invited review

The insulin receptor: structure, function, and signaling

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Lee, Jongsoon, and Paul F. Pilch. The insulin receptor: structure, function, and signaling. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C319–C334, 1994.—The insulin receptor is a member of the ligand-activated receptor and tyrosine kinase family of transmembrane signaling proteins that collectively are fundamentally important regulators of cell differentiation, growth, and metabolism. The insulin receptor has a number of unique physiological and biochemical properties that distinguish it from other members of this large well-studied receptor family. The main physiological role of the insulin receptor appears to be metabolic regulation, whereas all other receptor tyrosine kinases are engaged in regulating cell growth and/or differentiation. Receptor tyrosine kinases are allosterically regulated by their cognate ligands and function as dimers. In all cases but the insulin receptor (and 2 closely related receptors), these dimers are noncovalent, but insulin receptors are covalently maintained as functional dimers by disulfide bonds. The initial response to the ligand is receptor autophosphorylation for all receptor tyrosine kinases. In most cases, this results in receptor association of effector molecules that have unique recognition domains for phosphotyrosine residues and whose binding to these results in a biological response. For the insulin receptor, this does not occur; rather, it phosphorylates a large substrate protein that, in turn, engages effector molecules. Possible reasons for these differences are discussed in this review. The chemistry of insulin is very well characterized because of possible therapeutic interventions in diabetes using insulin derivatives. This has allowed the synthesis of many insulin derivatives, and we review our recent exploitation of one such derivative to understand the biochemistry of the interaction of this ligand with the receptor and to dissect the complicated steps of ligand-induced insulin receptor autophosphorylation. We note possible future directions in the study of the insulin receptor and its intracellular signaling pathway(s).

signal transduction; tyrosine kinase; diabetes

THE INSULIN RECEPTOR is one of the most studied proteins of the past 10–15 years, and illustrations of its one-dimensional structure (Fig. 1) are routinely included in current textbooks. There are at least two major reasons for this intense scrutiny. First, the interaction of the receptor with insulin initiates the cellular event(s) that regulate organismal glucose homeostasis. Disruptions of glucose utilization are extremely common in humans, leading to diabetes with an occurrence in North America of ~4% of the population. Thus a complete understanding of receptor function may ultimately be of therapeutic use in a common serious disease. Second, the insulin receptor is a receptor/tyrosine kinase, an enzyme family whose members play critical regulatory roles in development, cell division, and metabolism (42, 147, 188). Biochemically, the activation of receptor/tyrosine kinase family members by their cognate ligands results in phosphorylation of themselves (autophosphorylation) and selective protein substrates exclusively on tyrosine residues. This tyrosine phosphorylation mediates the interaction of receptor with effector, the details of which comprise a rapidly expanding area of investigation that will be discussed below. The insulin receptor is an experimental paradigm for receptor/tyrosine kinases that has the further advantage of a long history with regard to information about the structure and function of its cognate ligand, insulin. Also, insulin receptor signaling is unique among the tyrosine kinase family, in that its primary physiological role is in acute and chronic metabolic regulation, whereas other family members regulate cell growth and differentiation as noted above. This review will critically examine our current state of knowledge concerning the insulin receptor, and it will emphasize the areas where further progress is likely to increase our understanding of receptor structure and/or function.

PHYSIOLOGY OF INSULIN ACTION

Insulin is a very well-studied polypeptide hormone (181) produced by the β-cells of the pancreas in response to nutritional stimuli. Once in the blood, insulin controls glucose homeostasis by stimulating the clearance of glucose into skeletal muscle and, to a lesser degree, liver and adipose tissue. In muscle and adipocytes, insulin-stimulated glucose uptake is achieved by the translocation of the insulin-sensitive glucose transporter (GLUT-4) from intracellular storage vesicles to the cell surface (71, 214). This complex process is also under intense experimental scrutiny, but the biochemical connection between receptor and glucose transporter remains undescribed. Glucose homeostasis may also be regulated by alterations in glycogen metabolism in

