,24], and is best designated as IH-1^{PH} to emphasize this fact (Figs 2, 4*a*). The high degree of identity between the IH-1^{PH} domains in IRS-1/IRS-2 suggests a specific function for this region. Deletion of the IH-1^{PH} domain from IRS-1 significantly impairs coupling to an ordinary level of insulin receptors[25]. However, the IH-1^{PH} domain is not essential for IRS-1 phosphorylation, because a high level of insulin receptors in 32D cells rescues tyrosine phosphorylation of IRS-1 molecules lacking this domain[25].

Insulin-stimulated tyrosine phosphorylation of IRS-1 and Shc is mediated by an NPXY motif in the juxtamembrane region of the insulin receptor β-subunit[26,27]; a similar motif exists in the IL-4 receptor and is required for IRS phosphorylation[28]. A phosphotyrosine-binding (PTB) domain that recognizes phosphorylated NPXY motifs has been identified in Shc and other proteins[29,30]. Alignment of the IH-2 domain in IRS-1 and IRS-2 with the PTB domains revealed the presence of three conserved amino acids (Gly 204, Ser 314, and His 322 in IRS-2) surrounded by similar hydrophobic residues which are diagnostic for the PTB domain (Fig. 4*b*). We propose the designation, IH-2^{PTB}, to emphasize this similarity. Moreover, a fusion protein with glutathione-*S*-transferase that contained the IH-2^{PTB} domain of IRS-1 or IRS-2 bound specifically to the insulin-receptor-derived sequence, LYASS-NPE[pY_{960}]-LSASDV (data not shown). Moreover, deletion of the IH-2^{PTB} domain impairs IRS-1 phosphorylation, suggesting that this region is important for receptor coupling.

The identification of IRS-2 provides new insight into the modular structure and function of the IRS signalling proteins. Presumably, the IRS signalling system provides a means for signal amplification by eliminating the stoichiometric constraints encountered by receptors that directly recruit SH2 proteins to their autophosphorylation sites. Moreover, IRS proteins dissociate the intracellular itinerary of the signalling complex from the endocytic pathways ordinarily followed by the activated receptor. The shared use of IRS proteins by multiple receptors is likely to reveal important connections between various hormones and cytokines that were previously unrecognized, or observed but unexplained. □

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