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The insulin signalling system and the IRS proteins

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Summary During the past few years, the insulin signalling system has emerged as a flexible network of interacting proteins. By utilizing the insulin receptor substrate (IRS)-proteins (IRS-1 and IRS-2), the insulin signal can be amplified or attenuated independently of insulin binding and tyrosine kinase activity, providing an extensible mechanism for signal transmission in multiple cellular backgrounds. By employing IRS-proteins to engage various signalling proteins, the insulin receptor avoids the stoichiometric constraints encountered by receptors which directly recruit SH2-proteins to their autophosphorylation sites. Finally, the shared use of IRS-proteins by multiple receptors is likely to reveal important connections between insulin and other hormones and cytokines which were previously unrecognized, or observed but unexplained. [Diabetologia (1997) 40: S2–S17]

Keywords Insulin action, insulin signalling, insulin receptor substrate proteins, signal transduction.

Diabetes mellitus results from either a lack of insulin (insulin-dependent diabetes mellitus [IDDM]) or the failure to compensate for a diminished insulin response at various target tissues (non-insulin-dependent diabetes mellitus [NIDDM]). While there are important differences between IDDM and NIDDM, both diseases are characterized by high levels of circulating glucose, and both are accompanied in the long term by a set of debilitating sequelae, including retinopathy, nephropathy, neuropathy and vascular disease [1, 2]. Insulin also has dramatic effects on human embryonic development: maternal hyperinsulinaemia causes excess fetal growth, and insulin-resistant fetuses display growth retardation [3, 4]. These alone are good reasons to study the molecular mechanism of insulin signalling; however, the insulin-receptor tyrosine kinase and its cellular substrates are important signalling systems to understand, and in their details reveal common mechanisms and overlapping pathways controlling cellular growth and metabolism.

The mechanism of insulin action is a challenging problem. For 50 years since the discovery of insulin, the study of insulin action focused on the metabolic changes that occur during insulin stimulation – first at the whole animal level and later at the cellular level [3, 5]. These studies demonstrated that processes such as glucose uptake, lipid synthesis, protein synthesis, and glycogen deposition are controlled by insulin; but the principal cause of insulin resistance has not been identified. Recently, reduced insulin-receptor tyrosine kinase activity has been observed in cells from NIDDM patients, and various mutations have been identified in patients with severe insulin resistance [4–6]. As NIDDM appears to result from a complex set of genetic and environmental inputs, the principal causes of NIDDM have been difficult to discover by genetic means. A molecular understanding

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Abbreviations: MAP, mitogen activated protein; GTP, guanosine 5′-triphosphate; TNFα, tumour necrosis factor alpha; IH1, IRS homology-1; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; EGF, epidermal growth factor; PDGF, platelet derived growth factor; SH2, Src homology-2; PI, phosphatidylinositol; IGF-1, insulin-like growth factor-1; IRS, insulin-receptor substrate; IRR, insulin receptor-related receptor; GHR, growth hormone receptor; PTB, phosphotyrosine binding; IL, interleukin; IFN, interferon.

of the cellular mechanism of insulin action may ultimately elucidate the pathophysiology of NIDDM, and lead to the design of efficacious, safe and convenient interventions.

Assembly of signalling complexes by tyrosine kinase receptors

Following the initial discovery of tyrosine kinases, and their role in the regulation of cellular metabolism and growth, the search for order and specificity of this class of enzyme was confounded by their apparent catalytic promiscuity. The purified receptors for insulin, epidermal growth factor (EGF), platelet derived growth factor (PDGF) and others could phosphorylate almost any protein mixed with them in the presence of [^{32}P]ATP [7]. However, the specificity of various tyrosine kinases in cells became apparent during immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies (αPY). In cells, the insulin receptor was almost too restricted, and for many years the only tyrosine phosphorylated protein recognized during insulin stimulation was the β-subunit of the receptor itself [8, 9]. It was eventually appreciated that ligand-induced autophosphorylation of most growth factor receptors mediated the direct association of various signal transduction proteins [10, 11].

The common theme among all tyrosine kinase signalling mechanisms centres on hormone/growth factor/cytokine-induced dimerization, and downstream heterologous protein-protein interactions initiated by the activated receptor. Ligand binding mediates the dimerization of receptor subunits to juxtapose adjacent catalytic domains, so multiple tyrosine residues on the closely opposed receptors are phosphorylated, usually in *trans,* by the intrinsic or associated tyrosine kinase [12]. Signalling specificity begins with the selection of appropriate tyrosine residues. This step is largely directed by the specificity of the catalytic domain in the complex, and involves the interaction between positively charged residues in the catalytic pocket with negatively charged residues near the tyrosine residue [13]. Specificity is further controlled by the selective binding of the autophosphorylation sites to proteins with complementary Src homology-2 domains (SH2-protein) [14]. The SH2 domain is composed of approximately 100 amino acid residues which bind to phosphotyrosine in a specific amino acid sequence context [14]. Some proteins in this signalling complex are activated during binding to the phosphorylated motifs, whereas others are activated by tyrosine phosphorylation that occurs in the complex; the subcellular distribution of the SH2-protein may also contribute to signalling specificity. Irrespective of the exact mechanism, the characteristic biological response of each factor arises in large part from the cohort of interacting SH2-proteins [10, 14]. For example, the activated receptors for EGF and PDGF bind to the SH2 domains in the phosphatidylinositol (PI) 3′-kinase, p21^{ras}-GAP, phospholipase Cγ, Grb2/Sos, cytoplasmic tyrosine kinases like c-fyn and c-src, and other SH2-proteins in various cellular backgrounds [15].

The assembly of a similar signalling complex around the insulin receptor was always difficult to show. However, for insulin and the insulin-like growth factor-1 (IGF-1) receptor, autophosphorylation activates the catalytic domain, and the formation of the signalling complex occurs largely through the phosphorylation of the insulin-receptor substrate (IRS)-proteins, Shc, and other substrates. Phosphorylated IRS-1 activates the PI 3′-kinase during binding to the SH2 domains in its p85α regulatory subunit [16]. Other SH2-proteins, including SHP2 [17], GRB-2 [18], and nck [19], associate with IRS-1 to mediate the pleiotropic insulin response. Tyrosine phosphorylation of Shc stimulates the binding of Grb2/Sos, and contributes to the IRS-independent activation of p21^{ras} [20]. Moreover, other substrates such as pp60^{IRS} may also contribute to the insulin response in various tissues.