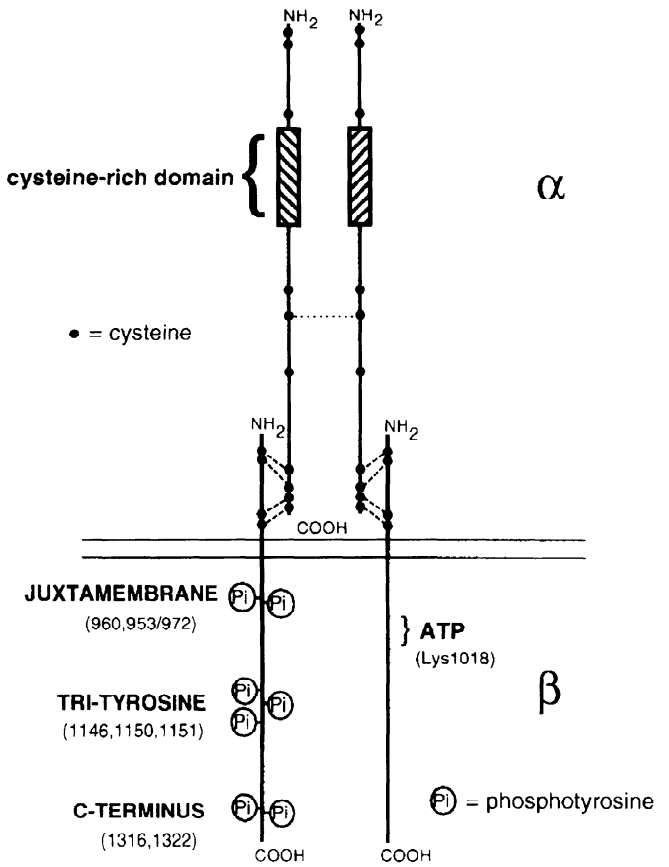

Fig. 1. Structure of the insulin receptor. The insulin receptor is depicted with numbering system of Ullrich et al. (186). The α-subunit is composed of 723 amino acids and has the insulin binding domain. •, cysteine residues; broken lines, disulfide bridges. The β-subunit has 620 amino acids and 3 compartmental domains: extracellular, transmembrane, and cytosolic. Cytosolic domain has 3 clusters of tyrosine residues that can be phosphorylated on insulin binding (represented as Pi in a circle) and exogenous tyrosine kinase activity. Both β-subunits are phosphorylated; phosphotyrosines are only depicted on one-half for convenience.

muscle and in liver and by decreased gluconeogenesis in the liver. The enzymes involved in the regulation of glucose metabolism by insulin appear to be regulated by phosphorylation and dephosphorylation on serine and/or threonine residues (for review see Refs. 64, 90). A critical and as yet incompletely resolved issue is the mechanism by which the tyrosine-specific kinase activity of the insulin receptor can activate serine- and/or threonine-specific phosphorylation. Some general progress is being made in this area, and it will be discussed below in POSTRECEPTOR SIGNALING PATHWAY(S). Another important function in the regulation of nutrient uptake is the increased amino acid uptake due to insulin, which occurs mainly in liver due to increased protein synthesis (reviewed in Ref. 116) and in muscle by a mechanism that is not well understood (56).

On a slightly slower time frame than for nutrient transport, insulin also effects changes in the amount of specific proteins (see Ref. 72 for a review) by inhibiting the rate of protein degradation and by regulating the rate of the transcription and/or translation (see review in Ref. 116). Perhaps the most notable example of transcriptional regulation is that of phospho*enol*pyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis. Transcription of this enzyme is rapidly decreased after insulin treatment, thus lowering protein levels and leading to diminished glucose synthesis (55). Enzymes involved in glucose production, such as fructose-1,6-bisphosphatase (39), are also downregulated by insulin at the level of transcription, whereas the transcription of enzymes involved in glucose utilization, such as the glucose transporter (16), glucose-6-phosphate dehydrogenase (168) and glyceraldehyde-3-phosphatase (1), is increased by this hormone. Of course, all these actions of insulin are initiated at the plasma membrane by insulin receptors responding to ligand binding. The remainder of this review will concentrate on the details of insulin receptor structure and/or function and its intracellular signaling pathway. A recently published review by Taylor et al. (182) addresses naturally occurring mutations in the human insulin receptor gene that disrupt receptor structure and function and, in some cases, may lead directly to diabetes in the affected individuals. Taylor et al. (182) discuss insulin receptor gene structure and receptor biosynthesis, and thus these topics will not be comprehensively treated herein.

INSULIN RECEPTOR SUBUNIT COMPOSITION

Figure 1 represents the characteristic two-dimensional structure of the insulin receptor as deduced from the cDNA sequence (37, 186) and numerous biochemical studies. The α-subunit is comprised of 723 amino acids and has an approximate molecular mass of 130 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37, 69, 127, 186, 205). The α-subunit is the only one detected by affinity-labeling protocols (69, 127, 205) and must therefore contain the site or sites for ligand binding [see INSULIN-INSULIN RECEPTOR INTERACTION (INSULIN BINDING)]. As noted in Fig. 1, the insulin receptor α-subunit has a cysteine-rich domain (hatched area), a feature it shares with the insulin-like growth factor I (IGF-I) receptor and members of the epidermal growth factor (EGF) receptor family (147). The β-subunit contains 620 amino acids and has an approximate molecular mass of 95 kDa in gels (37, 78, 186). Both subunits are glycosylated, thus accounting for most or all of the molecular mass difference in the observed vs. predicted values. The β-subunit is composed of three compartmentalized regions: the extracellular, transmembrane, and cytosolic domains. The cytosolic tyrosine kinase domain has an ATP binding consensus sequence and three clusters of tyrosine residues that can be phosphorylated in response to insulin. The three clusters are found in the juxtamembrane domain residues, the "tri-tyrosine" residues in the tyrosine kinase domain, and carboxy-terminal residues. The possible functional roles of these phosphorylated tyrosines will be discussed below.

