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INSULIN SIGNAL TRANSDUCTION AND THE IRS PROTEINS

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ABSTRACT

Insulin controls organismal and cellular physiology by initiating numerous intracelluar signals. Insulin first binds the extracelluar domain of the insulin receptor, which activates the receptor's intracellular tyrosine kinase. Receptor-mediated phosphorylation of the IRS proteins is required for the propagation of signals for mitogenesis, glucose transport, and numerous other biological and biochemical events during insulin signaling. IRS proteins also mediate signaling by a subset of other growth factor and cytokine receptors; recognition and phosphorylation by specific receptors appears to be mediated by the PH and PTB domains of the IRS proteins. The best understood mechainsm of IRS-protein–mediated signaling is the binding of SH2 domain–containing signaling molecules (such as PI 3′-kinase) by tyrosine phosphorylation sites on IRS proteins. Other paradigms of IRS-protein signaling are beginning to emerge, however, and these exciting molecules promise to teach us much in the next few years.

INTRODUCTION

Insulin, Metabolism, and Glucose Disposal

Insulin, discovered in 1922 by Banting & Best, is a crucial regulator of metabolic physiology (1). In response to increases in blood glucose concentration, the β-cells of the pancreatic islets release insulin into the bloodstream, through which it travels to its primary target tissues—fat, muscle, and liver. In these tissues, insulin promotes the influx of nutrients and blocks the release

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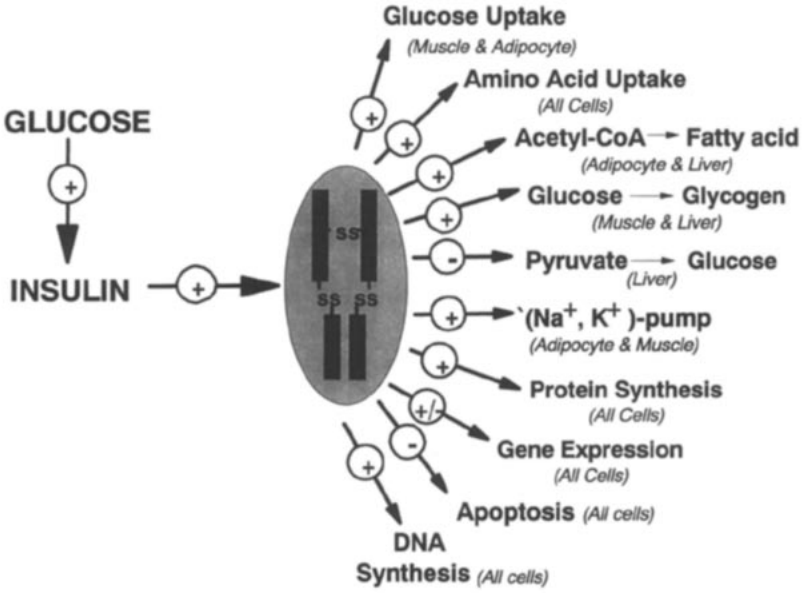

Figure 1 Insulin regulates diverse physiological processes. Insulin is secreted in response to increases in blood glucose levels. Insulin then moves through the circulation to bind and activate insulin receptors on various tissues. Receptor activation mediates the uptake and storage of energy in cells. It also controls gene expression, ion flux, cell proliferation, and apoptosis.

of stored forms of energy (2). Insulin promotes glycolysis and glycogen storage and suppresses glycogenolysis and gluconeogenesis in the liver. In striated and cardiac muscle, glycogen and protein synthesis are induced by insulin, and in fat, lipid storage is favored. Insulin mediates other diverse effects in a wide variety of cells and tissues (Figure 1).

Diabetes mellitus is a disease engendered by either lack of insulin [type I diabetes, or insulin-dependent diabetes mellitus (IDDM)] or the failure to compensate for a diminished insulin response [type II diabetes, or non–insulin-dependent diabetes mellitus (NIDDM). Although 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 (3, 4). Insulin also influences embryonic development, as maternal hyperinsulinemia results in increased fetal growth and insulin-resistant fetuses display developmental abnormalities, including growth retardation (1, 5).

The mechanism of insulin action is a challenging problem for scientists studying both cell biology and diabetes. For the first 50 years of insulin's history, the study of insulin action centered on the metabolic changes that occur during insulin stimulation—first at the whole animal level and later at the cellular level (1, 6). These studies demonstrated that processes such as glucose uptake, lipid synthesis, protein synthesis, and glycogen deposition are impaired in NIDDM, but none of these studies identified the principal cause of insulin resistance. Following the identification of the insulin receptor and the characterization of its intracellular enzymatic activity, we have begun to unravel the complex set of signaling cascades engaged in the cell by insulin. Even now, the molecular defects behind NIDDM remain undefined: Although the causes of a few rare types of insulin resistance [e.g. insulin receptor mutations and mature-onset diabetes in the young (MODY)] have been defined (5–7), the discovery of a defect underlying the bulk of NIDDM by genetic means appears unlikely, at least in the short term, since NIDDM results from a complex set of genetic and environmental inputs. A molecular understanding of the cellular mechanism of insulin action will contribute enormously to our understanding of this disease and should ultimately lead to the design of efficacious interventions.

THE INSULIN RECEPTOR

Structure of the Insulin Receptor

Following its release by the β-cells of the pancreas, insulin binds to receptors on the surface of most cells. Classic insulin-responsive cells—such as hepatocytes, adipocytes, and muscle cells—possess relatively high levels of receptors; all cells, however, possess some insulin receptors, and insulin may affect physiological processes in many of them. Insulin receptors are heterotetrameric transmembrane glycoproteins composed of two α- and two β-subunits (Figure 2) (8). The α-subunit is completely extracellular and mediates insulin binding, whereas the β-subunit has an extracellular domain, a membrane-spanning domain, and a 402–amino acid intracellular tail (8). This intracellular domain contains a tyrosine-specific protein kinase similar to that of certain cellular proto-oncogene products (e.g. $p60^{c\text{-}src}$, *c-ros*) and peptide growth factor receptors [e.g. platelet-derived growth factor receptor (PDGFr), epidermal growth factor receptor (EGFr)] (8).

Insulin binding to its receptor activates the tyrosine kinase, leading to autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit (9, 10): Tyr_{960} in the juxtamembrane region of the β-subunit; Tyr_{1146}, Tyr_{1150}, and Tyr_{1151} in the kinase domain; and Tyr_{1316} and Tyr_{1322} in the COOH-terminus (11–14) (Figure 2). The tyrosine kinase activity of the receptor is

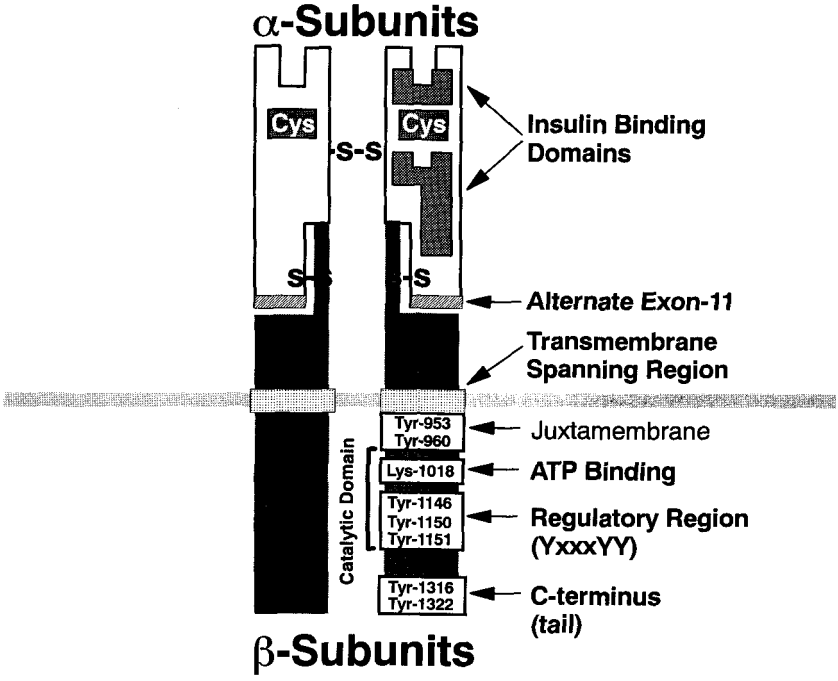

Figure 2 Important structural features of the insulin receptor. The insulin receptor is a disufide (-S-S-) bonded heterotetramer composed of two α- and two β-subunits. The α-subunits are extracellular and contain the insulin binding domains; an alternate form of the α-subunit contains an extra short exon. The β-subunits contain a short extracellular domain, a transmembrane-spanning region, and an intracellular tail. The intracellular tail is remarkable for an intrinsic tyrosine kinase containing an ATP binding site and autophosphorylation sites. The autophosphorylation sites are distributed in several regions: juxtamembrane, regulatory (kinase domain), and COOH-terminus.

crucial for insulin action: Site-directed point mutations of the insulin receptor that destroy ATP binding (and thus abolish kinase activity) also abrogate insulin signaling in cultured cells (15, 16). Naturally occurring mutations of the insulin receptor that inhibit kinase activity are associated with severe insulin resistance (17, 18). Thus, our current view focuses on the tyrosine kinase as the principal vehicle of insulin signal initiation and propagation.

Regulation of the Insulin Receptor Tyrosine Kinase

INSULIN BINDING Much early work on the insulin receptor focused on the regulation of its tyrosine kinase activity (12, 19). Before insulin binding, the extracellular domain exerts a negative effect upon the kinase: The tyrosine kinase is activated by enzymatic proteolysis, which removes the extracellular

insulin binding domain (20); the cytoplasmic domain of the insulin receptor when expressed alone (as in baculovirus) is more active than the intact receptor (21, 22). Although disulfide bonds stabilize the interactions between two α-subunits, insulin binding appears to activate the receptor by further cross-linking α-subunits within the receptor heterotetramer (23). The insulin receptor α-subunit (most notably an NH_2-terminal region) contains two distinct binding pockets that interact with two regions on the insulin molecule (23). Through this mechanism, insulin binding may bring adjacent α-subunits and other regions of the receptor closer together to mediate the so-called autophosphorylation reaction, which is actually transphosphorylation of β-subunits within the receptor tetramer (24–27). A close interaction between transmembrane-spanning regions of the β-subunits may also stabilize the active conformation (28). This view of insulin-stimulated activation is consistent with dimerization hypotheses proposed for other receptor systems (29).

ACTIVATION BY AUTOPHOSPHORYLATION The insulin receptor tyrosine kinase is activated by two separate events. First, insulin binding partially increases the activity of the tyrosine kinase and stimulates β-subunit autophosphorylation. Second, autophosphorylation of the insulin receptor fully activates the enzyme (30–34). In vitro, the two-step activation process can be resolved using peptide substrates, which, at high concentrations, act as competitive inhibitors of autophosphorylation as well as phosphate acceptors (30, 35). Incubation of the unstimulated or insulin-bound receptor with high concentrations of peptide substrates before autophosphorylation inhibits receptor autophosphorylation by competing with the tyrosine residues on the β-subunit for phosphorylation, effectively blocking the autophosphorylation-dependent component of insulin receptor kinase activity (30). Allowing the receptor to autophosphorylate before the addition of substrate inhibitors alleviates this inhibition of kinase activity. Whether in vivo substrates can inhibit receptor activity in the intact cell is unknown, but this represents a theoretical mechanism of receptor regulation and/or insulin resistance.

