# Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein

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**SINCE the discovery of insulin nearly 70 years ago, there has been no problem more fundamental to diabetes research than understanding how insulin works at the cellular level. Insulin binds to the $\alpha$ subunit of the insulin receptor which activates the tyrosine kinase in the $\beta$ subunit, but the molecular events linking the receptor kinase to insulin-sensitive enzymes and transport processes are unknown[1,2]. Our discovery that insulin stimulates tyrosine phosphorylation of a protein of relative molecular mass between 165,000 and 185,000, collectively called pp185, showed that the insulin receptor kinase has specific cellular substrates[3]. The pp185 is a minor cytoplasmic phosphoprotein found in most cells and tissues[4–10]; its phosphorylation is decreased in cells expressing mutant receptors defective in signalling[6,11]. We have now cloned *IRS-1*, which encodes a component of the pp185 band. IRS-1 contains over ten potential tyrosine phosphorylation sites, six of which are in Tyr-Met-X-Met motifs. During insulin stimulation, the IRS-1 protein undergoes tyrosine phosphorylation and binds phosphatidylinositol 3-kinase, suggesting that IRS-1 acts as a multisite 'docking' protein to bind signal-transducing molecules containing Src-homology 2 and Src-homology-3 domains[12–14]. Thus IRS-1 may link the insulin receptor kinase and enzymes regulating cellular growth and metabolism.**

We used the partial amino-acid sequence of rat liver pp185 (ref. 15) to prepare optimal complementary DNA probes[16]. Two partial cDNA clones (C18 and C19) were initially identified with these probes (Fig. 1*a*). Further screening yielded 14 overlapping clones that encode IRS-1. IRS-1 is a hydrophilic protein of relative molecular mass 131,000 ($M_r$, 131K) which contains nine of the original eleven tryptic peptide sequences obtained from rat liver pp185 (Fig. 1*b* and *c*). Northern analysis indicates that IRS-1 messenger RNA is about 9.5 kilobases (data not shown). No Src-homology domains 2 and 3 (SH2/SH3) have been identified in IRS-1. But it contains a potential ATP-binding site beginning with a glycine-rich motif ($Gly_{137}$-Val-Gly-Glu-Ala-Gly) and followed by an essential lysine residue 14 amino acids away (Ala-X-$Lys_{156}$-X-Ile, where X is any amino-acid residue) (Fig. 1*c*)[17,18]. However, IRS-1 lacks the Asp-Phe-Gly and Ala-Pro-Glu motifs diagnostic of a protein kinase[17]. Moreover, IRS-1 does not undergo autophosphorylation in immunecomplexes, suggesting that it is not a protein kinase (data not shown).

IRS-1 contains many potential phosphorylation sites. Based on typical motifs for cyclic AMP-dependent protein kinase(R/K-R/K-X-S/T; one-letter amino acid code), protein kinase C (S/T-X-R/K), casein kinase II (S/T-X-X-E/D) and the cdc2 kinase (S/T-P-X-K/R), 35 putative Ser/Thr phosphorylation sites are distributed throughout the protein (Fig. 1*b*). At least 10 potential tyrosine phosphorylation sites exist, six of which are located in the central region of IRS-1 and contain the YMXM motif (Fig. 1*b*); three others have a YXXM motif, and one site has the sequence EYYE. Synthetic peptides containing the YMXM motifs were phosphorylated by the purified

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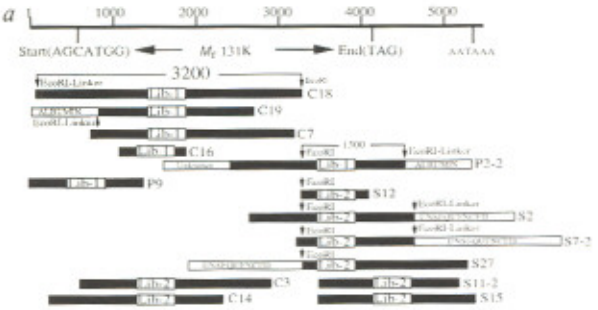
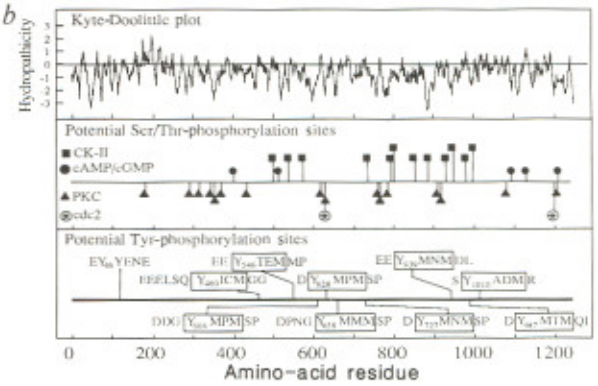