As depicted in Fig. 1, the physiologically relevant insulin holoreceptor is composed of two α-subunits and two β-subunits, linked by disulfide bonds into a tetramer that is functionally a dimeric protein complex. The

primary translation product of the insulin receptor is the linear αβ-sequence that is processed into individual subunits by proteolysis after disulfide linkage of two such translation products (70, 119, 140). The noncovalently linked dimeric nature of ligand-bound receptor/tyrosine kinases is a feature of the entire family (147, 188), but the insulin receptor and the closely related receptors for IGF-I receptor (187) and insulin-related receptor (IRR) (156), for which no ligand(s) is known, are unique in that the unliganded receptor is maintained as a covalent dimer by so-called class I (100) disulfide bond(s). For the insulin receptor, these linkages can easily be reduced under mild conditions, generating αβ-heterodimers that are functionally monomeric receptor species (19, 172). The half-receptors bind ligand with lower affinity than the holoreceptor (20, 172) but appear to be otherwise unaffected by mild chemical reduction because they can be induced to reassociate by insulin into holoreceptors concomitant with full retention of insulin-stimulated tyrosine kinase activity (18, 105, 173). Each α-subunit is linked to one β-subunit by so-called class II disulfide bond(s) (100). The exact pattern of the inter- and intrasubunit disulfide cross-linkage is largely unknown, and the published data on this matter concerning α-α linkages are not consistent (see below). For α-to-β disulfide linkages, site-directed mutagenesis has established an important role for Cys-647 [nomenclature of Ullrich et al. (186), see below]. When this residue is mutated to serine, no $\alpha_2\beta_2$-receptors can be detected by affinity labeling, only α_2-receptors are found (21).

The site of the inter-α-subunit linkages has been narrowed to one of the central cysteines (Cys-435, Cys-468, and Cys-524) by biochemical studies. Reduction of inter-α-disulfides, followed by labeling with *N*-ethylmaleimide, yielded data suggesting that a single cysteine mediates this linkage (24, 45). Partial chymotryptic digestion of the insulin receptor revealed a monomeric 55-kDa α-subunit domain that was resistant to further proteolytic digestion and that contained a site to which insulin could be chemically cross-linked (192). Microsequencing revealed this fragment to begin at the amino-terminal of the receptor, and Western blotting with an anti-peptide antibody established that it contained residues 240–252 and, most likely [see INSULIN-INSULIN RECEPTOR INTERACTION (INSULIN BINDING)], all of the cysteine-rich sequence. Thus the inter-α-linkage probably occurs to the carboxy side of the cysteine-rich region. Tryptic digestion of the holoreceptor in cells yields a monomeric fragment consisting of the entire β-subunit plus 30,000 of the α-subunit (161), and the four cysteines in this region of the α-subunit span only 72 amino acids from its carboxy-terminal. Therefore, the inter-α-linkage is most probably one of the cysteines of the Cys-435, Cys-468, and Cys-524 grouping. Recently, a study employed [^{35}S]cysteine labeling and tryptic digestion to identify receptor disulfides, and it was found that Cys-435 and Cys-468 were in the same high-performance liquid chromatography (HPLC) fraction, suggesting that they might be linked in an intrasubunit fashion (144), implicating Cys-524 as the residue for intersubunit linkage. However, it is not clear from this study that a single tryptic peptide was analyzed. Finally, another study of microsequenced tryptic-digested peptides concluded that there were two α-α interlinkages between amino acid sequence 122–270, part of the cysteine-rich domain, and there was one α-β class II disulfide linkage between residue 583 and the carboxy-terminal of the α-subunit and the extracellular domain of the β-subunit (amino acids 724–917) (199). The former conclusion is not entirely consistent with the other studies cited for reasons presently unclear. In any case, it is an interesting and open question as to why the insulin receptor and its two close relatives are maintained as covalent dimers, whereas all other members of the tyrosine kinase family must be dimeric to function but become so in a reversible noncovalent manner.