Autophosphorylation of the insulin receptor is required for the full kinase activity toward substrates (30–32). Three residues—Tyr_{1146}, Tyr_{1150}, and Tyr_{1151}—located in the so-called regulatory loop, appear to play a central role in this process (31, 32, 36, 37). Replacement of these autophosphorylation sites (alone or in combination) with phenylalanine decreases insulin-stimulated tyrosine kinase activity and renders the receptor incapable of mediating insulin signaling in cultured cells. Moreover, antiphosphotyrosine (αPY) antibodies trap the autophosphorylation cascade in the bisphospho-state—pY_{1146}/pY_{1150} or pY_{1146}/pY_{1151}—and inhibit kinase activation (30). Thus trisphosphorylation of the regulatory loop appears to be required for full activation of the insulin receptor kinase.

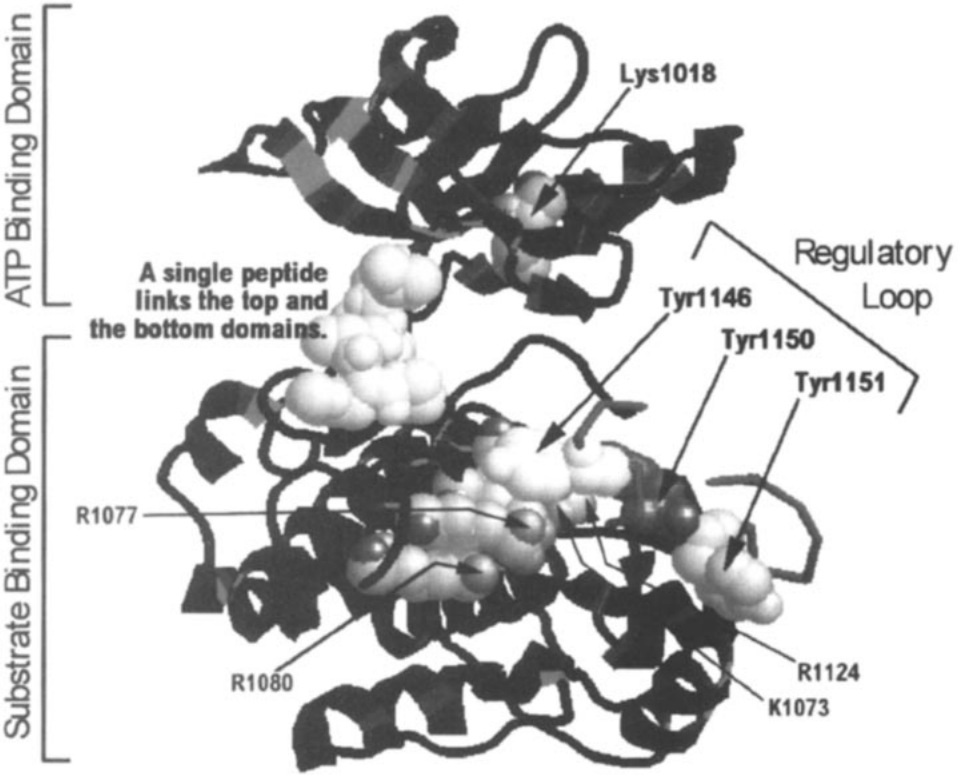

Figure 3 A three-dimensional structure of the insulin receptor tyrosine kinase domain rendered with RASMOL. The ATP-binding domain containing Lys_{1018} represented at a space-filling atom is indicated; the substrate-binding doman is indicated below it. The positions of the three tyrosine residues in the regulatory loop are shown, and the relative positions of four positively charged residues (Arg1077, Atg1080, Lys1073, and Arg1124) are indicated. This model is based on Brookhaven coordinates from Reference 38.

The crystal structure of the insulin receptor kinase domain provides new insight into the mechanism by which the regulatory-loop controls the kinase activity (38): Before phosphorylation, Tyr_{1150} blocks the active site, inhibiting access by potential substrates (Figure 3). During insulin binding, the juxtaposition of the regulatory loops on adjacent β-subunits may disengage Tyr_{1150} and allow ATP binding and transphosphorylation. It has been proposed that the phosphorylation of Tyr_{1150} keeps it out of the active site and allows further catalytic activity. Because phosphorylation of this residue alone is not sufficient to mediate full activity, the phosphorylation of all three tyrosine residues may guarantee full displacement of the regulatory loop.

INSULIN RECEPTOR SUBSTRATES The substrate specificity of the purified insulin receptor tyrosine kinase has been examined using protein and synthetic peptide substrates in vitro: The receptor prefers substrates in which the tyrosine residue is preceded by an acidic amino acid (Y^{-1} or Y^{-2} position) and is followed by a hydrophobic residue (Y^{+1}), although the receptor also phosphorylates tyrosines in other contexts (39, 40). Predictions from the crystal structure agree with the empirically determined substrate specificity of the insulin receptor (38): The negative charge of the acidic residue preceding the target tyrosine residue is attracted by the positively charged receptor residues Lys_{1073}, Arg_{1077}, and Lys_{1080} (Figure 3). Furthermore, the hydrophobic environment created by Val_{1161} and Leu_{1207} on the receptor favor the selection of a hydrophobic residue in the Y^{+1} position of the substrate (38). These predictions are consistent with the observed phosphorylation sites in insulin receptor substrate–1 (IRS-1), which contains 18 motifs that fit this general pattern for substrate selectivity (38–40).

CELLULAR SUBSTRATES OF THE INSULIN RECEPTOR

The Discovery of pp185

Early theories of tyrosine kinase signaling focused on intracellular substrate proteins as second messengers. While in many signaling systems the direct substrate hypothesis has been modified or set aside (41), it has held true in the case of the insulin receptor (42). The development of antiphosphotyrosine antibodies (αPY), which immunoprecipitated a high molecular weight phosphotyrosine-containing protein (pp185) from insulin-stimulated FAO hepatoma cells, provided the first evidence for the existence of a direct cellular substrate of the insulin receptor (43). The pp185 migrates between 165 and 210 kDa on SDS-PAGE in αPY immunoprecipitates from insulin-stimulated cells. It is located mainly in the cytoplasm and contains phosphotyrosine, phosphoserine, and phosphothreonine following insulin stimulation. Tyrosyl phosphorylation of pp185 occurs immediately after insulin stimulation, and gradually decreases during continuous insulin stimulation of many cell lines (44). The rapid tyrosine phosphorylation of this rare intracellular protein suggested immediately that it is closely coupled to the insulin receptor and should be important in the signaling mechanism.

Other Insulin-Stimulated Tyrosyl Phosphoproteins

Subsequent studies with αPY revealed that other cellular proteins become tyrosyl phosphorylated during activation of the insulin receptor in a variety of cells and tissues. The pp120 substrate is a liver-specific membrane glycoprotein that is tyrosine phosphorylated during insulin stimulation (45); it is now

known to be an Ecto-ATPase, perhaps involved in the active movement of small molecules across the liver cell membrane (46, 47). Several 60-kDa substrates molecules have been reported, including a phosphatidylinositol (PI) 3-kinase–associated molecule from adipocytes (48) and one that associates with $p21^{ras}$-GAP (49). pp15 is a 15-kDa protein that is tyrosine phosphorylated under certain circumstances during insulin stimulation of adipocytes; it is identical with 442(aP2), a myelin homologue (50, 51). Unfortunately, the signaling roles of these insulin-stimulated tyrosyl phosphoproteins during insulin stimulation remain enigmatic.

The discovery of tyrosine kinase substrates in other tyrosine kinase signaling systems has led to the enumeration of additional insulin-stimulated tyrosyl phosphoproteins. Vav, a 95-kDa proto-oncogene associates with the insulin receptor and is tyrosine phosphorylated during insulin stimulation (52). Although the role of Vav in insulin signaling is unclear, it contains several well-known associative domains [Src homology-2 and -3 (SH2 and SH3), see below] and regions that may contain guanine nucleotide–releasing activity for small GTP-binding proteins (52–54).

The Shc family is composed of at least three isoforms with molecular weights between 46 and 52 kDa (55). During stimulation of cells with various growth factors, including insulin, Shc is tyrosine phosphorylated (56). Shc contains domains that likely mediate signaling interactions, including an SH2 domain (see below) at its COOH-terminus and a region similar to α-1 collagen in the middle of the molecule that contains a binding motif for SH3 domains (55). During tyrosine phosphorylation Shc binds Grb-2/Sos, providing a link between receptor kinases and $p21^{ras}$ (57).

The IRS-Protein Family

IRS-1, THE FIRST IRS PROTEIN Using affinity chromatography on αPY antibodies, a constituent of the pp185 band (described above) was purified from the livers of insulin-treated rats and subsequently cloned (58, 59). This new protein, the first cloned insulin receptor substrate (IRS-1), has a predicted molecular weight of 131 kDa, although it migrates between 170 and 180 kDa on SDS-PAGE when expressed in CHO cells (59, 60). An identical protein was subsequently cloned from mouse and human tissues (61–63); recently, the *Xenopus laevis* IRS-1 protein was cloned, confirming the evolutionary conservation of IRS-1 (64). IRS-1 is highly serine phosphorylated even in the unstimulated state and is rapidly tyrosine and serine phosphorylated in response to insulin treatment of cells (59, 60).

THE DISCOVERY AND CLONING OF IRS-2 Although IRS-1 is an important lead, it is not the only IRS protein. Analysis of pp185/IRS-1 in FAO hepatoma cells

revealed that the pp185 band contains at least one other high molecular weight tyrosyl phosphoprotein in addition to IRS-1 (65). The lower portion of the pp185 band is composed of IRS-1, but the high molecular weight portion of pp185 is a distinct insulin receptor substrate protein, which we designated $pp185^{HMW}$ (65). An insulin receptor substrate resembling $pp185^{HMW}$ was observed in FDC-P2 myeloid cells during insulin stimulation (66, 67); this protein was originally designated 4PS (IL-4r phosphoprotein substrate), since it was initially observed during interleukin (IL)-4 stimulation (67). Like $pp185^{HMW}$, 4PS reacts poorly or not at all with most αIRS-1 antibodies, although it possesses many properties in common with IRS-1 (66, 67; and XJ Sun & MF White, in preparation). Most revealing was the finding that antibodies to the NH_2-terminal domain of IRS-1 (the pleckstrin homology domain, see below), react with 4PS. These data pointed the way to the second member of the IRS-protein family (68).