The insulin receptor

Introduction. The insulin receptor is present in virtually all vertebrate tissues, although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200 000 receptors on adipocytes and hepatocytes. The receptor gene is located on the short arm of human chromosome 19, is more than 150 kilobases in length and contains 22 exons which encode a 4.2 kb cDNA [21]. The insulin receptor is composed of two α-subunits that are each linked to a β-subunit and to each other by disulphide bonds (Fig. 1 A). Both subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of 4 basic amino acids. There is one site of alternative splicing surrounding exon 11 which results in two receptor isoforms differing by 12 amino acids near the COOH-terminus of the α-subunit (Fig. 1 A). The mature heterotetramer ($\alpha_2\beta_2$) contains complex N-linked carbohydrate side chains capped by terminal sialic acid residues and migrates with a molecular mass of 300–400 kDa by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE). The α-subunits are located entirely outside the cell and contain the insulin binding site(s), whereas the intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase (Fig. 1 A). The insulin receptor family contains two other structurally related molecules, the IGF-1 receptor, and the insulin receptor-related (IRR) receptor, an orphan receptor for which no ligand has yet been identified [22]. This family shares

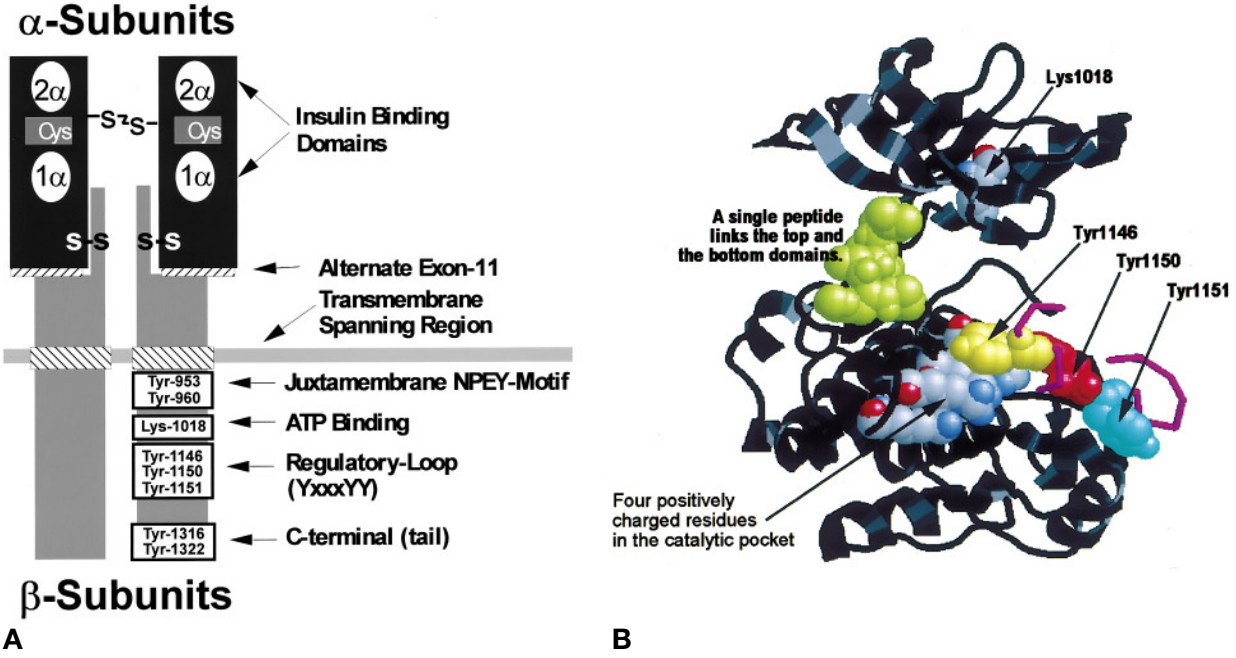

Fig. 1. A. Schematic diagram of the insulin receptor tetramer. The membrane is represented by a horizontal line. Insulin binding sites are shown in the α-subunit, and the autophosphorylation sites are listed in the β-subunits. **B.** Representation of the structure of the catalytic domain of the insulin receptor. The structure is composed of a small upper loop and a larger lower loop, which correspond to the ATP binding and catalytic domains, respectively. The lysine residue that binds ATP (upper loop), the three phosphorylation sites in the regulatory loop and the four positively charged residues that mediate phosphorylation site selection are indicated. A single peptide sequence links the upper and lower domains.

more than 80 % amino acid sequence identity in the kinase domain but has low amino acid sequence identity in the extracellular domain, consistent with their ligand preference.

Insulin binding. Following its release by the beta cells of the pancreas, insulin binds to its receptor on the surface of most cells. Before insulin binding, the α-subunits apparently exert a negative effect upon the kinase, as removal of the extracellular domain by enzymatic digestion or mutagenesis constitutively activates the kinase [23–25]. Although disulphide bonds stabilize the interactions between two α-subunits, insulin binding activates the receptor through a mechanism thought to be analogous to that for monomeric receptors. In particular, biochemical and structural studies on the growth hormone receptor (GHR) provide elegant support for this hypothesis: growth hormone behaves as a divalent ligand which crosslinks two GHR molecules [26, 27]. The notion that dimerization of adjacent receptor subunits leads to activation of the associated tyrosine kinases has gained general acceptance [28–30].

Although adjacent insulin receptor molecules are covalently linked during biosynthesis and processing, insulin binding apparently modifies the α-subunit dimer which mediates *trans*-autophosphorylation between the β-subunits [31–34]. Historically, the stoichiometry of insulin binding has been contentious, and reported to be between 1 and 2 insulin molecules per receptor tetrameter [35]. Recently, a conceptual model to explain these data has been proposed [35]. Each α-subunit contains two distinct insulin binding domains with a weak degree of internal redundancy flanking the single cysteine-rich domain, designated *site-1* α and *site-2* α (Fig. 2 B). The residues encoded by exon-2, especially Phe^{89} compose *site-2* α, and the residues encoded by exons 6 and 7 compose *site-1* α [35]. *Site-1* α and *site-2* α may fold as a β-sheet flanked by two α-helices, which associates as a pseudosymmetrical dimer within each α-subunit [36]. Experimental and naturally occurring mutations in the α-subunit suggest that both regions contribute independently to insulin binding [35]. To complement this hypothesis, the insulin molecule itself also has two distinct receptor binding domains: *site-1* is composed of a dozen invariant residues from both the A and B chain (G^{A1}, E^{A4}, Q^{A5}, Y^{A19}, N^{A21}, V^{B12}, T^{B16}, G^{B23}, F^{B24}, F^{B25} and Y^{B26}); *site-2* coincides with the hexamerization surface of insulin (L^{A13} and L^{B17}) (Fig. 2 A). Presumably, two independent sites each on insulin and the insulin receptor mediate crosslinking between the covalently linked α-subunits (Fig. 2 B). This hypothesis is fashioned after a similar model describing dimerization of the growth hormone receptor by growth hormone [35]. The model unifies the mechanism of insulin receptor activation with the general hypothesis proposed earlier for other systems [37]. The ligand sensitivity of chimeric receptors

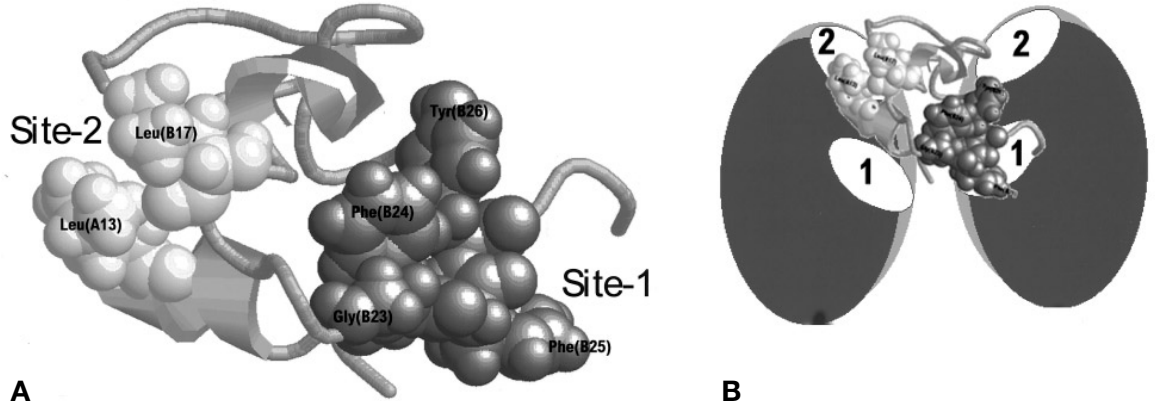

Fig. 2 A, B. Representation of the structure of insulin and its hypothetical binding to the α-subunits of the insulin receptor **A**. The insulin molecule is rotated to show the relative locations of the receptor binding sites: 1 and 2. Some amino acid residues in each site are shown for orientation. **B.** A cartoon depicting the hypothetical mechanism by which one insulin molecule is thought to modify the covalent insulin receptor dimer

composed of the ligand binding and catalytic domain of the EGF and insulin receptors supports this hypothesis [38, 39].