INSULIN RECEPTOR ISOFORMS: HIR-A (EXON 11−) AND HIR-B (EXON 11+)

Although the insulin receptor derives from a single gene, two isoforms are produced by alternative splicing of exon 11 (153). The insulin receptor with exon 11 (HIR-B or Ex11+) has 12 more amino acids at the carboxy-terminus of the α-subunit after Arg-723, compared with the other isoform (HIR-A or Ex11−). The tissue distribution established by Northern blotting suggests that Ex11− is the predominant species in liver and is very rare in leukocytes and muscle. Placenta and adipocytes express both isoforms of the insulin receptor at nearly equal levels (15). The biochemical properties and physiological functions of the isoforms exhibit some subtle and somewhat controversial differences. It has been reported that the insulin receptor without exon 11 shows higher insulin-binding affinity (~2-fold) and enhanced insulin-induced internalization (106, 191, 201). However, for insulin-stimulated receptor autophosphorylation and tyrosine kinase activity, one group showed no difference between the two isoforms (201). On the other hand, another group showed that the insulin receptor with exon 11 is more phosphorylated and is more active in an exogenous tyrosine kinase assay as a consequence of an increased maximum velocity (with no change in K_m toward substrates) (80). The other discrepancy between the two groups is the level of the two isoforms in patients with non-insulin-dependent diabetes mellitus (NIDDM). With the use of the polymerase chain reaction as an assay for receptor expression, it was reported that skeletal muscle from the control group had only insulin receptors without exon 11, but muscle from NIDDM patients showed dramatically increased levels of insulin receptor with exon 11 (107). However, a more recent study used immunoprecipitation by an anti-peptide antibody specific to insulin receptors expressing exon 11, and no difference in the amount of insulin receptor protein was found for the two isoforms from control and NIDDM patient (15). Thus the physiological role of the alternatively spliced receptors remains incompletely understood, and differences in isoform expression probably do not play a major role in diabetes.

INSULIN-INSULIN RECEPTOR INTERACTION (INSULIN BINDING)

As noted above, insulin itself has been intensively studied and was the first peptide hormone to be crystallized (17) and one of the first to be analyzed in receptor binding studies (28, 50). Historically, there has been great interest in describing the details of how insulin binds to its receptor, and this interest continues. Twenty years ago when no compositional or structural information was available for the insulin receptor, insulin binding to cellular membranes, as analyzed by Scatchard plots, was shown to produce upwardly curvilinear binding isotherms (33). These were interpreted as indicative of negatively cooperative binding (33), an interpretation that triggered numerous additional binding studies from many laboratories and considerable controversy over the years. The fact that the receptor is a functional dimer, thus possibly capable of binding two insulin molecules, has softened the controversy, and recently, molecular models of ligand binding to receptor have been postulated that can explain most or all of the binding data (see below and Fig. 2). Because all affinity labeling protocols for the insulin receptor identify only the α-subunit(s) of the receptor, it is within this subunit that ligand-receptor contact must occur. The exact regions of the receptor that directly contact hormone remain incompletely defined and are still the object of much scrutiny. The two general experimental approaches are being employed to define ligand-receptor contact regions, affinity labeling and mutagenesis.

Affinity labeling of the receptor with various insulin derivatives, and/or cross-linking of insulin to the receptor with bifunctional reagents, followed by isolation of receptor-insulin fragments has allowed the identification of three regions of the α-subunit as potential hormone-receptor contact sites. These are the amino-terminal amino sequence in the vicinity of residues 20–120 (194), the disulfide-rich region (192, 203), and the region just to the carboxy side of the disulfide-rich region around residue 390 (40). In fact, all of these sites may be encompassed in a 55-kDa domain biochemically generated by proteolysis of the covalent ligand-receptor complex that is resistant to further proteolytic digestion (192). This fragment comprises the amino-terminal, as determined by direct sequence analysis, residues 240–252 as verified by Western blotting, and enough additional protein and carbohydrate mass to migrate as 55 kDa on gels (192). Because this 55-kDa fragment contains the disulfide-rich region, it will migrate anomalously fast in the absence of reducing agents and hence probably does encompass at least 400 amino acids beginning from residue 1, in addition to some *N*-linked glycosylation. Our interpretation of these data is that this fragment, resistant to further proteolysis, represents a "ball" or a "fist" maintained by internal disulfide linkages that contains insulin and some part of its binding pocket. More detailed analysis will eventually be possible with structural data from crystallography and/or electron microscopy. In any event, the amino-terminal 400 amino acids of the α-subunit seem highly likely to contain some part of the ligand-receptor contact sites based on the these studies and those described in the next paragraph.