4PS was purified from lysates of insulin-stimulated FDC-P2 cells by affinity chromatography on phosphotyrosine-binding p85 SH2 domains. Digestion of the protein allowed sequencing of several peptides used for the cloning of the 4PS cDNA (68). Extensive homology between IRS-1 and 4PS suggested that they were members of the same family of signaling proteins (IRS proteins) (Figure 4); hence, 4PS was renamed IRS-2 (68). IRS-2 comprises at least part of the high molecular weight component of pp185 in FAO and other cell types (68). IRS-1 and IRS-2 appear to mediate similar signals (see below), but the possibility of important differences between these proteins exists and is beginning to be explored.

Although IRS-1 and IRS-2 contain common functional units, they may regulate unique signaling pathways, in part owing to their distinct cellular distribution. The expression of IRS-1 and IRS-2 was compared in various cell lines and tissues by immunoblotting or RT-PCR. Both IRS-1 and IRS-2 are detected in most cells and tissues, including lymphoid progenitor cells, B cells, carcinoma cells, fibroblasts, adipocytes, liver, skeletal muscle, and brain. However, IRS-2 predominates in cells of the myeloid lineage, some lymphoid cells, and two human colon carcinoma cells lines; only two tested cell lines (Daudi and EL4) expressed IRS-1 exclusively. Either IRS-1 or IRS-2 is expressed relatively highly in several mammary or colon carcinoma cells (XJ Sun & MF White, in preparation). Interestingly, during differentiation of 3T3-L1 cells into adipocytes, IRS-1 expression increased, whereas the IRS-2 level was unchanged; during retinoic acid–induced differentiation of P19 cells into neurons, IRS-1 protein levels increased, whereas IRS-2 decreased.

STRUCTURAL FEATURES OF IRS PROTEINS *Introduction* The identification of IRS-2 and its comparison with IRS-1 provides new insight into the modular structure and function of the IRS-signaling proteins (Figure 4). The IRS pro-

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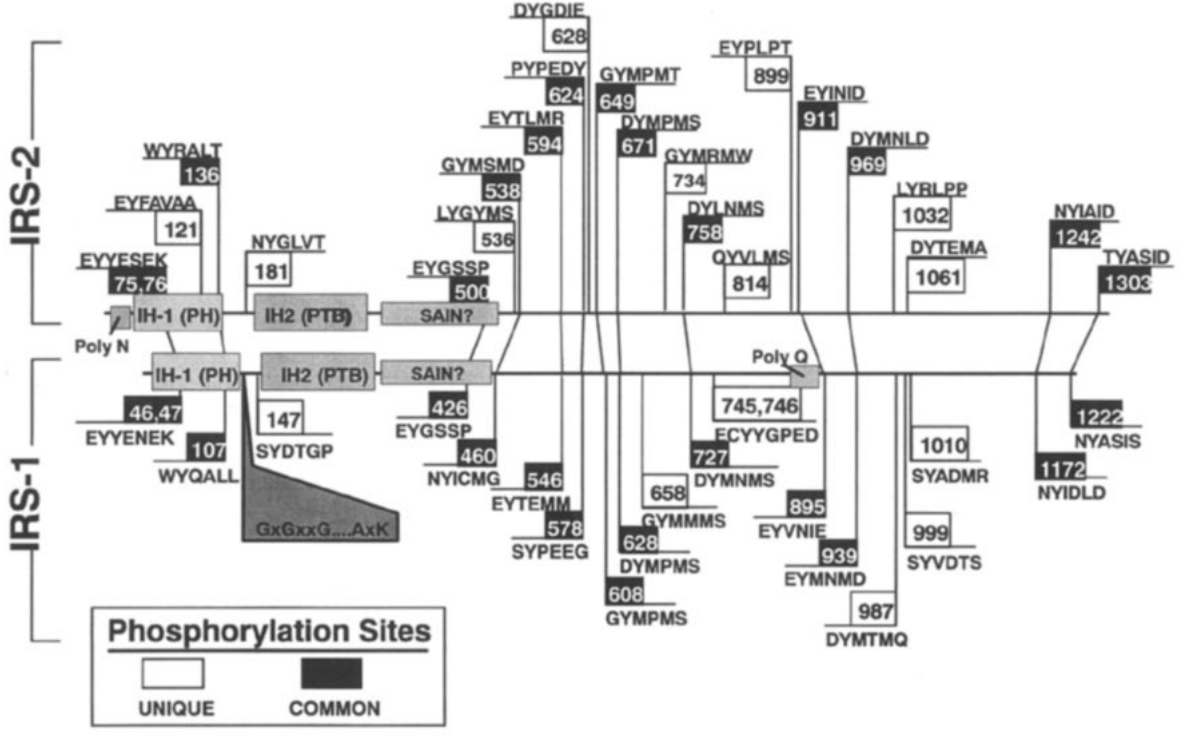

Figure 4 Alignment of IRS-1 and IRS-2. Linear diagrams of IRS-1 and IRS-2 amino acid sequence are shown with important strucutral features noted. The homology domains IH-1 (PH) and IH-2 (PTB) are shown, along with the putative SAIN domain. Potential tyrosine phosphorylation sites, along with surrounding amino acid sequences are shown. Sites in black boxes are common to IRS-1 and IRS-2, whereas those in white boxes are unique to one or the other of the proteins. Also shown are the potential ATP binding site on IRS-1 (GxGxxG...AxK) and the location of poly Q and poly N tracts on IRS-1 and IRS-2, respectively.

teins can be divided into two regions. The well conserved NH_2-terminal region may provide the molecular link to activated membrane receptors. This region contains a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain (68). In contrast, the amino acid sequence in the COOH-terminal region is poorly conserved, although it contains multiple tyrosine phosphorylation motifs that are specific substrates for the insulin receptor kinase and other various kinases and provides binding sites for signaling proteins with SH2 domains (Figure 4).

The pleckstrin homology domain: IH-1^{PH} The first homology domain in the IRS proteins is a pleckstrin homology (PH) domain: the IH-1^{PH} domain (Figure 5*A*). PH domains were first recognized as internal repeats in pleckstrin, the major substrate of PKC in platelets (69). The PH domain is a poorly conserved region of about 100 amino acids found in a variety of signaling proteins (69, 70). The structure of several PH domains is similar, although the amino acid identity is very low. PH domains contain six β-strands and an α-helix arranged around a hydrophobic core. One end of the structure is open and may represent a site of interaction with other molecules; the entire domain is electrically polarized. Loops between the elements of conserved structure may contribute to the binding specificity of different PH domains. The PH domains of IRS-1 and IRS-2 are 62% identical (68); this level of identity goes beyond the apparent requirement for similar folding, and suggests a conserved function between the PH domains of these two molecules. Unfortunately, knowledge of the PH domain structure has not improved our understanding of its role in signal transmission.

The phosphotyrosine binding domain: IH-2^{PTB} The second IRS homology (IH-2^{PTB}) domain lies immediately downstream of the IH-1^{PH} domain (Figure 5*B*). This region contains about 160 amino acids and is 75% identical between IRS-1 and IRS-2 (68). Interestingly, this region is similar to the phosphotyrosine binding (PTB) domain in Shc (68, 71–73). The IH-2^{PTB} domain binds to phosphorylated Asn-Pro-Xaa-Tyr (NPXY)-motifs, providing a mechanism for receptor engagement that is analogous to the role of SH2-domains (74, 75). The IH-2^{PTB} domain provides a molecular explanation for the requirement of Tyr_{960} during the insulin receptor signaling. Insulin stimulates autophosphorylation of Tyr_{960} located in the NPXY(Pi)-motif, which binds to the IH-2^{PTB} domain (68). A similar phosphorylation site in the IL-4 receptor provisionally explains the phosphorylation of IRS proteins during IL-4 stimulation (76). Although the role of the IH-2^{PTB} is clearly important, it is not absolutely required for the interaction between IRS proteins and the insulin receptor.

Tyrosine phosphorylation sites in IRS proteins The amino acid sequence identity in the COOH-terminal portion of IRS-1 and IRS-2 is only 35%;

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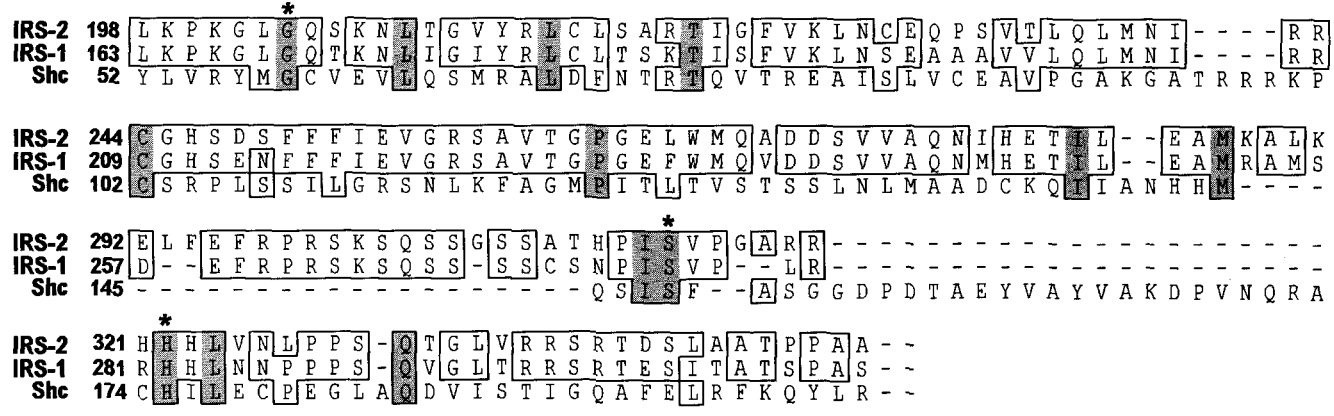
A.



B.

Figure 5 (*A*) Alignment of IRS protein PH domains with other PH domains. The amino acid sequence alignment of the PH domains of various proteins is shown. hbtk, human Bruton tyrosine kinase; makt, mouse c-akt proto-oncogene; hpleck-c, COOH-terminal PH domain from human pleckstrin; hpleck-n, NH_2-terminal PH domain from human pleckstrin; mIRS-1, mouse IRS-1; mIRS-2, mouse IRS-2; hdynam, human dynamin; hsos-1, human sos-1; hβark, human β adrenergic receptor kinase. Conserved residues are boxed. Gaps are represented by dashes. (*B*) Alignment of the PTB domains from IRS proteins and Shc. The amino acid sequences of the $IH\text{-}2^{PTB}$ regions of mouse IRS-1 and mouse IRS-2 are aligned with the PTB domain of human Shc. Conserved residues are boxed. Gaps are represented by dashes.

however, conserved phosphorylation motifs strongly suggest that the two isoforms have significant functional similarity (68). There are 21 tyrosine residues in the COOH-terminus of both IRS-1 and IRS-2, which represent likely sites of tyrosine phosphorylation, based on the criteria of insulin receptor substrate specificity (see above). About half of these phosphorylation motifs are similar and are conserved in both IRS proteins, whereas the others are unique: Four sites contain alternate surrounding sequences in IRS-2, three sites from IRS-1 are not found in IRS-2, and four novel sites exist in IRS-2 that are not found in IRS-1. Interestingly, the distribution of these sites along the length of IRS-1 and IRS-2 is not random, but organized in clusters that lie for the most part in the COOH-terminal two thirds of the molecule (Figure 4).