a
1000
2000
3000
4000
5000
Start(AGCATGG)
$M_r$ 131K
End(TAG)
AATAAA
3200
C18
C19
C7
C16
1500
P2-2
P9
S12
S2
S7-2
S27
C3
S11-2
C14
S15


b
Kyte-Doolittle plot
Hydropathicity
Potential Ser/Thr-phosphorylation sites
CK-II
cAMP/cGMP
PKC
cdc2
Potential Tyr-phosphorylation sites
0
200
400
600
800
1000
1200
Amino-acid residue


c
Met Ala Ser Pro
Pep-42: L
Pep-76b: N L I G I Y
Pep-138: S A
Pep-80: Y I P G A T M G T S P A L T G D
Pep-72b: X L P D A t m g X s p a X T
Pep-76a: S V S A P Q Q I I N


Pep-43a: E Q Q Q Q Q Q Q Q Q Q S I L
Pep-43b: L S S K T F S
Pep-72a: E E T G S E X Y M N M D L

insulin receptor with Michaelis constants, $K_m$, of about 50 μM, suggesting that these tyrosine residues are possible phosphorylation sites of IRS-1 *in vivo* (data not shown).

The predicted $M_r$ of IRS-1 (131K) is smaller than expected for a protein that migrates between 175 and 185K during SDS-PAGE. But expression of IRS-1 cDNA in CHO cells (CHO/IRS-1) revealed that IRS-1 migrates as a 180K phosphoprotein during insulin stimulation. IRS-1 was immunoprecipitated from insulin-stimulated CHO/IRS-1 cells with anti-phosphotyrosine antibody, and it migrated exactly with pp185 from CHO/IR cells (Fig. 2, lanes a–d). The pp185 was undetected in CHO/neo cells during insulin stimulation (Fig. 2, lanes e and f), suggesting that either overexpression of IRS-1 or the insulin receptor was necessary to obtain a detectable signal in this assay.

The juxtamembrane region of the insulin receptor β subunit is essential for signal transmission, and mutations in this region by substitution of Tyr 960 with phenylalanine (CHO/$IR_{F960}$), or deletion of 12 amino acids around Tyr 960 (CHO/$IR_{\Delta 960}$), impairs normal insulin-stimulated tyrosine phosphorylation of pp185 (refs 19, 20). As shown previously, the mutant receptors autophosphorylate normally during immunoprecipitation with the anti-phosphotyrosine antibody, but pp185 was immunoprecipitated only from the insulin-stimulated CHO/IR cells (Fig. 3*a*, lanes a and b). Using anti-IRS-1 antibody, a single [$^{32}$P]phosphoprotein was identified in each cell line before insulin stimulation which migrated at a position corresponding to an $M_r$ of 165–175K (Fig. 3*b*). This protein was not immunoprecipitated with anti-phosphotyrosine antibody as it