The transmembrane spanning region. The external ligand binding domain of the insulin receptor is linked to the tyrosine kinase by a single transmembrane segment which has broad tolerance for amino acid substitutions [40, 41]. However, the insulin receptor is constitutively activated by substitution in the transmembrane segment from the oncogene v-erbB-2 which contains a $Val_{664} \rightarrow Glu_{664}$ mutation [42]; an analogous point mutation ($Val_{938} \rightarrow Asp_{938}$) in the transmembrane segment of the insulin receptor also partially activates the receptor kinase [43]. Apparently, this region stabilizes the insulin-induced conformational change, which is analogous to the receptor-receptor contacts in the ligand activated growth hormone receptor [44]. Thus, mutations that stabilize an interaction between the membrane spanning region of adjacent receptors may cause constitutive activity.

The tyrosine kinase. Insulin binding activates the tyrosine kinase, leading to autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit, including Tyr_{960} in the juxtamembrane region; Tyr_{1146}, Tyr_{1150}, and Tyr_{1151} in the regulatory loop; and Tyr_{1316} and Tyr_{1322} in the COOH-terminus [45–48]. The tyrosine kinase activity of the receptor is crucial for insulin action: site-directed point mutations in the ATP binding domain destroy ATP binding (and thus abolish kinase activity) and abrogate insulin signalling in cultured cells [49, 50]; naturally occurring mutations of the insulin receptor which inhibit kinase activity are associated with severe insulin resistance [51, 52]. Thus, activation of the insulin receptor tyrosine kinase and the phosphorylation of cellular substrates predominates as an important mechanism of insulin signal transduction.

Autophosphorylation of all three tyrosine residues in the regulatory loop (Fig. 1 A, B) activates the kinase 10 to 20-fold [53]. Mutation at one, two or three tyrosine residues in this region progressively reduces insulin-stimulated kinase activity and results in a parallel loss of biological activity [54, 55]. In many cultured cell lines, the regulatory region is only *bis*-phosphorylated during insulin stimulation, which may limit the amplitude of the insulin response or the selection of phosphorylation sites in target proteins. However, *tris*-phosphorylation predominates in rat hepatocytes suggesting a mechanism for tissue-specific up-regulation of the insulin signal [56].

In addition to the regulation of the kinase, recent evidence suggests that the regulatory-loop may bind SH2-proteins. SHP2 associates with Tyr_{1146} in the regulatory loop of the β-subunit [57]. This may provide a more stable link between the insulin receptor and IRS-1 than can be obtained through other mechanisms. Furthermore, this association may play a role during insulin-stimulated glucose uptake [57].

The juxtamembrane region. The intracellular juxtamembrane region of the insulin receptor β-subunit is essential for signal transmission as it mediates substrate selection, presumably by engaging the phosphotyrosine binding (PTB) domains in the IRS-proteins and Shc (Fig. 3). This region is encoded by exon 16 of the insulin-receptor gene and contains several serine phosphorylation sites and at least one tyrosine autophosphorylation site in an $NPXY_{960}$-motif [45]. Substitution of Tyr_{960} with other amino acids impairs receptor signal transmission in vivo, even though the kinase activates normally; this appears to be due to an inability of these mutant receptors to mediate the phosphorylation of IRS-1 and Shc, as the tyrosine kinase is activated normally in all these cases [58, 59]. Overexpression of IRS-1 with these mutant receptors