Among the eight confirmed tyrosine phosphorylation motifs within IRS-1, six are well conserved in IRS-2 (Tyr_{649}, Tyr_{671}, Tyr_{911}, Tyr_{969}, Tyr_{1242}, Tyr_{1303}). The SH2 domains in p85 strongly bind to Y_{608}MPM and Y_{939}MNM in IRS-1 (77), which correspond to Y_{649}MPM and Y_{969}MNL in IRS-2 (Figure 4); however, the substitution of Met_{972} with Leu may alter the interaction. Nevertheless, there are eight other YXXM motifs in IRS-2 that may bind p85 (Figure 4). The SH2 domain of GRB-2 binds to a Y_{895}VNI motif in IRS-1 (77, 78), which corresponds to Y_{911}INI in IRS-2 (Figure 3). IRS-1 also binds to SH-PTP2 at the Y_{1172}IDL motif and the Y_{1222}ASI motif (77, 79, 80), which corresponds to the Y_{1242}IAI and Y_{1303}ASI motifs in IRS-2; however, the spacing between these motifs in IRS-1 and IRS-2 is different, which may alter the regulation of the engaged SH2 proteins (Figure 4). Several other potential tyrosine phosphorylation motifs that occur in either IRS-1 or IRS-2 may provide unique signaling potential (Figure 4). Moreover, the COOH-terminus contains other conserved motifs that may mediate novel protein-protein interactions independently of tyrosine phosphorylation.

Serine phosphorylation of IRS proteins Although IRS-1 is best known as a substrate for tyrosine phosphorylation, it is mainly a phosphoserine-containing protein. IRS-1 and IRS-2 each contain over 30 potential serine/threonine phosphorylation sites with homologies to casein kinase II, PKC, the MAP kinases, cdc 2, and cyclic AMP- and cyclic GMP-dependent protein kinase consensus phosphorylation sites (59, 68). Before insulin stimulation, IRS-1 is strongly serine phosphorylated and weakly threonine phosphorylated. Insulin stimulates significant serine phosphorylation of IRS-1, but the consequences of this modification are generally unknown. One possibility is that serine phosphorylation sensitizes IRS-1 to the action of PEST proteases, mediating the degradation of IRS-1 and downregulating its signal (81). Casein kinase II, MAP kinase, and PI 3′-kinase are responsible for phosphorylation of some of these residues, although further work is required to fully characterize the serine phosphorylation of IRS-1 (82, 83).

Other motifs in the IRS proteins There are two potential nucleotide binding motifs in rat IRS-1 [i.e. GXGXXG...(12–25 aa)...AXK]—one near the amino terminus and one close to the carboxyl terminus of the molecule (Figure 3) (59, 84). Although the COOH-terminal motif is not present in all IRS-1 isoforms, the NH_2-terminal site is conserved between the rat, mouse, and human IRS-1 proteins (85). Other sequences predictive of protein kinases are not present in IRS-1, however (59), suggesting that this motif is not part of a kinase catalytic site; nor is either motif found in IRS-2 (68). Furthermore, we have mutated the lysine in the potential ATP-binding site without effect upon any parameter of IRS-1 phosphorylation or signaling in vitro or in vivo (MG Myers Jr, EM Glasheen, & MF White, unpublished data). The high content of glycine and proline in IRS-1 and IRS-2 suggests that these sites may merely form collagen-like α-helices and not represent actual nucleotide binding motifs (68).

Rat IRS-1 contains a series of 11 glutamine residues homologous to the polyglutamine tracts found in proteins such as the glucocorticoid receptor (Figure 3), but only 6 of these residues are conserved in human IRS-1, and this region is not present in IRS-2, suggesting that it plays no conserved role in signal transmission (59, 61, 68, 85).

IRS-1 and IRS-2 each contain a single proline-rich motif, located in different regions of the two proteins. Proline-rich motifs bind to Src homology-3 (SH3) domains in various signaling molecules (86, 87). In IRS-1, the PQPPPPPP-PHQPL motif (human isoform) is located in the COOH-terminus between two tyrosine phosphorylation site. In contrast, this motif is not conserved in IRS-2; however, a proline-rich motif (PLPPGSHPHLPHPLHHP) is located just after the IH-2^{PTB} domain. The sequences of these domains are different, and the distinct locations may provide signaling specificity.

IRS PROTEINS ARE COMMON SIGNALING ELEMENTS IN DISTINCT RECEPTOR SYSTEMS

IRS-1 Is Engaged by a Subset of Cell Surface Receptors

Although IRS proteins are prominent insulin receptor substrates, other cytokine receptors also mediate their tyrosine phosphorylation. Several cytokines that bear little relationship to insulin/IGF-1 at any level engage IRS proteins. These include the growth hormone (GH) receptor (88–90), several interleukin receptors (IL-4, IL-9, and IL-13), leukemia inhibitory factor (LIF), and interferon (IFN) receptors (88, 91). Thus, IRS proteins appear to be more general mediators of cellular signaling than we originally thought. This is not to say that they are entirely without specificity, as most receptors (those for PDGF and EGF, for instance) do not engage them.

NPXY-motifs in the β-Subunit of the Insulin Receptor Provide Specific Recognition for Substrates

Compelling evidence for a role of substrate phosphorylation during insulin signaling was obtained by the use of mutant insulin receptors. Although mutations in the regulatory loop or the ATP-binding domain have the expected inhibitory effect on autophosphorylation and substrate phosphorylation, mutations in the intracellular juxtamembrane region (outside the catalytic domain) also inhibit biological response during insulin stimulation (92). The juxtamembrane region is not essential for catalytic activity, as substitution of Tyr_{960} with other amino acids or deletion of 12 amino acids around this residue has no effect on insulin-stimulated autophosphorylation of the regulatory loop in vivo and in vitro. However, these mutants poorly mediate tyrosyl phosphorylation of IRS-1/IRS-2 (pp185) and Shc (92–94). These observations provided one of the early clues that insulin receptor autophosphorylation was not sufficient for signaling and that substrate phosphorylation would play an important role.

For various technical reasons, it was difficult to prove that Tyr_{960} is a phosphorylation site; however, recent experiments indicate that it is one of the first and possibly most significant tyrosine phosphorylation events that occurs on the receptor in the intact cell (11). Thus, Tyr_{960} must be an important mediator of IRS protein/insulin receptor recognition (92). The yeast two-hybrid system has also been used to analyze the insulin receptor–IRS-1 interaction; this interaction requires the presence of phosphotyrosine and Y_{960} on the insulin receptor, suggesting that an element on IRS-1 binds the insulin receptor's juxtamembrane $NPXY_{960}$ motif when phosphorylated (95). This element on IRS-1 localizes to amino acids 160–546, which contains the IH-2^{PTB} region and a second region called the Shc and IRS interaction (SAIN) domain, which was aligned with minimal similarity to the Shc PTB domain (71). The SAIN domain, however, was identified by assuming that a Phe-Leu-Val-Arg (FVLR) motif (also found in the Shc PTB domain) was required for function. Furthermore, the similarity between the IRS-1 SAIN and the corresponding region in IRS-2 is very low, and this region does not mediate IGF-1 receptor–IRS-1 interactions in the yeast two-hybrid system (96).

The IH-2^{PTB} Domain Links IRS Proteins to Activated Receptors

Analysis of IRS-1 by deletional mutagenesis identified two regions in the NH_2-terminus of IRS-1 that are required for insulin receptor–mediated tyrosyl phosphorylation of IRS-1. The first of these deletions contains the IH-1^{PH} domain; the second deletion immediately downstream includes the IH-2^{PTB} domain and sequences immediately COOH-terminal to the IH-2 region (68, 97). These regions likely perform different functions in insulin receptor–IRS-1 interactions. Similarity between the IH-2^{PTB} domain of IRS proteins and the

PTB domain in Shc suggests a common mechanism by the insulin receptor engages these substrates (68, 71, 73, 95, 98–100) (Figure 5*B*).

The IH-2^{PTB} regions of IRS-1 and IRS-2 are 75% identical and may function in mediating insulin receptor–IRS protein interaction by binding to the phosphorylated NPXY motif in the juxtamembrane region of the insulin receptor (68). Indeed, the IH-2^{PTB} region [expressed as a glutathione-*S*-transferase (GST) fusion protein] binds peptides containing insulin receptor sequences if they include the tyrosyl phosphorylated NPXY$_{960}$ motif (K Makatai, L Yenush & MF White, unpublished data). The requirement for IRS-1 phosphorylation of an intact NPXY motif in the IL-4 receptor suggests that many receptors that engage IRS-1 employ a similar NPXY-PTB interaction as part of the recognition signal (76).

Although the PDGFr contains a tyrosine phosphorylation site in an NPXY motif that recruits Shc via its PTB, it does not recruit IRS-1. The tighter receptor specificity of the IRS-1 compared to Shc may be due to greater selectivity of the IH-2^{PTB} domain relative to the PTB domain of Shc. The IH-2^{PTB} domain in IRS-1 recognizes a PLXXXXNPXYXSXSD sequence conserved in the insulin/IGF-1 and IL-4 receptors, whereas the Shc PTB domain requires only NPXY(Pi) motif (76). Although a common mechanism appears to be involved, the insulin receptor phosphorylates Shc and IRS-1 with dramatically different kinetics. Although the affinity of the IH-2^{PTB} domain is 10- to 20-fold lower than that of the Shc PTB domain, IRS-1 reaches maximal phosphorylation immediately after insulin stimulation, whereas Shc requires 5–10 min (78). Hence, other components likely play an important role to mediate a rapid receptor–IRS protein recognition.

Role of the IH-1^{PH} Domain in IRS Protein Phosphorylation

Deletion analysis of IRS-1 demonstrates that the IH-1^{PH} domain plays a role in the mediation of insulin receptor–IRS protein interactions (97). Thus, the IH-1^{PH} and downstream sequences, including the IH-2^{PTB}, appear to cooperate in mediating the insulin receptor–IRS-1 interaction in vivo. The mechanism for recognition between the IH-1^{PH} and the insulin receptor is unknown. The entire domain is polarized, and the structure of several unrelated PH domains suggests that one end of the structure is open and may represent a site of interaction with other molecules (101). Loops between the elements of conserved structure may contribute to the binding specificity of different PH domains. Indeed, the function of the PH domains is quite sensitive to even small changes; a single missense mutation in the PH domain of the Bruton tyrosine kinase (Btk) disrupts Btk function and causes X-linked immunodeficiency in mice (102, 103) (Figure 5). The IH-1^{PH} domains of IRS-1 and IRS-2 are 62% identical (68); this level of identity suggests a unique conserved function (Figure 5).

Although the mechanism by which the IH-1^{PH} domain functions in insulin receptor–IRS protein recognition is unclear, several intriguing possibilities exist. One hypothesis that fits with most data is that the PH domain binds phospholipids, which may target IRS proteins to the plasma membrane (104, 105). Indeed, a fraction of IRS-1 associates with intracellular membranes, even in the absence of insulin stimulation (106) and (B Cheatham, unpublished observations); therefore, some phosphotyrosine-independent motif on IRS-1 must mediate membrane association (directly or indirectly). Thus, the IH-1^{PH} domain could constrain IRS protein to the membrane, providing a kinetic advantage in recognition of IRS-1 by the insulin receptor compared to Shc.