◀ FIG. 1 *a*, IRS-1 cloning strategy. The cDNA clones C18 and C19 were identified from Lib-1 (see Methods) with a mixture of optimal cDNA probes constructed from the partial amino-acid sequence of pp185 (ref. 15). The remaining clones were identified by screening Lib-1 and Lib-2 with the radiolabelled 3,200-base pair (bp) *Eco*RI insert of clone C18, or the 1,300-bp *Eco*RI fragment from clone P2-2. Overlapping cDNA sequences are indicated by the solid bars, and concatamers formed between cDNAs encoding IRS-1 and albumin or unknown sequences are indicated by the open bars. The *Eco*RI site is found in the cDNA, whereas the *Eco*RI-linkers were contributed by the λZapII vector. The partial cDNA is 5,365 bp long and contains an open reading frame which extends from a Kozac start site at nucleotide 589, to the first TAG stop codon at nucleotide 4,293. *b*, IRS-1 structural features. The hydropathicity of the deduced sequence of IRS-1 was analysed by the Kyte–Doolittle algorithm. The relative location of potential Ser/Thr phosphorylation sites for casein kinase II (CK-II) (S/T-X-X-E/D) cAMP/cGMP kinase (R/K-R/K-X-S/T), protein kinase C (PKC) (S/T-X-R/K), and *cdc*2 kinase (S/T-P-X-K/R) are shown. Potential tyrosine phosphorylation sites and the surrounding amino-acid sequence is shown to illustrate the distribution of YMXM or YXXM motifs. *c*, Complementary DNA and deduced protein sequence of IRS-1. The partial cDNA sequence of IRS-1 is shown with the deduced amino-acid sequence of its 131 K open reading frame. In-frame stop codons in the 5′-untranslated end are indicated in lower-case letters, and the Kozak initiation site, CAGCATGG, is underlined in bold starting at nucleotide 585. The locations of the tryptic peptides obtained from the pp185 band[15] are indicated in bold (single-letter code) under the corresponding amino-acid sequence. The putative ATP-binding site beginning at Gly137 is shown in italic under the corresponding amino-acid sequence. Potential tyrosine phosphorylation sites are indicated by bold typeface in the deduced amino-acid sequence; potential sites of glycosylation are underlined. In the 3′-untranslated region, inframe stop codons are indicated in lowercase, a putative poly(A)$^+$ adenylation signal, TATAAA, is underlined in bold, and the mRNA destabilization consensus sequence, ATTTA, is shown in bold italics. Disagreements between the nucleotide sequence of Lib-1 and Lib-2 are indicated by the discordant nucleotide from Lib-2 above the consensus sequence.

METHODS. Two oligo(dT) and random-primed bacteriophage cDNA libraries, Stratagene 936507 (Lib-1) and 936512 (Lib-2) were screened with optimal oligonucleotide probes to Pep 80 and Pep 138. A pair of oligonucleotides (Oligos Etc., Connecticut) with a 12-nucleotide overlap (underlined sequence) were synthesized for Pep 80 (TCTGCTGTGACAGGCCCCGGCGAGTTCTGGATGCAGGTGG and CATGTTCTGGGCCTTCACAGAGTCATCCACCTGCATCC), and Pep 138 (CCGGGCCTCATCGCCTGTCAGGGCAGGGGAGGTGCCCAT and TACATCCCTGGCGCCACCATGGGCACCTC). Each pair of oligonucleotides (0.6 pmol) was annealed in 10 μl labelling buffer (Amersham). [$^{32}$P]dCTP (210 μl 1 mCi ml$^{-1}$, 3,000 Ci per mmol) and [$^{32}$P]dGTP (21 μl 20 mCi ml$^{-1}$, 6,000 Ci per mmol) were mixed and lyophilized in microfuge tubes, followed by addition of 26 μl $H_2O$, 4 μl of 5×labelling buffer, 4 μl dATP and dTTP, 10 μl annealed oligos, and extended with excess Klenow (Amersham). The mixture was incubated at room temperature for 2 h and then at 37 °C for 30 min. The labelled probes were separated from free dNTPs using an Elutip (Schleicher & Schuell). Specific activity was $2\times10^9$ c.p.m. per pmol for probe 80 and $2.25\times10^9$ c.p.m. per pmol for probe 138. About $1.5\times10^6$ plaques were plated at a density of 50,000 plaques per 150-mm plate, transferred to nitrocellulose filters (Schleicher & Schuell), and screened with an equimolar mixture of probes 80 and 138 ($3\times10^6$ c.p.m. per ml). Hybridizations were performed overnight in 5× Denhardt's solution containing 20% formamide, 10% dextran sulphate, 6×SSC, and 50 mM sodium phosphate (pH 6.8) containing 100 μg ml$^{-1}$ salmon sperm DNA. Filters were washed 3 times with 2×SSC containing 0.1% SDS at 22 °C for 30 min, then with 0.2×SSC and 0.1% SDS for 30 min at 37 °C. Dried blots were exposed to Kodak XAR-5 film with a Quanta 111 itensifying screen at −70 °C. The pBluescript SK$^-$ plasmid containing the cDNA inserts that remained positive after two rounds of plaque purification were released from the λZapII vector by *in vivo* excision with the helper phage R408, as described in the manufacturers instructions (Stratagene). Inserts were sequenced on both strands with Sequenase (USB) using specific primers selected at convenient intervals. The sequence was confirmed by sequencing the coding strand of independent cDNA inserts obtained from Lib-2. Sequences were aligned and analysed using the EUGENE and SAM programs (Molecular Biology Computing Research Resource, Dana Faber Cancer Institute and Harvard School of Public Health).