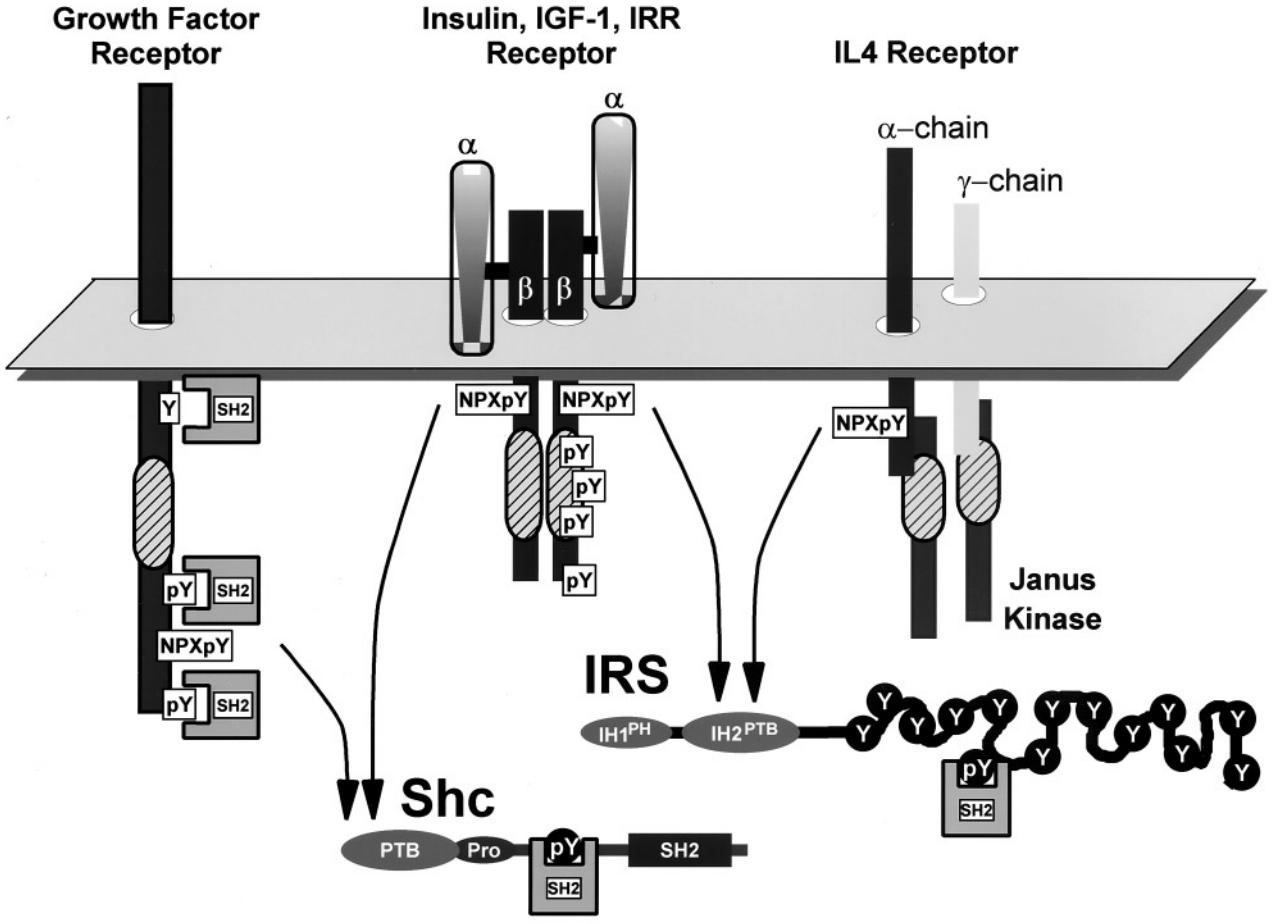

Fig. 3. Schematic diagram depicting three different mechanisms used by the receptor to transmit signals to SH2-proteins. Many growth factor receptors associate directly through their autophosphorylation sites to SH2-proteins. The insulin receptor and several cytokine receptors engage IRS-proteins, phosphorylating multiple tyrosine residues which bind SH2-proteins. Like the IRS-proteins, Shc also associates with receptor to provide signalling potential in addition to the endogenous autophosphorylation sites

h-IL4r	487	TPLVIAGNPATRSFSNSL
m-IL4r	490	VPLVLADNPAYRSFSDCC
r-IL4r	483	VPLVISDNPAYRSFSDFS
h-INSr	959	GPLYASSNPEYLSASDVF
m-INSr	979	GPLYASSNPEYLSASDVF
r-INSr	990	GPLYASSNPEYLSASDVF
h-IGF1r	941	GVLYASVNPEYFSAADVY
g-IRRr	950	GTLYTSVNPEYPSASDMY

Fig. 4. Alignment of the NPXY-motifs in the insulin and IL4r family of receptors

rescues the insulin response, suggesting that the NPXY-motif plays a non-obligatory, albeit very beneficial role to sensitize receptor/substrate coupling [60].

Alignment of the NPXY-motif in the receptors for insulin, IGF-1, and interleukin 4 (IL4), all of which phosphorylate IRS-1, reveals a longer sequence, the LxxxxNPXYxSxSD-motif, which may be the preferred recognition site for IRS-proteins (Fig. 4) [61]. EGF and PDGF receptors lack an NPXY-motif in this context and do not phosphorylate IRS-proteins [62]. Recent studies suggest that the phosphorylated LxxxxNPXYxSxSD-motif binds to the PTB domain in the NH_2-terminus of the IRS-proteins and Shc [63, 64].

The COOH-terminal tail. Protein tyrosine kinases invariably contain a COOH-terminal tail extending beyond the end of the tyrosine kinase homology region (Fig. 1 A). In the EGF and PDGF receptors, the tail contains autophosphorylation sites that bind to SH2-proteins, but a similar function for the insulin receptor tail occurs variably in vivo [65, 66]. The COOH-terminus of the insulin receptor contains two autophosphorylation sites at Tyr_{1316} and $Tyr_{1322,}$ as well as the threonine and serine phosphorylation sites. Short deletions in the COOH-terminus which remove the tyrosine and threonine residues ($IR_{\Delta 43}$) either have no effect on or enhance insulin-stimulated autophosphorylation in the other regions; the same applies to insulin-stimulated receptor kinase activity and

biological activity [67–69]. Even the deletion of 82 amino acids from the COOH-terminus ($IR_{\Delta 82}$) has little effect on substrate phosphorylation and downstream biological effects [70]; however, insulin-stimulated autophosphorylation decreases significantly. Thus, the COOH-terminus appears to regulate insulin signals rather than to recruit SH2-proteins to the receptor.

One of the insulin receptor autophosphorylation sites in the COOH-tail is in a YXXM-motif. This phosphorylation site is a preferred binding motif for the SH2 domains in the PI-3 kinase. Several reports suggest that the PI-3 kinase binds to this site in the activated insulin receptor [71, 72]. However, in cells lacking IRS-proteins, PI-3 kinase does not associate with the insulin receptor kinase, suggesting that in vivo this does not occur [73].

The substrate hypothesis for insulin signal transduction

Introduction. Early theories of tyrosine kinase signalling focused on intracellular substrate proteins as likely candidates for second messengers. However, it was difficult initially to identify physiologically relevant substrates, and the direct substrate hypothesis was largely set aside [74]. During this interval, the notion that autophosphorylation of the receptor itself was sufficient for signalling was popular. This paradigm was strengthened with the discovery that SH2-proteins bind directly to these autophosphorylation sites [11, 75]. However, the discovery of Shc and our work with IRS-1 reasserted the central role of the substrate hypothesis in several signalling systems [14, 76, 77].

Insulin receptors phosphorylate multiple proteins. IRS-1 is the best characterized insulin receptor substrate [78]. Although IRS-1 was discovered as a substrate for the insulin receptor, we now know that it is a substrate for several other receptor systems as well, including those in the IL6 family (growth hormone, oncostatin) the IL2 family (IL2, IL4, IL9, IL13 and IL15), and interferons (IFNα/β and IFNγ) (Fig. 3). Recently, we purified and cloned the cDNA for IRS-2, which has provided considerably more information regarding the essential features of the IRS-protein substrate family [63]. With the discovery of IRS-2, several additional receptor systems are likely to be discovered that utilize the IRS-proteins to mediate some of the biological responses.

In addition to the IRS-proteins, several other cellular substrates have been described for the insulin receptor. One of the first was pp120, a liver-specific membrane glycoprotein that is tyrosine phosphorylated during insulin stimulation [79]; it is now known to be an ecto-ATPase, perhaps involved in the active movement of small molecules across the liver cell membrane [80, 81].

An adipose-specific substrate, pp15, is tyrosine phosphorylated under certain circumstances during insulin stimulation. The pp15 was initially interesting as its phosphorylation was inversely correlated to the rate of glucose uptake; however, it is now known to be identical with 442(aP2), and is phosphorylated at very low stoichiometries [82, 83]. The roles of pp120 and pp15 in insulin action remain enigmatic.

The discovery of tyrosine kinase substrates in other signalling systems has led to the enumeration of other insulin-stimulated tyrosyl phosphoproteins. Vav, a95-kDa proto-oncogene associates with the insulin receptor and is tyrosine phosphorylated during insulin stimulation of certain haematopoietic cells [84]. While the role of Vav in insulin signalling is unclear, it contains several well-known domains for protein-protein interaction, including SH2 and SH3 domains, and regions which may contain guanine nucleotide releasing activity for small guanosine 5′-triphosphate (GTP)-binding proteins [84–86].

Shc plays an important role during insulin signalling. The Shc family is composed of at least three isoforms with molecular weights between 46 and 52 kDa [87]. During stimulation of cells with various growth factors, including insulin, Shc is tyrosine phosphorylated [88]. Shc contains several protein-protein interaction domains that mediate signalling, including an SH2-domain at its COOH-terminus, a PTB-domain at its NH_2-terminus, and a collagen-like proline-rich region in the middle of the molecule that contains a tyrosine phosphorylation site in a YINI-motif [77]. The phosphorylated YINI-motif binds to the SH2-domain in Grb-2 and mediates $p21^{ras}$ GTP-loading [87]. Through this interaction, Shc plays a role in the activation of the mitogen activated protein (MAP) kinase cascade by several growth factor and cytokine receptors, including the insulin receptor [11, 20].