The second major approach to binding site mapping employs recombinant DNA technology to interchange sequences between the insulin receptor and its close relative, the receptor for IGF-I. Various of these studies have supported numerous α-subunit sequences as having a role in the recognition of insulin. These are the amino-terminal region of residues 1–68 (82), the cysteine-rich region (57), and the residues from 325 to 524 (149). The disulfide-rich region of the IGF-I receptor, when introduced into the insulin receptor, confers high-affinity IGF-I binding to the chimera (3, 148, 208). Additional information relative to the ligand binding site of the insulin receptor is that site-directed mutation of Phe-89 essentially abolishes insulin binding (32). Antibodies to the receptor α-subunit sequence from 450 to 601 inhibit insulin binding (209), and an antibody against a sequence from the disulfide-rich region implicates this part of the receptor in ligand recognition (202). Insertion of 12 amino acids near the carboxy-terminal of the α-subunit encoded by exon 11, the insulin receptor splice variant discussed above (153), also modestly alters ligand binding affinity (201). These facts taken together, and with the additional fact that only truncated receptors having essentially the entire α-subunit sequence are still capable of binding insulin (143), lead one to conclude that the entire α-subunit is important in some way for insulin binding. The data and references concerning insulin receptor residues or regions thought to be important for insulin binding are summarized in Table 1.

We believe that the binding of one insulin molecule to the holoreceptor is the normal situation at physiological concentrations of insulin and that this is sufficient to activate autophosphorylation and the exogenous kinase activity of the receptor. This conclusion derives in part from our recent demonstration that covalent coupling of one molecule of BBpa-insulin is sufficient to activate the receptor (160). Three previously published lines of evidence support a stoichiometry of one insulin molecule bound per holoreceptor at physiological relevant hormone levels. *1*) As noted previously, insulin binding to cells and membranes was shown to exhibit negative cooperativity as determined by Scatchard analysis, indicating that binding of one insulin molecule to the receptor makes the binding of the second molecule more difficult (33). Furthermore, supraphysiological concentrations of hormone are required for the second molecule to bind. *2*) In confirmation of this hypothesis, purified αβ heterodimers prepared by mild reduction of the class I disulfides show only low-affinity linear-binding isotherms with a stoichiometry of one insulin per αβ heterodimer, whereas the purified $\alpha_2\beta_2$ holoreceptor exhibits negative cooperativity and only one high-affinity insulin binding site (20, 172). *3*) Double probe analysis using two different insulin analogues showed that only one analogue at a time could bind to the receptor with high affinity (120, 121). Recently, another group concluded that insulin receptors are bivalent

Table 1. *Summary of receptor residues possibly involved in insulin binding*

Residue(s)	Method	Characteristics	Reference
Asn-15 → Lys	A	Decreased processing and insulin binding	(74)
Arg-31 → Gly	A	No cleavage, no processing, and no insulin binding	(189)
Phe-89 → Leu	A	Decreased insulin binding	(32)
Leu-233 → Pro	A	Decreased processing and insulin binding	(97)
Arg-723 → Ser	A	No cleavage and decreased insulin binding	(65, 75)
Δ486–569	B	Decreased insulin binding	(73)
1–68	C	Increased insulin binding and decreased IGF-I binding	(82)
1–137	C	Increased insulin binding and decreased IGF-I binding	(148)
20–120	D	Labeled fragment	(194)
38–68	C	Increased insulin binding and decreased IGF-I binding	(3)
Exons 2 and 3	C	Low affinity for insulin and high affinity for IGF-I	(2)
191–297	C	No change in insulin binding and increased IGF-I	(208)
205–316	D	Labeled fragment	(203)
241–251	D	Labeled fragment	(202)
230–285	C	Increased insulin binding and decreased IGF-I	(57)
325–524	C	Specific binding for insulin	(149)
390–401	D	Labeled fragment	(40)
450–601	E	Antibody inhibits binding	(209)
469–592	D	Antibody inhibits binding	(130)
55 kDa (1–400)	D	Labeled fragment	(192)

A, point mutation; B, deletion; C, domain swapping between insulin receptor and insulin-like growth factor I (IGF-I) receptor; D, affinity labeling with insulin and/or insulin analogues; E, site-specific antibody.

(204), but the receptor source was human placenta, where others have shown insulin IGF-I receptor hybrids to be present (108, 185). The experimental protocol of Yip and Jack (204) is consistent with the interpretation that hybrid receptors were being analyzed. Therefore almost all data support the notion that one molecule of insulin binding to its receptor is the greatly predominant, if not exclusive, situation under all but the most unusual physiological conditions.