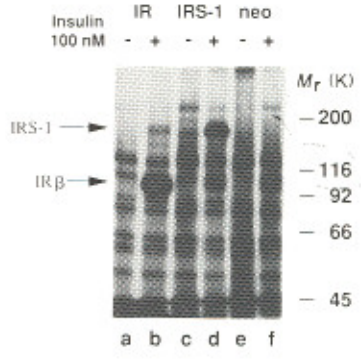



FIG. 2 Expression and tyrosine phosphorylation of IRS-1 in CHO cells. CHO cells expressing $10^6$ insulin receptors (CHO/IR) (lanes a and b), IRS-1 (CHO/IRS-1) (lanes c and d), or no exogeneous cDNA (CHO/neo) (lanes e and f) were labelled with [$^{32}$P]orthophosphate[19] and incubated without (a, c and e) or with 100 nM insulin for 1 min (b, d and f). Phosphotyrosine-containing proteins were immunoprecipitated from cell extracts with antiphosphotyrosine antibody separated by SDS-PAGE under reducing conditions, and detected by autoradiography[19]. The migration positions of the β subunit of the insulin receptor and IRS-1 are indicated.

METHODS. The CHO-IR and CHO/neo cells have been described[19]; CHO/IRS-1 cells were prepared by calcium phosphate-mediated transfection of the IRS-1 cDNA in the expression vector pCMVhis (M. Birnbaum, Harvard Medical School) which contains a histidinol resistance gene[28]. The noncoding region of the IRS-1 cDNA contains several inframe start and stop codons which might interfere with the efficient translation. These regions were removed as follows: the sequence of 553 to 997, including the start codon at 588 and *Bst* EII sit at 642 was amplified by polymerase chain reaction (PCR). A 5′-end primer located at position 553 adapted with an *Spe*I site (TCAACTAGTTTTTCGACACCTCCCTCTGCT) and 3′-end primer at nucleotide 997 (CAGAGCTGCCGCTGCA) in the IRS-1 cDNA were synthesized (Oligos Etc.). PCR was performed in 100 μl 100 mM Tris-HCl, pH 8.3, containing 40 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTP, 50 pmol of primers, 0.5 μg full-length IRS-1 cDNA in pBluescript-II and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus). The reaction was cycled 10 times at 94 °C (1 min), 55 °C (2 min) and 72 °C (1.5 min). Both PCR products and full-length pp185 cDNA in pBluesccript (Stratagene) were cut with *Spe*I and *Bst*EII. The 5′-end region in the full-length IRS-1 cDNA was replaced by the fragment of 553–642 released from the PCR product. The new vector carrying the modified IRS-1 cDNA was confirmed by sequencing and restriction mapping. The complete IRS-1 cDNA was confirmed by DNA sequencing and released from pBluescript by digestion with *Spe*I and *Eco*RV, blunt-end inserted into pCMVhis at a blunt-ended *Hin*dIII site. CHO cells were co-transfected with this plasmid (10 μg) and pSVEneo (0.5 μg) selected with 800 μg ml$^{-1}$ G418 (ref. 19), and then with 10 mM histidiniol (Sigma). Four surviving clones were selected and all expressed raised levels of IRS-1.