Recently, a 115 kDa protein was found in 3T3-L1 adipocytes associated with SHP2 during insulin stimulation [89]. Semi-quantitative analysis by immunoblotting indicates that pp115 may be a major target of SHP2 during insulin stimulation of these cells. By contrast, only a small fraction of SHP2 binds to IRS-1. The structure and function of pp115 is unknown, but it may be a novel docking molecule similar to the IRS-1 protein. In this case, pp115 may couple to the insulin receptor through a pleckstrin homologue domain or a phosphotyrosine binding domain with similarity to those in the IRS-proteins.

Finally, adipocytes contain a 60 kDa protein that undergoes tyrosine phosphorylation during insulin stimulation. Previous attempts to clone pp60 were unsuccessful, and considerable confusion has arisen about the nature of this substrate [90–93]. In Chinese hamster ovary (CHO) cells two 60 kDa proteins exist

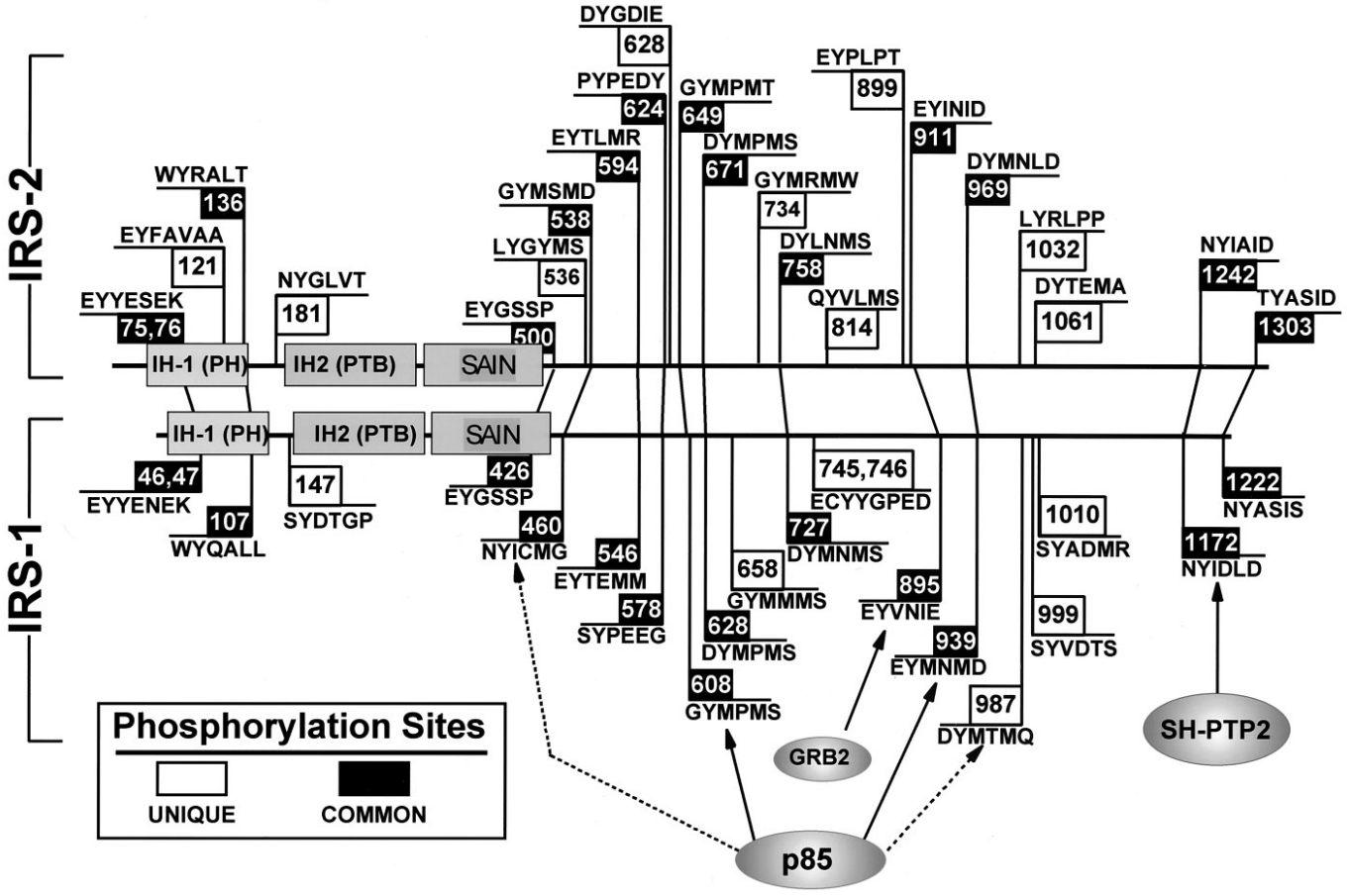

Fig. 5. Schematic diagram of IRS-1 and IRS-2. Two and possibly three conserved modules thought to mediate receptor or other interactions are indicated at the NH_2-terminus: $IH1^{PH}$ and $IH2^{PTB}$ and SAIN. Putative tyrosine phosphorylation sites are indicated in the COOH-terminal region. Entirely unique sites are in open boxes, whereas relatively (but not absolutely) conserved motifs are shown in black boxes. IRS-2 is about 100 residues longer than IRS-1

which bind to $p21^{ras}$GAP or the PI-3 kinase [94]. However, in (HTC) hepatoma cells, pp60 binds strongly to p85 and can be eluted from the p85 immunoprecipitates with phosphopeptides containing YMXM-motifs [95]. These results suggest that pp60 may be a 'mini' IRS-protein, and could be designated $pp60^{irs}$. Based on our understanding of the structure and function of IRS-1 and IRS-2, $pp60^{irs}$ is expected to contain either a PTB domain or a pleckstrin homology domain, which are similar to those in the IRS-proteins. The recent clanking of $pp62^{dok}$ and IRS-3 supports this hypothesis.

The IRS-proteins

Structure. IRS-1 was initially detected in insulin-stimulated Fao hepatoma cells by immunoprecipitation with antiphosphotyrosine antibodies; it was originally called pp185 based on migration during SDS-PAGE [96]. It was purified on immobilized antiphosphotyrosine antibodies (αPY) from insulin-stimulated rat liver [97] and 3T3-L1 cells [98], and its cDNA was isolated from rat [76], human [99, 100] and murine [101] sources. The open reading frame predicts a molecular weight of 131 kDa; its slow migration on SDS-PAGE is due largely to the high level of phosphorylation of the protein. The IRS-1 gene contains the entire 5′-untranslated region and protein coding region in a single exon and is localized on chromosome 2q36-37 in humans [99]. IRS-1 mRNA is of low abundance and consists of 6.9 kb and 6 kb species in human tissues, and a 9.5 kb species in rodent tissue. IRS-1 mRNA is detectable in most human fetal tissues by quantitative PCR after reverse transcription of total RNA [99].