PH domains are also thought to function in the recognition of β- and γ-subunits of heterotrimeric G proteins (Gβ and Gγ) (107). Several PH domains, including the IH-1^{PH} expressed as bacterial fusion proteins bind Gβ and Gγ in vitro (107). Furthermore, overexpression of these PH domains in COS cells interferes with Gβ- and Gγ-mediated signaling (108, 109). Interestingly, the PH domains may coordinately bind Gβ, Gγ, and phospholipids: The COOH-terminus of PH domains appears to mediate Gβ-Gγ interactions and the NH_2-terminus may bind phospholipids (110). Indeed, specific phospholipids enhance the ability of PH domains to activate the β-adrenergic receptor kinase (105). Thus, as well as mediating lipid binding, PH domains may mediate cross-talk between the tyrosine kinase and G-protein signaling systems.

The role of the IH-1^{PH} and IH-2^{PTB} domains remains to be elucidated in noninsulin systems, such as IL-4 and GH. Given the importance of the NPXY motif within the IL-4 receptor (α chain) (76), the mechanism by which this receptor interacts with and phosphorylates IRS-1 is probably similar to that used by the insulin receptor. However, other interleukin receptors and interferon receptors and the growth hormone receptor may differ in interesting ways, as these molecules and their known accessory molecules contain no NPXY motif (88).

SH2 DOMAINS COUPLE IRS PROTEINS TO MULTIPLE SIGNALING PATHWAYS

SH2 Domains

Several years ago it became clear that a major link between tyrosine phosphorylation and downstream signaling is mediated by SH2 domains, which bind to phosphotyrosine in the context of certain amino acid motifs (41). At the core of the SH2 domain lies a FLVR(D/E) sequence. Crystallographic data demonstrate that the conserved Arg at the base of the SH2 domain binding pocket makes important contacts with the phosphate group on the phosphotyrosine residue (111–114). Crystallographic data also demonstrate a second

binding region that varies among SH2 domains; the environment provided by these pockets or grooves appears to define the binding specificity for sequences near of the phosphotyrosine residue. Analysis of the *abl* SH2 domain in vitro demonstrates that SH2 domains bind phosphotyrosine residues alone with low affinity but require sequences surrounding the phosphotyrosine residue for high affinity interactions (41, 115).

In vivo, certain tyrosine phosphorylation sites on the PDGFr bind specific SH2 proteins (116, 117). The sequence requirements for phosphopeptide association with several SH2 domains were determined by binding a biased random peptide library [peptides containing the sequences GDY(P)XXX] to the immobilized SH2 domains from these proteins and directly sequencing the bound peptides (118). While this study determined the preferences of SH2 domains for certain amino acid motifs, it also demonstrated that there is flexibility built into this preference. The specificity of motif selection by SH2 domains can be altered with single amino acid alterations (119). Some SH2 domains may bind more than one motif, and conversely, one motif can bind multiple SH2 domain isoforms: In vivo, the SH2 domains of p85 recognize motifs other than the canonical YMXM motif (120), and the Y_{751}MXM motif in the PDGFr binds Nck as well as p85 (121). Furthermore, single motifs in the EGF and hepatocyte growth factor (hGF) receptors bind multiple SH2 proteins (122, 123). Most SH2 domains prefer phosphotyrosine residues with some form of hydrophobic downstream motif, which fits well with the substrate specificity of the insulin receptor and other tyrosine kinases for tyrosine residues followed immediately by hydrophobic residues (118).

IRS Proteins as Multisite SH2-Docking Proteins

IRS proteins interact with many SH2 proteins with diverse phosphotyrosine motif requirements (59, 61). Shortly after the cloning of IRS-1, PI 3′-kinase was shown to bind tyrosine phosphorylated IRS-1 (59, 124, 125); recently PI 3′-kinase was found to bind IRS-2 as well (68). PI 3′-kinase is a dimeric enzyme composed of a catalytic subunit (p110α, p110β) and regulatory subunit (p85α, p85β, $p55^{PIK}$, see below) containing two SH2 domains (126, 127). Several other IRS-binding SH2 proteins have been discovered, including GRB-2 (an upstream regulator of $p21^{ras}$), nck (a small SH2-SH3 domain–containing adapter protein of unknown function), SHPTP2 (PTP1D, Syp) (an SH2 domain–containing tyrosine phosphatase), and fyn (a Src-family tyrosine kinase) (85; XJ Sun & MF White, unpublished data). Novel proteins with SH2 domains that bind IRS proteins are likely to exist. Preliminary data suggest that IRS-1 and IRS-2 bind some common SH2 proteins (68); however, IRS-1 and IRS-2 display different affinities for a variety of the SH2 protein (XJ Sun & MF White, in preparation), perhaps reflecting slight variations in the sequences surrounding their tyrosine phosphorylation sites.

Phosphatidylinositol 3′-Kinase

BINDING AND ACTIVATION OF PHOSPHATIDYLINOSITOL 3′-KINASE PI 3′-kinase plays a central role in a broad range of biological effects (126). PI 3′-kinase is composed of a catalytic subunit and a regulatory subunit. The first examples of these proteins were p110α and p110β (catalytic subunits) and p85α and p85β (regulatory subunits) (128–130). The p85 regulatory subunits have no discernible catalytic activity but possess two SH2 domains, an SH3 domain, and a BCR-like region (126) (Figure 6). The SH2 domains of p85 bind to phosphotyrosine residues in the context of the motif YXXM or YMXM (125, 131). Interestingly, six of the potential tyrosine phosphorylation sites in IRS-1 lie in the amino acid motif YMXM, and a further three lie in YXXM motifs (Figure 2) (59). Furthermore, eight sites on IRS-2 fall into YXXM motifs (68). Several sites of tyrosine phosphorylation on IRS-1 have been identified by peptide microsequencing: These include five in YXXM motifs (Tyr_{460}, Tyr_{608}, Tyr_{628}, Tyr_{939}, and Tyr_{987}) and three others (Tyr_{895}, Tyr_{1172}, and Tyr_{1222}) (77). Thus, IRS proteins have extensive potential to bind PI 3′-kinase.

A region between the two SH2 domains of p85 binds to the NH_2-terminus of p110, mediating the constitutive association of the two subunits (132, 133). Binding of p85 to p110 partially activates p110 (135). These well-known isoforms of p85 (α and β) are highly homologous and appear to contain similar structural and functional domains, but there is evidence for functional differences between the p85 isoforms (136, 137).

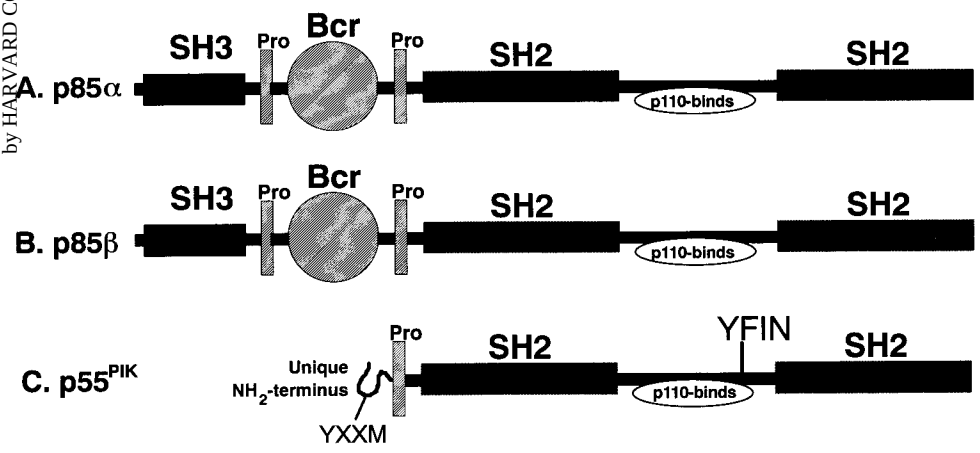


Figure 6 Structural motifs of PI 3′-kinase regulatory subunit isoforms. The three known isoforms of p85 (p85α, p85β, and $p55^{PIK}$) are shown. All three contain two similar SH2 domains flanking the p110 catalytic subunit binding site. In their NH_2-termini, p85α and -β contain an additional SH3 domain, Bcr/GAP-like domain, and two proline-rich motifs. $p55^{PIK}$ has a shortened NH_2-terminus containing only a single proline-rich motif.

More diverse isoforms of p85 also exist (Figure 6): By screening expression libraries with tyrosine phosphorylated IRS-1, we recently identified a novel 55-kDa PI 3′-kinase regulatory subunit (p55^{PIK}) (138). Like p85 isoforms, p55^{PIK} contains two SH2 domains that recognize phosphotyrosine in YMXM and YXXM motifs and possesses an inter-SH2 region with a p110 interaction motif identical to that found in p85α and -β; thus it interacts with p110 and mediates binding to IRS proteins (138). Unlike the p85α and -β, however, p55^{PIK} lacks an NH_2-terminal SH3 domain and BCR-like region; instead, it has a unique 30–amino acid NH_2-terminus and a proline-rich motif (138). p55^{PIK} is also tyrosine phosphorylated during insulin stimulation of cells.

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. Activated PI 3′-kinase also colocalizes with a 60-kDa phosphotyrosine-containing protein (pp60) that immunoprecipitates with αp85 antibodies (48, 139): Although pp60 is poorly characterized, it is possible that pp60 and p55^{PIK} are the same protein.

IRS PROTEINS BIND AND REGULATE PI 3′-KINASE DURING INSULIN SIGNALING

Many growth factor receptors, such as the PDGFr, bind PI 3′-kinase directly via phosphorylated YMXM motifs intrinsic to the receptor, but the insulin receptor and various cytokine receptors employ IRS-1 (140). While the insulin receptor has been shown to associate with PI 3′-kinase during insulin stimulation, several lines of evidence argue that this association occurs indirectly, via IRS-1, which binds PI 3′-kinase directly and associates weakly with the insulin receptor. Overexpression of IRS-1 in CHO cells increases the amount of PI 3′-kinase bound to the insulin receptor (141); no PI 3′-kinase associates with the insulin receptor in cells that lack IRS proteins (140). Although the insulin receptor binds PI 3′-kinase in vitro, this is at most a minor pathway in the intact cell (140, 142).

Insulin stimulates PI 3′-kinase activity through tyrosine phosphorylation of IRS-1 rather than through direct tyrosine phosphorylation of p85 (124). PI 3′-kinase, immunoprecipitated from quiescent cells, is activated in vitro during association with recombinant IRS-1 that has been tyrosine phosphorylated by the insulin receptor (124). The activation is completely inhibited by blocking the phosphotyrosine residues in IRS-1 with excess bacterial fusion proteins containing the SH2 domains of p85α (125). Synthetic peptides containing a single phosphorylated YMXM motif based on IRS-1 sequences activate PI 3′-kinase (124). However, longer peptides containing two phosphorylated YMXM motifs are more potent activators and closely mimic the ability of recombinant IRS-1 to activate the kinase in vitro (143). These results suggest that both SH2 domains in p85 must be occupied in order to fully activate the

kinase, a function well performed by IRS-1, which contains numerous YXXM motifs (59, 77).