Insulin itself has no obvious intrinsic symmetry (63) but must interact with two identical αβ-receptor halves for high-affinity binding, suggesting that asymmetry may be induced in the receptor by insulin binding. For this, insulin may need to make contact with both α-subunits, a situation generally analogous to that of growth hormone (GH), where one molecule of GH contacts two molecules of the soluble portion of the GH receptor, the GH binding protein (29). Recently, the structure of this ligand-receptor complex was solved by X-ray crystallography (35). Although GH is not symmetrical, it makes contact with the same region of the receptor on both receptor halves. Similarly, we propose a model of insulin binding to both α-subunits with two primary insulin contact sites per $\alpha_2\beta_2$-receptor as illustrated in Fig. 2, but we propose that insulin makes contact with different sites on each half of the receptor. At low physiologically relevant hormone concentrations, insulin can contact both binding sites 1 and 2, each on separate halves of the receptor, resulting high-affinity insulin binding. Site 1 on the receptor might be interacting with residues in the carboxy-terminal of the insulin B chain (102). As illustrated, at higher concentrations of insulin, a second insulin molecule may also make contact with site 1, resulting in low-affinity binding. Obviously, when the receptor is chemically reduced to make αβ-half-receptors, contact with insulin can only exist for one receptor site, thus leading to the observed low-affinity binding (20, 172). Moreover, the proximity of the four possible

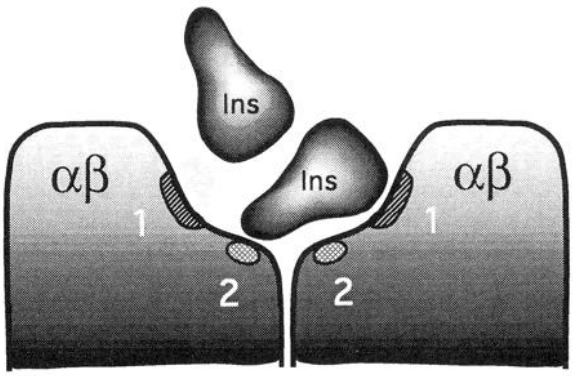

Fig. 2. Model for insulin binding to its receptor. See text for details.

contact sites are such that one insulin can interfere with binding of a second insulin in such a way that negative cooperativity may occur. Thus this model can explain much of the observed data for insulin binding to its receptor. A similar model for insulin binding has recently been proposed (31, 150) based on binding studies of insulin analogues to insulin and/or IGF-I receptor chimeras similar to those referenced above (151).

INSULIN RECEPTOR SUBUNIT INTERACTIONS

Insulin binding to its receptor rapidly causes receptor autophosphorylation, an event that appears to be essential both for activation of the exogenous tyrosine kinase activity, in vitro, and insulin action, in vivo (135), although there is some evidence against this point (see below). Nevertheless, the importance of kinase activity is supported by several independent lines of evidence. *1*) Microinjection of an antibody that inhibits the tyrosine kinase activity of the insulin receptor in vitro, into the *Xenopus* oocyte (103), Hep G2, or TA1 mouse adipocytes (104), inhibits respective mitogenic and metabolic insulin responses. *2*) Mutated receptors totally lacking tyro-

sine kinase activity in vitro are devoid of biological activity when transfected into cells (reviewed in Ref. 182). *3*) The addition of tyrosine kinase inhibitors to cells can block or decrease the actions of insulin (141, 157). Exactly how insulin binding to the α-subunit can transduce signals to the β-subunit to activate the tyrosine kinase activity is not understood at the level of detail resembling, for example, the differently phosphorylated conformations of glycogen phosphorylase *a*, the structure of which is known from X-ray crystallography (164). However, some hypotheses and experimental models exist and are being tested.

It has been postulated that the general role of the α-subunit in the absence of insulin is to negatively regulate or inhibit the kinase activity of the β-subunit kinase domain. This notion derives in part from experiments with trypsin-treated cells that, after immunoprecipitation, yield a kinase active receptor fragment consisting of an intact β-subunit (95 kDa) disulfide linked to the carboxy-terminal of the α-subunit (25 kDa) (62, 161) as discussed above in another context. Trypsin treatment of fat cells activates glucose transport in a trypsin concentration-dependent manner, where the degree of receptor proteolysis, as assessed by affinity labeling, parallels transport activation (126), a result consistent with this hypothesis. On the other hand, the fragment generated by trypsin (158), as well as the expressed intracellular portion of the β-subunit kinase domain (27, 59), exhibit slower autophosphorylation and exogenous kinase kinetics that closely resemble the kinetic behavior of the insulin holoreceptor basal state rather than that of the insulin-stimulated receptor kinase (84). If the role of the α-subunit was only to inhibit kinase activity of the β-subunit, then the above-described constructs should behave as activated kinases, but they do not. Thus it is unclear whether this attractive and simple hypothesis is correct.