Shortly after the cloning of IRS-1, analysis of pp185 in Fao hepatoma cells revealed another high molecular weight tyrosyl phosphoprotein, designated $pp185^{HMW}$ [102]. An IRS resembling $pp185^{HMW}$ was also observed in FDC-P2 myeloid cells which was designated 4PS (IL4r **P**hosphoprotein **S**ubstrate), since it was initially observed during IL4 stimulation [103, 104]. An NPXY-motif in the IL4r was essential for IL4 stimulated tyrosine phosphorylation of 4PS, suggesting that it shared a common recognition mechanism with IRS-1 [61].

The purification and cloning of 4PS revealed discrete regions of functional homology between IRS-1 and 4PS, suggesting that they were members of the same family of signalling proteins (IRS-proteins); since antibodies against 4PS cross reacted with $pp185^{HMW}$, both were renamed IRS-2 [63]. The 43% overall identity between IRS-1 and IRS-2 is not distributed evenly throughout the molecules: two extended 100–150 amino acid regions in the NH_2-terminus, designated the IRS homology-1 (IH1) and IH2 domains, contain higher degrees of identity than the rest of the molecule (65 and 75%, respectively) (Fig. 5).

The IRS-proteins contain multiple tyrosine phosphorylation motifs in the COOH-terminal portion of the molecule (Fig. 5). IRS-1 contains 21 putative tyrosine phosphorylation sites based on general tyrosine kinase specificity (nearby acidic residues and downstream hydrophobic residues) [13, 76]. IRS-1 and IRS-2 show about 35% identity in this region, and with a few exceptions, the similarity is restricted to the potential tyrosine phosphorylation sites [63]. Fourteen of the 21 potential tyrosine phosphorylation sites from IRS-1 are conserved in IRS-2; four sites contain alternate surrounding sequences; three sites from IRS-1 are not found in IRS-2; and four novel sites exist in IRS-2 (Fig. 5).

Detailed studies of tyrosine phosphorylation have been conducted with IRS-1 during insulin stimulation [105]. At least eight tyrosines on IRS-1 undergo phosphorylation by the activated insulin receptor, including residues 608, 628, 939 and 987 which are in YMXM motifs (Fig. 3). The methionine residues at the Y^{+1} and Y^{+3} positions facilitate phosphorylation by the insulin receptor [106]; however, other motifs are phosphorylated including Y_{460}ICM, Y_{895}VNI, Y_{1172}IDL, and Y_{1222}ASI [105]. Thirteen additional tyrosine residues may be phosphorylated by the insulin receptor based in proximity to an aspartate or glutamate residue, or by other tyrosine kinases with different selectivity (Fig. 2).

The IRS-proteins also contain over 30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases. Before insulin stimulation IRS-1 is strongly serine phosphorylated [76]. Casein kinase-2 phosphorylates rat IRS-1 at Ser-99 and Thr-502 [107], and preliminary data suggest that purified MAP kinase also phosphorylates recombinant IRS-1 (White, M. F. et al, unpublished). Serine and threonine phosphorylation of IRS-1 appears to inhibit its tyrosine phosphorylation during insulin stimulation [108].

Distribution of IRS-1 and IRS-2. Although IRS-1 and IRS-2 contain common functional domains, they may regulate unique signalling pathways, in part owing to their distinct cellular distribution. IRS-proteins are thought to play an important role during insulin-stimulated glucose transport [109–111]. The 3T3-L1 adipocyte is frequently used to study the mechanism of insulin-stimulated glucose uptake, as several metabolic responses acquire insulin sensitivity during their differentiation into adipocytes [112]. Before differentiation, IRS-1 and IRS-2 were barely detectable in the 3T3-L1 fibroblasts by immunoblotting with αPY during insulin stimulation. After differentiation, the amount of IRS-1 increased dramatically, and insulin strongly stimulated its tyrosine phosphorylation; however, insulin-stimulated tyrosine phosphorylation of IRS-2 remained low. Consistent with this observation, IRS-1 was the predominant phosphorylated IRS-protein in isolated rat adipocytes during insulin stimulation and strongly associated with p85. Based on these results, IRS-1 may be the predominant isoform responsible for metabolic signals.

Interaction between the insulin receptor and IRS-proteins. The interaction of the insulin receptor with a limited number of cellular proteins, including IRS-1/2 and Shc, suggests that a common mechanism may exist. Recently, the NH_2-terminal portion of IRS-1 was shown to mediate an interaction with Tyr_{960} of the insulin receptor [113–115]. An alignment of the predicted amino acid sequence of murine IRS-1 and IRS-2 revealed two conserved regions at the NH_2-terminal end which we call IRS-homology (IH) domains [63]. We designated these IH domains as $IH1^{PH}$ and $IH2^{PTB}$, to reflect their similarity to pleckstrin homology domains or PTB domains in various proteins (Fig. 5). The $IH1^{PH}$ domain contains 109 amino acid with 69% identity (83% similarity) between IRS-1 and IRS-2. The $IH2^{PTB}$ is separated from the $IH1^{PH}$ domain by a 40-residue variable region and is distantly similar to the PTB domain of Shc [63]. The alignment of highest quality defines a region of 160 amino acids with 75% identity (85% similarity) between IRS-2 and IRS-1, and 15% identity to the PTB domain in Shc (Fig. 6).

A third region, between residues 313 and 462 of IRS-1 was aligned recently to the PTB domain in Shc, and designated as the 'SAIN' domain [114–116] (Fig. 5). However, this region is poorly conserved between IRS-1 and IRS-2, and appears to be a collection of short conserved motifs including known tyrosine phosphorylation sites. We suspect that this region mediates downstream signalling rather than upstream receptor recognition.

The $IH2^{PTB}$ domain binds to the phosphorylated NPXY-motif in the insulin and IL4 receptors. Recombinant $IH2^{PTB}$ from IRS-1 binds specifically to the $_{NH2}$-LYASSNPE(pY_{960})LSASDV-$_{COOH}$, previously predicted to mediate the interaction of the insulin receptor and the IL4 receptor with IRS-1 (Fig. 2). Similar results have been observed with these regions of IRS-2 (data not shown). Thus, the $IH2^{PTB}$ in the