PI 3′-KINASE AND p21^{ras} Recent reports suggest that PI 3′-kinase and p21^{ras} modulate each other's signals. Overexpression of activated p21^{ras} in PC12 cells increases PI 3′-kinase activity and stimulates the accumulation of 3′-phosphorylated inositol lipids in the cells (144). GTP-bound p21^{ras} also directly binds and activates PI 3′-kinase in vitro (144). Expression of activated PI 3′-kinase in NIH 3T3 cells apparently potentiates p21^{ras}-dependent signaling events (145). Such cross-talk between p21^{ras} and PI 3′-kinase may be cell-type specific, however; in many systems, PI 3′-kinase–dependent events (such as activation of p70^{s6k} and glucose transport) do not require p21^{ras} activation and are not blocked by dominant negative mutants of p21^{ras} (140, 146–150).

PI 3′-KINASE CATALYTIC SUBUNITS The first recognized form of the PI 3′-kinase enzymatic subunit was p110, which associates with p85α and -β or p55^{PIK} (127). Since the original cloning of p110α, several other p110 isoforms have subsequently been cloned, including p110β and most recently p110γ. While the p110α- and -β-isoforms are relatively similar and contain similar structural motifs, p110γ differs by the addition of a PH domain at its NH_2-terminus (151). Since PH domains are thought to bind lipid and Gβ and Gγ, the p110γ PH domain could provide a mechanism to mediate PI 3′-kinase membrane targeting and activation by heterotrimeric G proteins. A PI 3′-kinase activity that is regulated by β and γ subunits was originally identified in myeloid cells (152).

The lipid kinase domain of the p110 catalytic subunit of PI 3′-kinase contains similarity to a variety of proteins, now known to be PI 3′-kinases, which regulate signaling and vesicle transport and sorting (153). VPS34, a protein involved in protein-vesicle trafficking in *Saccharomyces cerevisiae* was the first of these p110 homologues demonstrated to have a PI 3′-kinase activity (129, 154, 155). The human form of VPS34 has recently been cloned (156). Other family members include the TOR (target of rapamycin) group (157); these yeast proteins bind to FKBP12 in complex with rapamycin, which blocks cell-cycle progression and a variety of biochemical signaling events in the cell. A mammalian homologue (mTOR, FRAP, RAFT1, RAPT1) of the yeast TORs has been cloned by several labs (158–161). The recently cloned ataxia telangiectasia disease gene is a member of a third group of PI 3′-kinases, including the *ESR1* gene important for DNA repair and recombination in yeast (153); outside of the lipid kinase domain, this group of proteins also contains similarity to rad3, a DNA damage repair monitor at the G2-M checkpoint in *Schizosaccharomyces pombe*. p110α and -β are most similar to the VPS34 vesicular trafficking family of PI 3′-kinases, suggesting that the signal transmitted by the p85 and p110 PI 3′-kinase could function by targeting an acti-

vated signaling complex to important locations within the cell. Indeed, PI 3′-kinase binding is required for correct targeting of the internalized PDGFr during PDGF stimulation (162, 163).

PI 3′-kinase phosphorylates the lipid PI on the 3 position of the D-myoinositol ring, yielding phosphatidylinositol 3-phosphate (PI-3-P) (164). Because the enzyme can use alternatively phosphorylated forms of phosphatidylinositol (PI-4-P, PI-4,5-P_2) as substrates, activation of the enzyme also leads to the formation of PI-3,4-P_2 and PI-3,4,5-P_3. How the PI 3′-kinase products generate a signal is not known, especially as they are present in minute quantities in comparison to other inositol phospholipids in the cell. These lipids may directly activate downstream signaling molecules; for instance, PI-3,4,5-P_3 has been shown to activate PKC isoforms in vitro (165, 166). Furthermore, PI 3′-kinase binding sites on the PDGFr are required for the activation of Akt, a PH domain-containing serine-threonine kinase (167). Akt is activated by incubation with PI-3-P in vitro, suggesting that this kinase may mediate signaling downstream of PI 3′-kinase. Its activation requires the integrity of its PH domain, suggesting that PI-3-P binding to the lipid-binding element of its PH domain mediates enzymatic activation of Akt. Another possibility is that the lipid kinase activity of p110 is not the important mediator of signaling but that a serine kinase activity (mediated by the same kinase domain as the lipid kinase) of p110 is (168, 169). This p110 serine kinase is known to phosphorylate at least two important signaling mediators—p85 and IRS-1 (83, 169); p110 likely serine phosphorylates other molecules as well.

PI 3′-KINASE MEDIATES INSULIN-STIMULATED $p70^{s6k}$ Although several signaling events may converge to mediate the activation $p70^{s6k}$ during insulin stimulation, PI 3′-kinase appears to be a requisite player (170). Similar conclusions were reached for PDGF stimulation, since inhibitors of PI 3′-kinase or mutations in the receptor that diminish PI 3′-kinase binding inhibit activation of $p70^{s6k}$ (147, 150). Concordant with the requirement for IRS proteins in the activation of PI 3′-kinase by insulin, IRS proteins are required for the activation of $p70^{s6k}$ during insulin stimulation (Figure 7).

In order to directly address the importance of tyrosine phosphorylation of IRS-1 and the role of PI 3′-kinase binding to IRS-1 in insulin signaling, we constructed a mutant IRS-1 in which all functional tyrosine phosphorylation sites were replaced by phenylalanine (IRS-1^{F18}). We further generated two derivatives of this mutant containing three tyrosine phosphorylation sites in PI 3′-kinase binding YMXM motifs (Y_{608}, Y_{628}, Y_{658}) (IRS-$1^{Y608-658}$) or the two tyrosine phosphorylation sites predicted to bind SHPTP2 (IRS-$1^{Y1172, 1222}$) (MG Myers Jr, & MF White, unpublished data). IRS-1^{F18} fails to undergo tyrosyl phosphorylation; some tyrosyl phosphorylation is recovered in IRS-$1^{Y608-658}$ and IRS-$1^{Y1172, 1222}$. IRS-1^{F18} and IRS-$1^{Y1172, 1222}$ fail to bind PI 3′-kinase or

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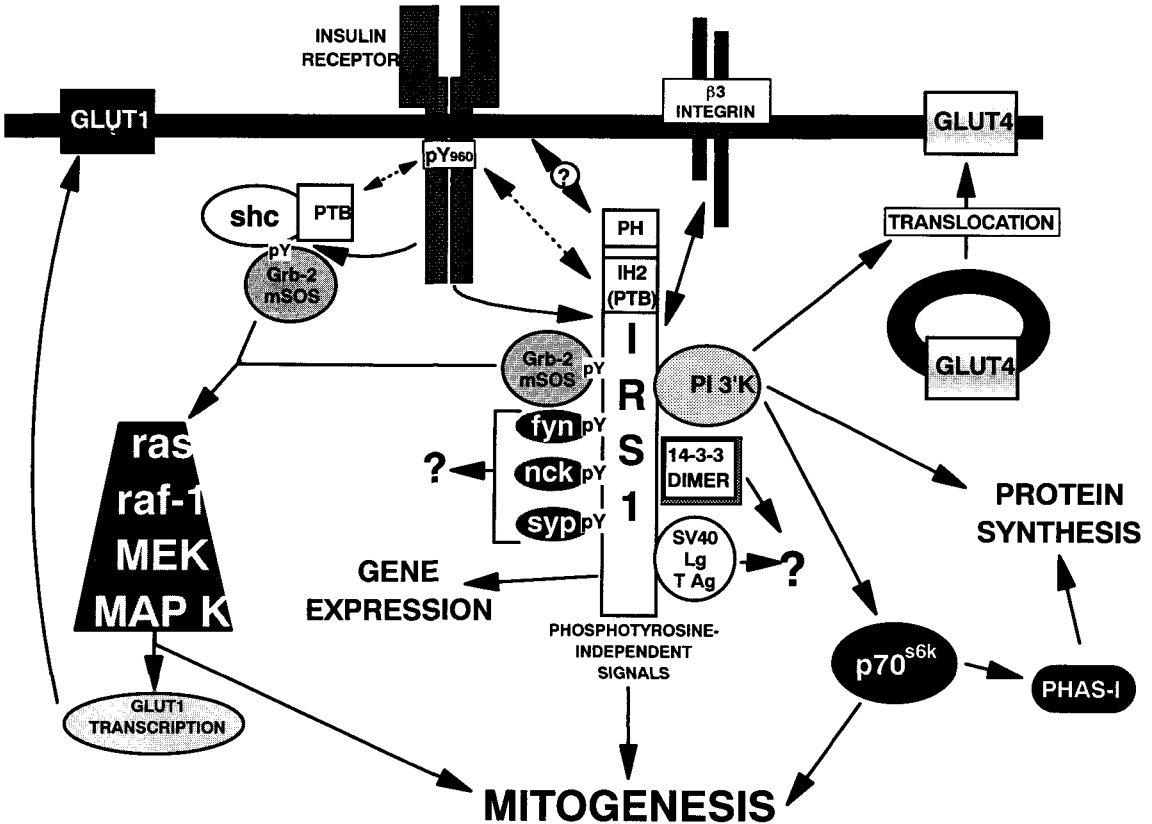

activate $p70^{s6k}$; IRS-$1^{Y608-658}$ binds PI 3′-kinase and mediates $p70^{s6k}$ activation. Thus, PI 3′-kinase binding to IRS-1 is a crucial mediator of $p70^{s6k}$ activation during insulin stimulation (Figure 7).

MITOGENIC SIGNALING BY PI 3′-KINASE PI 3′-kinase is an important element in growth factor– and cytokine-regulated mitogenesis (85, 126). PDGFr's containing mutations at the five major tyrosine autophosphorylation–SH2 domain binding sites are deficient in mediating cellular proliferation in response to PDGF stimulation (although the tyrosine kinase activity of these receptors is similar to wild-type PDGFr) (131, 154, 171). The mitogenic activity is rescued by reversing the mutations at the p85 binding sites. Moreover, transformation defective mutants of polyoma middle T, *v-src*, *v-abl*, and *v-fms* lack the ability to bind PI 3′-kinase (126). Experiments using PI 3′-kinase inhibitors and mutants of the PDGFr suggest that as well as regulating cell proliferation and activation of Akt and $p70^{s6k}$, PI 3′-kinase may act upstream of chemotaxis, receptor internalization, vesicle sorting, and glucose transport, among other things (85, 154). The apparent involvement of PI 3′-kinase in so many disparate signaling pathways suggests that it may provide a more general, facilitative, signaling function, such as targeting an active complex, rather than directly controlling these myriad events. Alternately, the effects of PI 3′-kinase signaling may be cell type specific, as the effects of IRS-1 appear to be.