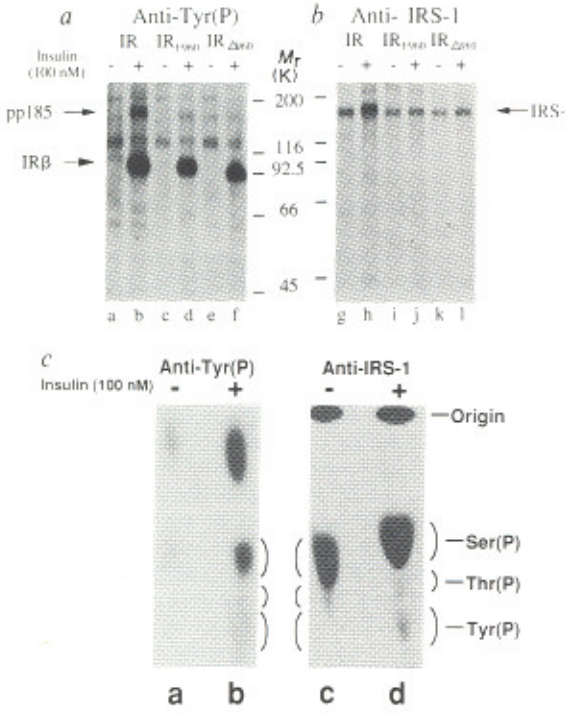



FIG. 3 Insulin-stimulated phosphorylation of pp185 and IRS-1 in CHO/IR cells, or CHO cells expressing mutant receptors ($IR_{F960}$ or $IR_{\Delta 960}$) has been previously described[19,20]. CHO/IR cells (lanes a, b, g and h), CHO/$IR_{F960}$ cells (lanes c, d, i and j) and CHO/$IR_{\Delta 960}$ cells (lanes e, f, k and l) were labelled with 0.5 mCi $ml^{-1}$ [$^{32}$P]orthophosphate for 2 h, and then incubated without (−) insulin or with (+) 100 nM insulin for 1 min[19]. Cell extracts were prepared and immunoprecipitated with anti-phosphotyrosine antibody (*a*) or the specific IRS-1 antibody (*b*), separated by SDS-PAGE under reducing conditions, and the phosphoproteins detected by autoradiography. *c*, Phosphoamino-acid composition of pp185 and IRS-1 immunoprecipitated from CHO/IR cells with anti-phosphotyrosine antibody (lanes a and b) or anti-IRS-1 (lanes c and d), respectively. Phosphoamino-acid analysis was carried out on tryptic digests[29]. The anti-IRS-1 antibody was prepared in rabbits with synthetic Pep80 (provided by S. Shoelson, Joslin Diabetes Center) coupled to keyhole limpet haemocyanin[30].

contained predominantly phosphoserine and a small amount of phosphothreonine (Fig. 3*c*). After insulin stimulation of the CHO/IR cells, phosphorylation of IRS-1 doubled and it migrated with a higher $M_r$ (175–185K) (Fig. 3*b*, lanes g and h). IRS-1 and pp185 from insulin-stimulated CHO/IR cells contained phosphotyrosine (Fig. 3*c*). Insulin had little or no effect on the phosphorylation of IRS-1 in the CHO/$IR_{F960}$ and CHO/$IR_{\Delta 960}$ cells, which is consistent with the absence of pp185 in the corresponding anti-phosphotyrosine immunoprecipitates (Fig. 3*a* and *b*). These results show that IRS-1 has characteristics similar to those of pp185, suggesting that they are related proteins.

A phosphatidylinositol 3′-kinase (PtdIns 3′-kinase) associates with certain phosphotyrosine-containing proteins, including receptors for platelet-derived growth factor (PDGF), epidermal growth factor, colony stimulating factor-1 and insulin[12,21–23]. These ligands also increase the amount of PtdIns 3-phosphate in intact cells[12] which may be important in growth control[12]. As previously described[22,24], PtdIns 3′-kinase was detected in anti-phosphotyrosine and anti-insulin receptor immunoprecipitates after stimulation of CHO/IR cells, but eight times more PtdIns 3′-kinase activity was found in anti-IRS-1 immunoprecipitates (Fig. 4). Immunoprecipitation of PtdIns 3′-kinase by anti-IRS-1 antibody was blocked by the peptide antigen and did not occur with nonspecific rabbit antibodies; HPLC analysis confirmed that the principal product was phosphatidylinositol 3-phosphate (data not shown).

The association of PtdIns 3′-kinase with IRS-1 during insulin stimulation supports the hypothesis that IRS-1 binds signal transduction molecules during insulin-stimulated tyrosine phosphorylation. It is unlikely that IRS-1 itself is a PtdIns 3′-kinase, because the purified PtdIns 3′-kinase exists as a 110K catalytic subunit and an 85K subunit which contains two SH2 domains and one SH3 domain[13,21,25,26]. The association between PtdIns 3′-kinase and IRS-1 probably occurs through phosphorylated YMXM motifs on IRS-1 and the SH2/SH3 domains on the 85K subunit. Insulin increases the cellular concentration of PtdIns 3-phosphate, suggesting that the PtdIns 3′-kinase is activated during insulin stimulation[22]. Tyrosine phosphorylation of the 85K subunit may activate the kinase[27], but there is no

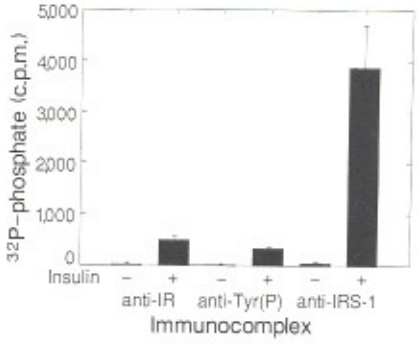



FIG. 4 Insulin stimulation of phosphatidyl inositol 3′-kinase. CHO/IR cells were stimulated with 100 nM insulin for 10 min and extracted . The PtdIns 3′-kinase activity was assayed in immunecomplexes prepared with antiphosphotyrosine antibody (anti-Tyr(P)), anti-insulin receptor antibody (anti-IR)[31], and anti-IRS-1 antibody.