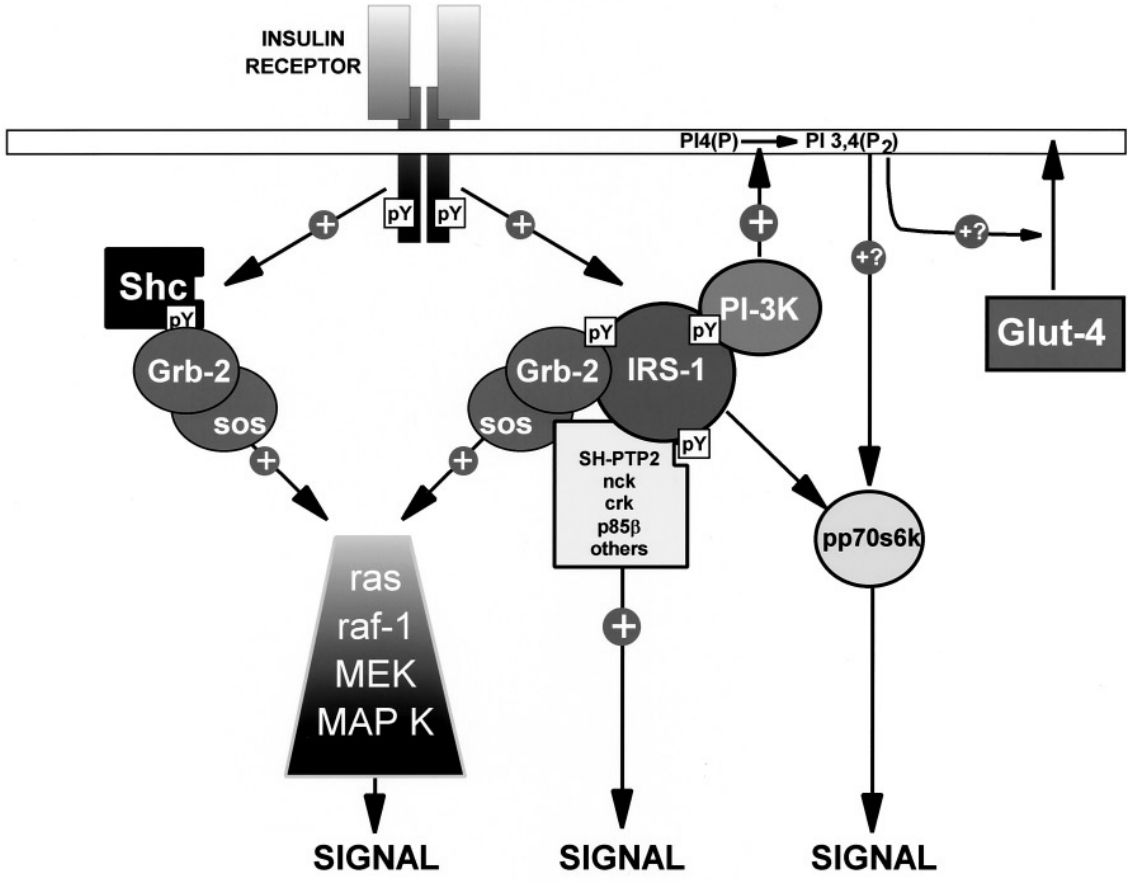

Fig. 6. Schematic diagram of the insulin receptor signalling mechanism. Largely through IRS-proteins, but also involving Shc, the insulin receptor controls various enzyme pathways, including the $p70^{s6k}$, MAP kinase and glucose transporter translocation

IRS-proteins may mediate interactions with receptors by association with phosphorylated Tyr_{960}.

Role of SH2-proteins during insulin signalling

Introduction. Unlike many growth factor and cytokine receptors, the insulin receptor binds poorly to SH2-proteins [78]; however, the IRS-proteins, Shc, and possibly a 60-kDa protein that binds to p85 in rat adipocytes, and pp115 which associates with SHP2 in 3T3-L1 adipocytes, function as interfaces between the receptor and various SH2-proteins. The binding of SH2-proteins to IRS-1 serves several purposes (Fig. 6): first, enzymes associated with IRS-1 through their SH2 domains may be activated. This is observed in the case of PI-3 kinase and SHP2. On the other hand, IRS-1 may serve to juxtapose heterogeneous signalling molecules in one location to generate a composite signal. Finally, IRS/SH2-protein complex is mobile and free to move independently of the internalized receptor.

Phosphatidylinositol-3 kinase. The PI 3-kinase was the first SH2-protein found to associate with IRS-1 [76]. It is normally composed of two subunits, a 110-kDa catalytic (p110) subunit and an 85-kDa (p85α) regulatory subunit containing two SH2 domains. In neuronal cells, $p55^{PIK}$ also regulates p110 [117]. Both SH2 domains in p85 and $p55^{PIK}$ associate specifically with phosphorylated IRS-1 in the intact cells and with recombinant IRS-1 in vitro. IRS-1 contains at least four sites that interact with the SH2 domains of p85: $Y_{608}MPM > Y_{939}MNM > Y_{987}MTM$ and $Y_{460}ICM$ [105]. The association of p85 with tyrosine phosphorylated IRS-1 activates the PI-3 kinase [118, 119]. Synthetic peptides containing a single phosphorylated YMXM-motif also activate PI 3′-kinase but are less potent than IRS-1; synthetic peptides containing two phosphorylated YMXM-motifs are equipotent with recombinant IRS-1 [120]. Blood glucose levels are maintained around 5 mmol/l through the actions of several hormones, including insulin and IGF-1. Glucose stimulates insulin release which promotes glucose transport in skeletal muscle, cardiac myocytes, and adipocytes. Insulin increases glucose transport by causing translocation of GLUT4 from intracellular compartments to the plasma membrane [121]. The effect on GLUT4 translocation reaches a maximum within minutes after insulin stimulation. Two distinct experimental approaches have converged recently to the conclusion that PI-3 kinase is

necessary, although not necessarily sufficient for insulin-stimulated GLUT4 (and GLUT1) translocation. First, insulin-stimulated glucose uptake is inhibited in fat cells by wortmannin (ED_{50} = 50 nmol/l), a poorly characterized inhibitor of the PI-3 kinase [110]. Alone, these results are questionable because it is impossible to know whether other unknown kinases are also inhibited. However, a p85 mutant (Δp85) lacking the binding site for p110 inhibits insulin-stimulated glucose uptake (translocation of GLUT1 to the plasma membrane in this case) in CHO cells [109]. Together, these results suggest that the activation of the PI-3 kinase is necessary for insulin-stimulated glucose uptake. However, the activation of PI-3 kinase through other receptors without the stimulation of glucose uptake emphasizes our incomplete state of knowledge [122, 123].

Since IRS-1 is an important upstream element in the regulation of PI-3 kinase by insulin and IGF-1, IRS-1 may play an obligatory role in insulin-stimulated GLUT4 translocation [124]. Disruption of the IRS-1 gene in mice partially supports this conclusion, since insulin-stimulated glucose uptake is reduced by 50 % [125, 126]. In normal adipocytes, insulin-stimulated PI-3 kinase activity is found in a very low density membrane fraction which also contains a highly phosphorylated cohort of IRS-1 [124]. Interestingly, p85 remains largely in the cytosol during insulin-stimulation, and the total amount in the subcellular fractions is unchanged [124]. The activated PI-3 kinase co-localizes with a 60 kDa phosphotyrosine-containing protein R3-3 which immunoprecipitates with p85 antibodies [90, 124]. Together with IRS-1, pp60 may regulate PI-3 kinase and glucose influx in adipocytes (126 a).

Phosphotyrosine phosphatase, SHP2. SHP2 is an SH2-containing tyrosine phosphatase expressed ubiquitously in mammalian cells and tissues [127]. It is homologous to *Drosophila csw,* which mediates the signals from the *torso* receptor (a PDGF receptor homologue) [128]. Several growth factor receptors, including the EGFr, the PDGFr, and c-kit bind specifically to the SH2 domains in SHP2 [129–132]. Interestingly, the phosphorylated SHP2 serves as an adaptor molecule between the activated PDGFr and the Grb2-Sos complex [133]. During association with these receptors, SHP2 is tyrosine phosphorylated which may increase its catalytic activity. SHP2 is also a target for several non-receptor tyrosine kinases including IL3 and granulocyte/macrophage colony-stimulating factor in murine myeloid progenitor-like cells [134]. During association with $p210^{bcr\text{-}abl}$ in several murine cell lines, SHP2 is highly and constitutively tyrosyl phosphorylated [135]. Similarly, SHP2 is constitutively tyrosyl phosphorylated in cells transformed with v-Src, although it does not physically associate with v-Src [129].