GRB-2, an SH2 Domain–Containing Adapter Protein

Growth factor receptor bound (GRB)-2 regulates Son of Sevenless (Sos), a $p21^{ras}$ guanine nucleotide exchange factor (172–174); $p21^{ras}$ is a proto-oncogene and a small GTP binding protein that is active when bound to GTP and inactive when GDP bound. 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 (175): 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 (Figure 7) (175). Thus, engagement

Figure 7 A model of the role of IRS-1 in insulin-regulated signaling. Insulin binding activates the insulin receptor tyrosine kinase, which recognizes and phosphorylates IRS proteins and Shc. The recognition of IRS-1 is mediated by IH-2^{PTB}–pY_{960} interaction and some PH domain–mediated component. Recognition of Shc depends only upon the PTB-pY_{960} interaction. IRS proteins mediate signals controlling mitogenesis, gene expression, protein synthesis, and glucose transport by binding signaling proteins. Some of these proteins contain SH2 domains and bind tyrosine phosphorylation sites (pY) on IRS proteins; these SH2 proteins include SHPTP2 (syp), nck, and fyn, as well as Grb-2 (which mediates ras signaling) and PI 3′-kinase (PI 3′K) (which controls a variety of signaling events). IRS proteins also associate with 14-3-3 proteins, SV40 large T antigen (Lg T Ag) and β_3 integrin. IRS proteins mediate some as yet undefined phosphotyrosine-independent signals.

of GRB-2–Sos by tyrosyl phosphorylated signaling proteins is expected to activate $p21^{ras}$ and the downstream MAP kinase cascade (176).

Our work with single Tyr → Phe point mutants 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 (78). 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, although IRS proteins enhance the insulin-stimulated activation of MAP kinase, they are not required for phosphorylation of Shc or for activation of $p21^{ras}$ and MAP kinase (Figure 7). 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 (52).

SHPTP2, an SH2 Domain–Containing Tyrosine Phosphatase

SHPTP2 is an SH2 domain–containing tyrosine phosphatase that associates with the COOH-terminal tyrosine phosphorylation sites of IRS-1 and with the tyrosine phosphorylation sites in some growth factor receptors, such as the PDGFr (85, 177, 178). Synthetic peptides containing the tyrosine phosphorylated motif thought to mediate PDGFr-SHPTP2 or the IRS-1–SHPTP2 association increase the phosphatase activity of SHPTP2 in vitro (79, 179). Thus, SHPTP2 is probably activated during association with IRS-1. While the effect of IRS-1–SHPTP2 interaction on signal transmission is unknown, many intriguing possibilities exist: SHPTP2 may dephosphorylate signaling intermediates located either in the IRS-1 signaling complex or at distant sites, thus downregulating signaling. Also, SHPTP2 is a homologue of the *Drosophila melanogaster* tyrosine phosphatase *corkscrew* (180), which is implicated in the control of $p21^{ras}$ and MAP kinase signaling during activation of the *torso* pathway in fly development.

The role of SHPTP2 in insulin signaling has been examined by several approaches: Microinjection of the SH2 domains of SHPTP2 or antibodies against SHPTP2 block insulin-stimulated DNA synthesis (181, 182). Similarly, overexpression of a catalytically inactive mutant SHPTP2 molecule inhibits mitogenesis and $p21^{ras}$ and MAP kinase activation (183–185). It is not clear how SHPTP2 transmits signals to $p21^{ras}$ and MAP kinase or mediates mitogenesis during insulin signaling. Furthermore, it is not apparent whether IRS proteins are responsible for engaging SHPTP2 to mediate these signals: MAP kinase is activated in the absence of IRS proteins (78, 186–188), and our mutagenic analysis demonstrates that SHPTP2 binding to IRS-1 is not required for mitogenic signaling by insulin (MG Myers, P Shi, & MF White, unpub-

lished data). The insulin receptor may directly engage SHPTP2 (189), or an unknown pathway may exist between the insulin receptor and SHPTP2. The insulin signaling system may also engage SHPTP1, a related tyrosine phosphatase, as insulin stimulates the tyrosine phosphorylation of this protein under some conditions (190).

IRS PROTEINS BIND NON-SH2 SIGNALING ELEMENTS

SV40 Large T Antigen

As well as associating with SH2-proteins during insulin signaling, IRS proteins bind other cellular signaling elements (Figure 7). Large T antigen (Lg T Ag), the transforming protein of the SV40 tumor virus, binds to IRS-1 in an apparently phosphotyrosine-independent manner; this interaction is at least in part mediated by the 250–amino acid COOH-terminal of Lg T Ag (191). This association appears to mediate the transformation of cells by Lg T Ag and suggests that a cellular protein may exist that functions homologously to Lg T Ag in this manner (191).

14-3-3 Proteins

During expression screening with IRS-1 protein, several 14-3-3 isoforms bind to IRS-1 independently of tyrosine phosphorylation (T Asano & MF White, unpublished data) (Figure 7). 14-3-3 proteins are small 25- to 30-kDa intracellular proteins found in a variety of species from yeast to mammals. The function of 14-3-3 proteins in the cell is poorly understood, but many cellular functions have been ascribed to them, including the regulation of tryptophan hydroxylases, cdc25 phosphatase, PKC, PLA_2, and raf-1 kinase and the activation of MAP kinase-kinase (192–203). Furthermore, disruption of a *Saccharomyces cerevisiae* 14-3-3 homologue (BMH1) had no detectable effect on cell physiology, perhaps due to the presence of redundant homologues: Disruption of both *rad24* and *rad25* (14-3-3 homologues) from *Schizosaccharomyces pombe* is lethal (204), and disruption of either of these genes alone interferes with the DNA damage checkpoint in mitosis (204). How different 14-3-3 isoforms influence cellular processes in mammalian cells and the consequences of 14-3-3–IRS-1 interactions is unclear.

The crystal structures of 14-3-3 τ and ζ have recently been solved. The 14-3-3 protein consists of nine antiparallel α-helices that form two large domains (205, 206). In the crystal, 14-3-3 forms a dimer in which the NH_2-terminal domains of the two monomers interact to form a channel; the COOH-terminal domains form surrounding structures. Interestingly, the residues lining the channel are highly conserved among 14-3-3 proteins and consist of largely acidic residues. IRS-1 binds to the more variable COOH-terminal region of 14-3-3 proteins.

β3 *integrins*

IRS-1 also binds complexes containing β3 integrin during insulin stimulation (207), suggesting that IRS-1 binds integrins or associated proteins in the focal adhesion. Integrins are important mediators of cellular morphology, migration, proliferation, and apoptosis, as well as physical (cell-cell and cell-matrix) interaction (208). How the presence of IRS-1 in integrin-containing protein complexes may effect insulin or integrin signaling is unknown, but it may act to bring important signaling molecules into the adhesion complex. The interaction between IRS proteins and integrins may be indirect.

BIOLOGICAL FUNCTIONS OF THE IRS PROTEINS

IRS-1 Knockout Mice

Disruption of the IRS-1 gene in mice (IRS-1$^{-/-}$) has provided new insight into the physiologic role of IRS-1 in an intact organism (209, 210). While these animals survive and breed, they demonstrate dramatically altered physiology. These mice display hyperinsulinemia and have impaired glucose tolerance compared to controls. Thus, IRS-1 is an important mediator of insulin signaling and glucose homeostasis. Furthermore, insulin-stimulated glucose uptake is reduced by 50% in tissues from these mice, suggesting that IRS-1 is an important mediator of insulin-stimulated glucose transport. While it was at first surprising that IRS-1$^{-/-}$ mice survive and were not frankly diabetic, the subsequent discovery of IRS-2 suggests an explanation—that the ubiquitous expression of this IRS-1 homologue can partially substitute for the absent IRS-1 (68, 209).

IRS-1$^{-/-}$ mice are 50% smaller than wild-type and heterozygous littermates beginning early in development, suggesting an important role for IRS-1 in development (68, 209). This reduction in size may be due to increased apoptosis during fetal development, suggesting that IRS-1 is an important controller of this important process as well. These mice may have other defects that have yet to be discovered; the generation of IRS-2$^{-/-}$ mice and their interbreeding with IRS-1$^{-/-}$ mice will answer many more questions about the role of these important proteins in insulin signaling and organismal physiology.

IRS-1 Mediates the Proliferative Response to Insulin

The role of IRS protein for cell growth and mitogenesis has been investigated in several systems. The overexpression of IRS-1 in CHO cells (CHO-IRS) and in CHO cells overexpressing the insulin receptor (CHO^{IR}-IRS) causes an increased insulin response (59, 60). Following insulin stimulation, the amount of tyrosine phosphorylated IRS-1 is increased in these cells compared to controls, as is the amount of PI 3′-kinase activity associated with IRS-1 (59, 60). Interestingly, while insulin stimulates thymidine incorporation in CHO-

IRS cells more effectively than in CHO parental cells, the maximal DNA synthesis response to insulin in CHO^{IR}-IRS cells is blunted compared to the parental CHO-IR cells, with higher levels of IRS-1 expression correlating with increased inhibition (60). These data provided the first evidence that IRS-1 plays a role in the proliferative response to insulin (Figure 7). Interestingly, CHO cells transiently transfected with IRS-1 display a similar effect on transcription: Insulin-stimulated transcription mediated by the Fos SRE or collagen AP-1 element increases with IRS-1 expression in CHO but decreases in CHO^{IR} cells (211). Thus, IRS-1 controls gene expression as well as cell proliferation (Figure 7). The mechanism by which IRS-1 enhances these insulin effects with a low level of endogenous insulin receptors and blunts the response in the context of overexpressed human insulin receptor remains unclear but may involve the engagement of negative signals by a highly phosphorylated IRS-1 molecule.

The positive role for IRS proteins in mitogenic signaling is further supported by data derived from cells in which IRS-1 signaling is blocked: Microinjection of αIRS-1 antibodies into 3T3 cells overexpressing the insulin receptor abrogates insulin-stimulated mitogenic signaling (212). Furthermore, expression of antisense IRS-1 in CHO cells overexpressing the insulin receptor inhibits insulin-stimulated mitogenesis (213). Interestingly, neither of these techniques completely abrogated the insulin signal; this likely reflects the continued presence of IRS-2 in these cell lines (68, 209).

The fortuitous absence of IRS-1 and IRS-2 from the 32D myeloid progenitor cell line provides an ideal system for the analysis of IRS-1 in insulin–IL-4 signaling. 32D cells grow in the presence of IL-3, but unlike ordinary myeloid progenitor cells, they are insensitive to insulin because they contain few endogenous receptors and no detectable IRS-1 or IRS-2 (66, 68). Expression of insulin receptors in the absence of IRS-1 has little effect on the mitogenic response. Alone, expression of IRS-1 or IRS-2 has little effect; however, expression of either IRS protein in 32D cells expressing receptors for insulin allows mitogenesis and sustained growth in response to insulin (66, 68). Thus, IRS proteins are indispensable for insulin-stimulated mitogenesis and cell-cycle progression in a variety of cell systems. Interestingly, the mitogenic response in 32D cells appears to have two components—proliferation and protection from apoptosis (L Yenush, MG Myers Jr, & MF White, unpublished data); thus, IRS proteins perform multiple roles in mitogenic signaling.