METHODS. *In vitro* phosphorylation of phosphatidylinositol was carried out in the immunecomplexes as described[22]. Subconfluent CHO cells grown in 100-mm dishes were made quiescent by an overnight incubation in F-12 medium containing 0.5% BSA. The cells were then incubated in the absence or presence of insulin (100 nM) for 10 min, and washed once with ice-cold PBS and twice with 20 mM Tris (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM $Na_3VO_4$ (buffer A). The cells were solubilized in 1 ml buffer A containing 1% N-P40 (Sigma) and 10% glycerol, and insoluble material was removed by centrifugation at 13,000*g* for 10 min. Immunecomplexes were precipitated from the supernatant with protein A/Sepharose (Pharmacia) and washed successively in (1) PBS containing 1% N-P40 and 100 μM $Na_3VO_4$ (3 times); (2) 100 mM Tris (pH 7.5) containing 500 mM $LiCl_2$ and 100 μM $Na_3VO_4$ (3 times), and (3) 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA and 100 μM $Na_3VO_4$ (twice). The pellets were resuspended in 50 μl 10 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EDTA. To each pellet was added 10 μl 100 mM $MgCl_2$ and 10 μl phosphatidylinositol (Avanti) (2 μg $\mu l^{-1}$) sonicated in 10 mM Tris (pH 7.5), 1 mM EGTA. The reaction was started by the addition of 10 μl 440 μM ATP containing 30 μCi [$^{32}$P]ATP. After 10 min at 22 °C, the reaction was stopped by the addition of 20 μl 8 M HCl and 160 μl $CHCl_3$: methanol (1:1). Samples were centrifuged, and the lower organic phase removed and applied to a silica gel thin-layer chromatography plate (Merck) which had been coated with 1% potassium oxalate. Thin-layer chromatography plates were developed in $CHCl_3$:$CH_3OH$:$H_2O$:$NH_4OH$ (60:47:11.3:2), dried and visualized by autoradiography. The radioactivity in spots which co-migrated with PtdIns-4P standard (Sigma) was measured by Cerenkov counting as before[22].

evidence for phosphorylation of an 85K band during insulin stimulation. Activation may occur during the binding of the PtdIns 3'-kinase to IRS-1, but there is no direct evidence for this. The PtdIns 3'-kinase binds weakly to the insulin receptor, which may be an important step to recruit the enzyme to the plasma membrane. Recovery of the PtdIns 3'-kinase in anti-insulin receptor immunoprecipitates, however, could be due to an association between the insulin receptor and the IRS-1/PtdIns 3'-kinase complex, rather than direct binding of the insulin receptor to the PtdIns 3'-kinase.

Other signal transduction proteins, including the phosphoinositide-specific phospholipase $C_{\gamma 1}$, the GTPase-activating protein and various Src-like tyrosine kinases, contain SH2/SH3 domains and associate strongly with certain membrane-bound phosphotyrosine-containing proteins, such as the PDGF receptor[12-14]. The PDGF receptor contains a YMXM and a homologous YVXM motif in its kinase-insert region which is essential for binding PtdIns 3'-kinase[12]. The association between the PDGF receptor and the PtdIns 3'-kinase, but not phospholipase $C_{\gamma 1}$ or GTPase-activating protein, is blocked with phosphopeptides containing the YMXM motif, suggesting that phosphorylated YMXM motifs form specific recognition sites for proteins containing certain isoforms of the SH2/SH3 domain[12,21]. The presence of nine potential tyrosine phosphorylation sites in YMXM and YXXM motifs suggests that IRS-1 may act as a multisite 'docking' protein which binds a variety of signal-transducing molecules that contain the appropriate SH2/SH3 domains. Other tyrosine kinases may also phosphorylate specific sites in IRS-1, and serine/threonine kinases could regulate association between IRS-1 and other molecules. This model may begin to explain the pleotropic effects of insulin, especially if the activity or cellular location of various signal transduction molecules is altered during association with tyrosine-phosphorylated IRS-1. We have been unable to identify an intrisic enzymatic activity for IRS-1, but if one exists it could also play an important part in insulin signalling. □

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