The precise role of SHP2 in insulin signalling remains unclear, but several reports suggest that it is an essential component in the pathway. Inactive SHP2 inhibits insulin-stimulated MAP kinase and c-fos transcription [136, 137]. This dominant negative effect of inactive SHP2 is partially reversed by co-expression of v-ras or Grb2, indicating that SHP2 may act upstream of Ras [137].

Recently, we discovered that SHP2 associates with Tyr_{1146} in the regulatory loop of the β-subunit [57]. This may provide a more stable link between the insulin receptor and IRS-1 that can be obtained through the $IH1^{PH}$ or $IH2^{PTB}$ domain alone. Furthermore, this association may play a role during insulin-stimulated glucose uptake [57]. We have also found that IRS-1 mediates insulin-stimulated tyrosine phosphorylation of SHP2 (Shi P, White MF et al, unpublished results). Co-expression of the human insulin receptor with IRS-1 or IRS-2 substantially increases insulin-stimulated phosphorylation of SHP2, as compared to those cells overexpressing insulin receptor alone. Mutations of the binding sites in IRS-1 abolish the phosphorylation of SHP2 and its in vivo association with IRS-1. These results suggest that SHP2 is placed in close proximity to an activated tyrosine kinase during insulin stimulation. The binding to Tyr_{1146} in the receptor regulatory loop may facilitate an interaction with the insulin receptor itself. Phosphorylation of SHP2 may further modulate its phosphatase activity or provide a binding site for additional downstream signalling molecules. The phosphorylation of SHP2 by insulin may help clarify its role during insulin signalling.

GRB-2, the insulin-stimulated ras pathway, and the activation of MAP kinase. Grb-2 is a small cytoplasmic protein that contains two SH3 domains and one SH2 domain that bind to IRS-1 or Shc. GRB2 is an 'adapter molecule' that links mSOS, a guanine nucleotide exchange factor for $p21^{ras}$, to tyrosyl phosphoproteins such as the EGF receptor, IRS-1 and Shc [133, 138]. During insulin stimulation, both IRS-1 and Shc bind Grb-2; however, the predominant mechanism employed in various insulin-stimulated tissues may depend on the level of insulin receptors and the competition between phosphorylated Shc or Tyr_{895} in IRS-1 [20, 139, 140].

The $p21^{ras}$ is active when bound to GTP and inactive when GDP-bound, and while associated with Grb-2, Sos promotes the release of GDP from $p21^{ras}$ enabling GTP binding. While $p21^{ras}$ has many cellular functions, its ability to control the activation of the MAP Ser/Thr kinases in many cell systems is well established [141]: active $p21^{ras}$ associates with and activates the raf-1 kinase, which phosphorylates and activates MAP kinase-kinase (MEK), which in turn phosphorylates and activates MAP kinase [141]. Thus, engagement of GRB-2/Sos by tyrosyl

phosphorylated signalling proteins is expected to activate $p21^{ras}$ and the downstream MAP kinase cascade [142].

Our work with single Tyr → Phe point mutations of IRS-1 reveals that the binding of GRB-2 by IRS-1 mediates activation of MAP kinases by insulin in some cells; $IRS\text{-}1^{F895}$, which does not associate with GRB-2, does not mediate the IRS-1-dependent component of MAP kinase activation [20]. The association of Grb-2/Sos with phosphorylated Shc, may provide an alternate pathway for the insulin receptor and a common link for many growth factor receptors. Indeed, while IRS-proteins enhance the insulin-stimulated activation of MAP kinase, they are not required for phosphorylation of Shc, or activation of $p21^{ras}$ and MAP kinase (Fig. 6). The relative contributions of Shc and IRS-proteins to this cascade are likely to be cell and tissue specific. The IRS-independent tyrosine phosphorylation of Vav may also play a role in the regulation of these pathways [84].

Serine phosphorylation, TNFα, and the regulation of insulin signalling

The regulation of the insulin receptor kinase by serine phosphorylation is an enigma. Although it is generally accepted that serine phosphorylation inhibits insulin-stimulated tyrosine phosphorylation of the receptor, the mechanism for this effect is not understood [143, 144]. Several serine kinases have been found to mediate serine phosphorylation of the insulin receptor, but protein kinase C has received the most attention [145–149]. Several sites of serine phosphorylation have been identified in the β-subunit, but none of them appear to inhibit the receptor kinase.

Serine phosphorylation also plays a role in the regulation of IRS-1 signalling potential. Okadaic acid, a serine phosphatase inhibitor increases serine phosphorylation of IRS-1 in 3T3-L1 adipocytes, which appears to inhibit insulin-stimulated tyrosine phosphorylation; however, the insulin receptor is unaffected [108]. Recently, a role for serine phosphorylation in the regulation of the insulin receptor kinase has emerged from studies on the mechanism of tumour necrosis factor-α (TNFα) inhibition of the insulin receptor kinase [150].

Recent studies have demonstrated that overexpression of TNFα in adipose tissue is a common feature of many different rodent models of obesity [151]. Elevated TNFα expression in adipose tissue is also present in human obesity and correlates strongly with the degree of obesity and the level of hyperinsulinaemia, an indirect measure of insulin resistance [151]. Moreover, neutralization of TNFα in obese and insulin-resistant rats improves insulin signalling and sensitivity of peripheral tissues, demonstrating that TNFα interferes with insulin action in this disease [152].

The mechanism of TNFα-inhibited insulin signalling may occur through its ability to inhibit the tyrosine kinase activity of the insulin receptor [152, 153]. Interestingly, IRS-1 mediates the inhibition of insulin receptor tyrosine kinase activity in vitro, and this effect is dependent upon serine phosphorylation of IRS-1 [150]. The presence of IRS-1 is necessary for the inhibition of insulin receptor signalling by TNFα in intact 32D myeloid cells. 32D cells which lack endogenous IRS-1 are resistant to the effect of TNFα on insulin receptor signalling, whereas cells expressing IRS-1 are very sensitive to TNFα. An inhibitory form of IRS-1 is also observed in obese animals, but only when IRS-1 is isolated from the major sites of insulin resistance, muscle and fat. TNFα induces serine phosphorylation of IRS-1 in cultured adipocytes and Fao cells; this appears to inhibit the insulin signal [150, 154]. These results provide both biochemical and genetic evidence for a novel mechanism by which TNFα induces insulin resistance and demonstrates an unexpected role for IRS-1 in the attenuation of the insulin receptor signalling, possibly through serine phosphorylation.

Conclusions and perspectives

With the recent identification of cellular substrates such as the IRS-proteins, and the recent discovery of additional substrates we are in a much better position to understand the role of insulin in molecular terms. But more importantly, as we learn more about the details of the signalling pathways, we begin to appreciate new relationships that may explain puzzling observations. The discovery that TNFα contributes to insulin resistance and that this may be mediated by IRS-protein adds an intriguing twist to the link between obesity and NIDDM. Moreover, the growing list of cytokine receptors that use IRS-proteins to mediate their signals adds another dimension to our understanding of the potential complexity of insulin signalling and the consequences of resistance at the IRS-proteins.

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