There is considerable evidence that various conformational changes are involved in the multiple steps of receptor activation, as would be expected, from the following experimental data. Receptor treated with a chemical homobifunctional cross-linker was shown to undergo a more facile cross-linking of one α-subunit to the other in the presence of insulin compared with the absence of ligand (193). A similar approach was employed with phosphorylated receptor to show that the insulin-treated receptor generated more cross-linked β-β complex than receptor without insulin (145). Because two αβ-heterodimers are necessary for ligand-activated autophosphorylation, these data suggest that the following sequence of events takes place. The initial conformational change in the receptor alters the interface between the two α-subunits, and this leads to a change in the β-β subunit interaction, such that autophosphorylation takes place. Other evidence documenting conformational changes in the insulin receptor β-subunit following ligand binding comes from the studies of antibodies that discriminate between different states of the receptor. Antibodies have been raised that document conformational changes due to autophosphorylation of the tri-tyrosine site (60, 98, 124) and the carboxy-terminal (13, 14). Taken together, these data suggest that conformational changes in the receptor can be driven by each step of ligand binding, ATP binding, and autophosphorylation and lead stepwise to tyrosine kinase activation and signal transduction.

The transmembrane domain of the insulin receptor physically links the ligand-binding domain on the outside of the cell to the kinase domain in the cell cytoplasm and may therefore be involved in signal transduction. A series of deletion or insertion mutants in this sequence did not show any differences from the native receptor in functions including processing, insulin binding, autophosphorylation, and tyrosine kinase activity or metabolic and mitogenic pathway activation (48, 200), suggesting a passive role for the transmembrane domain. However, the substitution of Val-938 by a charged amino acid has given conflicting data from different labs. Two groups reported no difference from wild type for this mutation (48, 200), whereas another laboratory reported increased basal and no insulin-dependent kinase activation for this construct (95). More recently, substitutions of the transmembrane domain that optimize helicity have been shown to accelerate receptor internalization (53), and a substitution of the Neu oncoprotein (22) transmembrane domain is fully activating for the receptor kinase (200) and alters the biological activity of the receptor in transfected cells (22). We interpret these data to mean that, normally, the transmembrane domain probably plays a passive role in subunit communication from α to β but mutations that potentially alter the interactions between the two β-subunits can have marked biochemical consequences.

INSULIN RECEPTOR PHOSPHORYLATION AND SIGNAL TRANSDUCTION

As noted in the introduction, insulin primarily regulates nutritional metabolic pathways, whereas other growth factors that activate receptor/tyrosine kinases primarily regulate cell growth and differentiation. Two biochemical features also distinguish the insulin receptor from most of its relatives. The first, also noted previously in this review, is that the insulin receptor (and its two close relatives, the IGF-I receptor and the IRR) is a covalent dimer, whereas other receptor/tyrosine kinase family members need to form dimers for activation but do so noncovalently. The second is that most receptor/tyrosine kinases form relatively stable complexes directly with substrates and/or effector molecules following receptor autophosphorylation. They do so via high-affinity interactions of receptor phosphotyrosine residues with *src* homology domains (SH2) in the effector proteins (83, 101). Individual effectors such as phosphoinositol-3-kinase (PI-3-K), Ras GTPase-activating protein, and phospholipase Cγ (PLCγ) have been shown to associate with a different phosphotyrosine residue in the platelet-derived growth factor (PDGF) receptor (42). On the other hand, it is generally thought that the insulin receptor does not form such complexes. Rather, the activated insulin receptor phosphorylates its principal substrate, insulin receptor substrate 1 (IRS1), and the phosphotyrosine residues generated by this

process engage and activate signaling proteins such as PI-3-K [reviewed in Ref. 113 and see POSTRECEPTOR SIGNALING PATHWAY(S)]. The fact that the ligand-stimulated exogenous kinase activity of the insulin receptor has been repeatedly seen in multiple laboratories using various reporter substrates (77, 78, 114, 125, 136, 137, 154, 155, 190, 207, 212, 213), whereas this augmented exogenous kinase activity is difficult to demonstrate for EGF and PDGF receptors (147), supports the notion that there may be some fundamental biochemical differences in the activation mechanism for insulin and IGF-I receptors compared with the monomeric receptor/tyrosine kinases. On the other hand, there are also some common features such as the recent discovery that both EGF receptors (123, 139) and insulin receptors (87, 131) can phosphorylate the SH2 domain-containing transforming protein, Shc (SH2 domain-containing protein with sequence homology to α-1 collagen). The paradox of receptors that activate different physiological responses, apparently by using common signaling pathways, is discussed in detail [POSTRECEPTOR SIGNALING PATHWAY(S)].