Mutational Analysis of IRS-1 Function in Mitogenic Signaling

By creating a panel of deletion mutants spanning the full length of IRS-1, we investigated the requirement for large regions of IRS-1 in mediating insulin signaling (MG Myers Jr, & MF White, unpublished data). No deletion mutant

of IRS-1 entirely abrogates mitogenic signaling by insulin, suggesting either that the element(s) in IRS-1 responsible for the propagation of this signal is present in multiple copies or that distinct regions mediate complementary functions. Deletions within the middle 30% of IRS-1 decrease the sensitivity of insulin-stimulated mitogenesis, suggesting that elements within this region of IRS-1 are important mediators of signaling. Conversely, deletion of the IH-1^{PH}, of the IH-2^{PTB}–SAIN region, or within the COOH-terminus of IRS-1 does not detectably alter mitogenic signaling in the 32D cells. This analysis suggests that PI 3′-kinase may be an important mediator of insulin mitogenic signaling, as many YXXM-motifs (which mediate binding of PI 3′-kinase) lie in this middle 30% of the IRS-1 molecule. However, this region may also contain other elements important for signaling.

We also used the IRS-1^{F18} (which is not tyrosine phosphorylated during insulin stimulation) and IRS-1$^{Y608-658}$ (which has only PI 3′-kinase–binding YMXM motif tyrosine phosphorylation sites) to examine mitogenesis. While IRS-1^{F18} confers only a diminished proliferative response to insulin and fails to signal long-term growth, IRS-1$^{Y608-658}$ mediates the full mitogenic response to insulin (albeit with decreased sensitivity than wild-type IRS-1) and mediates long-term growth in insulin (MG Myers Jr, JH Pierce, & MF White, unpublished data). Two conclusions stem from this analysis: First, because a nontyrosine phosphorylated IRS-1 molecule is not devoid of signaling potential, IRS-1 engages some signals in a phosphotyrosine-independent manner (Figure 7). Second, the full mediation of mitogenic signaling is possible from an IRS-1 molecule containing only one type of tyrosine phosphorylation site—the YMXM motif. It is unlikely that the three tyrosine residues which we restored in IRS-1$^{Y608-658}$ mediate signaling differently than any of the other YMXM motifs in IRS-1, since these sites can be mutagenized individually or even deleted without destroying the ability of IRS-1 to signal in 32D cells. Thus, although other signaling proteins may bind to these sites, these data cast PI 3′-kinase as a crucial regulator of insulin mitogenic signaling. Whether the restoration of additional YXXM motifs is required for the fully sensitive insulin response or whether this requires some other motif is unknown.

The nature of the IRS-1–mediated phosphotyrosine-independent signal(s) is at present unclear, as is its role in a normal, fully functional IRS-1 molecule. Because sequences in the middle of IRS-1 mediate signals important for the full mitogenic response, these signals may function independently of IRS-1 tyrosine phosphorylation, although further studies will be required to determine whether this is actually the case.

IRS Proteins and Glucose Transport

The movement of glucose into cells from the circulatory system is largely accomplished by the action of a family of facilitated glucose transporters—the

GLUTs (2, 214). These are proteins containing 12 membrane-spanning domains that passively transport hexoses down their concentration gradient. GLUT1 and GLUT4 are responsible for removing the bulk of glucose from the bloodstream. These molecules differ importantly in their physiologic role. GLUT1 is present in most cells and tissues, is distributed predominantly to the plasma membrane, and is relatively insulin insensitive. It is therefore thought to function predominantly as a "housekeeping" transporter, providing the basal hexose uptake required for all cells. GLUT4 is present exclusively in insulin target tissues (adipose and striated muscle) and resides in an intracellular vesicular compartment in the basal state. During insulin stimulation, a small amount of intracellular GLUT1 translocates to the plasma membrane, but the bulk of insulin-stimulated glucose uptake is mediated by the translocation of the intracellular GLUT4 to the plasma membrane (215).

Two distinct experimental approaches have recently converged on the conclusion that PI 3′-kinase is necessary, although perhaps not sufficient, for insulin-stimulated GLUT4 (and GLUT1) translocation. First, insulin-stimulated glucose uptake is inhibited in fat cells by wortmannin and LY90024, two inhibitors of PI 3′-kinase (150, 216–218). Alone, these results are questionable because it is impossible to know whether other unknown signaling molecules 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 (148). Together, these results suggest that the activation of PI 3′-kinase is necessary for insulin-stimulated glucose uptake. Because IRS proteins are upstream regulators of PI 3′-kinase during insulin stimulation, they likely play a role in insulin-stimulated GLUT4 translocation (59, 124, 140).

This likelihood is supported by the impaired insulin-stimulated glucose transport and disposal exhibited by IRS-1$^{-/-}$ mice (209, 210). These mice display hyperinsulinemia and have impaired glucose tolerance compared to controls. Furthermore, insulin-stimulated glucose uptake is reduced by 50% in tissues from these mice even in the face of persistent IRS-2 expression, suggesting that IRS-1 is an important mediator of insulin-stimulated glucose transport. Thus, IRS-1 is an important mediator of insulin signaling and glucose homeostasis.

Recent data also suggest that the increased glucose uptake characteristic of transformed or rapidly proliferating cells is controlled by a distinct set of signals. Activation of the p21^{ras} → raf-1 → MAP kinase pathway activates the transcription of the GLUT1 gene, thus increasing intracellular levels of GLUT1 and increasing hexose uptake (219). Because control of this serine-threonine kinase cascade occurs independently of IRS-1 (78, 186, 188), regulation of GLUT1 expression levels may be independent of IRS-1 in some cells and tissues. In some systems, activated p21^{ras} mediates the translocation of GLUT4

to the cell membrane, although this appears to represent a pathway parallel to, not engaged by, the insulin signal (220, 221).

Control of Protein Synthesis by Insulin

One cardinal effect of insulin is the stimulation of protein synthesis, partially through increased translation of mRNAs; much of this is controlled at the level of translation initiation (222, 223). Dephosphorylation of eIF-2B increases the supply of methionyl-tRNA, increasing the overall rate of initiation. Additionally, $p70^{s6k}$-mediated increases in phosphorylation of the S6 protein in the 40S ribosomal subunit may selectively increase binding of mRNAs with polypyrimidine tracts to ribosomes (222). Much recent excitement has been generated by our increased understanding of the processes involved in mRNA cap binding: Insulin increases the phosphorylation of PHAS-I, causing it to dissociate from eIF-4E (222, 224, 225). This free eIF-4E mediates increased mRNA cap binding along with the rest of the eIF-4F complex, increasing the translational rate of capped mRNAs.

While the upstream signaling elements controlling these translational events remain for the most part unexplored, MAP kinase is reported to phosphorylate PHAS-I in vitro; thus, MAP kinase may be involved in this regulation, and IRS proteins may not be required (223, 226). Other data suggest that MAP kinase is not the crucial player in PHAS-I phosphorylation in vivo; however, inhibitors of MEK, the MAP kinase activator, do not inhibit PHAS-I phosphorylation, nor does activation of pathways that inhibit MAP kinase (227). Wortmannin and rapamycin (inhibitors of PI 3′-kinase and $p70^{s6k}$, respectively) do block PHAS-I phosphorylation, however, suggesting that the PI 3′-kinase $\rightarrow$ $p70^{s6k}$ pathway regulates protein synthesis by controlling PHAS-I phosphorylation (228).

Our own data from 32D cells show that expression of neither insulin receptor nor IRS proteins alone suffices for insulin-stimulated protein synthesis (measured by methionine incorporation); rather high levels of both insulin receptor and IRS-1 are required (R Mendez, MG Myers Jr, MF White, & RE Rhoades, submitted for publication). Similarly, the presence of IRS-1 is required for eIF-4E phosphorylation. Like PHAS-I phosphorylation, protein synthesis in 32D cells is inhibited by wortmannin; it is not inhibited by rapamycin, however. Thus, while this stimulation of global protein synthesis requires PI 3′-kinase, it does not require $p70^{s6k}$, unlike PHAS-I phosphorylation. Thus, the majority of insulin-stimulated protein synthesis likely does not require PHAS-I phosphorylation, and PHAS-I may regulate transcription of a relatively minor portion of mRNAs in these cells.

Phorbol esters stimulate about 50% of the protein synthesis observed during insulin stimulation of the 32D cells, and can rescue insulin-stimulated protein

synthesis to normal levels during wortmannin inhibition. Thus, PKC also regulates protein synthesis in these cells. While the phorbol ester-dependent rescue of protein synthesis in wortmannin-treated cells could reflect the PKC-dependent enhancement of residual insulin signaling, these data suggest that a PI 3′-kinase–regulated PKC signal may operate in insulin-stimulated protein synthesis.

FUTURE PROSPECTS

Serine Phosphorylation of IRS Proteins

In the absence of insulin, IRS proteins are strongly serine phosphorylated and weakly threonine phosphorylated, and following insulin stimulation there is an increase in serine as well as tyrosine phosphorylation. Casein kinase II, MAP kinase, and PI 3′-kinase are responsible for phosphorylation of some serine residues, although further work is required to fully characterize the serine phosphorylation of IRS proteins (82, 83; MF White, unpublished data). Our own analysis of the nontyrosine phosphorylated IRS-1^{F18} suggests that most insulin-stimulated serine phosphorylation of IRS-1 occurs without engagement of PI 3′-kinase by IRS-1, since IRS-1^{F18} exhibits normal serine phosphorylation (MG Myers Jr, & MF White, unpublished data).

There are 81 conserved serine residues among mammalian IRS-1 and IRS-2 isoforms. The distribution of these sites is nonrandom; they are excluded from the PH domain and clustered in the downstream IH-2 region and just beyond (59, 68). Thus, while PH domain function may not be regulated by serine phosphorylation, the role of the phosphotyrosine binding region of IRS proteins could be regulated by phosphorylation. Indeed, treatment of cells with okadaic acid increases the serine phosphorylation of IRS proteins and interferes with the tyrosine phosphorylation of IRS proteins and insulin signal transmission (230), suggesting that serine phosphorylation decreases the sensitivity of receptor–IRS proteins coupling. Furthermore, TNF-α, which stimulates cellular serine kinases, impairs insulin signaling, potentially through impairment of IRS-1 function (231, 232).

Why an Accessory Signaling Protein? Wherefore IRS-1?

Although the nature of the phosphotyrosine-independent signals is presently unclear, their existence sheds light on the ontological question, Why are there accessory signaling proteins (IRS proteins) in some signaling cascades when so many growth factor receptors rely on intrinsic SH2 binding sites? The transmission of phosphotyrosine-independent signals contributes a dimension to the signals generated by IRS-engaging growth factors that is potentially absent from the signals mediated by receptors that do not utilize IRS proteins, such as the PDGFr.

Of course, other reasons may exist for a growth factor receptor to use an IRS protein instead of engaging SH2 proteins directly. Such a mechanism acts to amplify a signal by allowing a single receptor to create a large number of SH2-protein docking platforms (tyrosine phosphorylated IRS-1 molecules). Furthermore, since multiple receptors share IRS-1, each receptor need not contain the functional units within IRS-1, only units specific to that particular receptor. Thus, the use of redundant genetic material and protein sequence is eliminated.

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