What then is the functional role of the phosphotyrosine residues generated on the insulin receptor as a result of autophosphorylation? There are 13 tyrosine residues in the cytoplasmic portion of each insulin receptor half, and the individual autophosphorylation site(s) have been identified and correlated with the exogenous tyrosine kinase activity and biological activity of the receptor. Phosphopeptide mapping techniques (43, 85, 178, 184, 197) have resolved 5–7 tyrosine residues (amino acids 960, 953 and/or 972, 1146, 1151, 1152, 1316, and 1322) out of the 13 possible tyrosines predicted from the cDNA clone (37, 186). These tyrosine residues occur in three clusters in the primary structure, in the juxtamembrane region (amino acids 953, 960, and 972), the tri-tyrosine region of the catalytic domain (1146, 1151, and 1152), and in the carboxy-terminal (1316 and 1322). The most important domain for the autophosphorylation and exogenous tyrosine kinase activity seems to be the tri-tyrosine domain (amino acids 1146, 1151, and 1152), which lies in the tyrosine kinase catalytic domain. Phosphorylation of these sites, as determined by peptide mapping, temporally correlates with the acquisition of exogenous kinase activity (91, 197). Substitution of any of these tyrosine residues with phenylalanine affects receptor autophosphorylation and insulin action when the resultant mutant is transfected into cells, although the degree of inhibition is dependent on the site and/or the number of mutations and the cell type (38, 198, 206, 210). When all three tyrosine residues are changed to phenylalanine or serine, the effects of insulin are totally abolished, including endogenous substrate (IRS1) phosphorylation (110, 198). Analysis of various single and double mutations in the tri-tyrosine region have given somewhat conflicting data concerning the role of an individual tyrosine within this region (38, 198, 206, 210), but there is unanimity concerning the overall importance of this region. Interpretation of the effects of receptor tyrosine mutations on the biological activity of insulin in cells, including those in the tri-tyrosine region, has resulted in conflicting data, possible reasons for which are discussed in the next paragraph.

The tyrosine residues at the carboxy-terminal region (amino acids 1316 and 1322) are also heavily phosphorylated and account for 30–40% of total phosphate incorporation into the β-subunit (91, 178, 184, 197). The biochemical and biological consequences of phosphorylating these residues are unclear for various reasons including conflicting data generated in various laboratories. Carboxy-terminal deletions lacking either 43 amino acids (99) or mutants with tyrosine residues 1316 and 1322 changed to phenylalanine (4, 111, 174) were reported to have normal tyrosine kinase activity and diminished autophosphorylation due to the loss of two phosphorylatable tyrosine residue in vitro. However, these mutants, when transfected into the fibroblast cell line called Rat 1, show diminished (99, 183) or unchanged (4, 174) glucose uptake and glycogen synthase activity ("metabolic signaling"). However, mitogenesis, as determined by thymidine incorporation into DNA, was enhanced (4, 99, 183). Chinese hamster ovary (CHO) cells transfected with receptor lacking the same 43 amino acids were reported to behave exactly like cells transfected with wild-type receptor (111). Transfection into CHO cells of a receptor lacking 69 carboxy-terminal amino acids resulted in a more insulin-sensitive activation of mitogen-activated protein kinase (MAP kinase) (36), a possible indicator of mitogenic potential (122). However, biochemically, this construct was shown to have markedly activated basal kinase activity that could be further activated to the level of wild-type receptor when exposed to insulin (179). The 43-amino acid deletion and phenylalanine substitution mutants, however, apparently have normal basal kinase activity in vitro (4, 99, 111, 174). Thus no clear picture emerges as to the role of the carboxy-terminal autophosphorylation sites in insulin action. They may indeed play a negative regulatory role in insulin receptor-mediated mitogenesis in the context of certain cells, but, as emphasized already, the insulin receptor is not primarily involved in regulating mitogenesis, particularly in the physiological context of the whole organism. The "metabolic response" of cell lines such as Rat 1 and CHO is also dramatically different from normal insulin target tissues, and they lack the expression of genes such as GLUT-4 that mediate insulin-responsive glucose transport in muscle and fat (71). Thus transfection experiments into cells such as these can be very useful for obtaining biochemical information about receptor behavior but can be problematic for physiological studies concerning insulin action

There are three tyrosine residues in the juxtamembrane region of the β-subunit (amino acids 953, 960, and 972) that are possible phosphorylation sites. One of these, Tyr-960, was mutated to phenylalanine, and this receptor construct, when transfected into CHO cells, implicated the importance of this residue for IRS1 phosphorylation and for metabolic and mitogenic responses in this cell type (195). Autophosphorylation and exogenous kinase activity for this mutant was the same as for wild-type receptor. Additional substitution and

deletions that include Tyr-953 and Tyr-960 have established a role for this region in receptor endocytosis (6, 132), as this conformation of the tyrosines in the two aforementioned residues (8) corresponds to consensus endocytosis signals for internalization via clathrin-coated pits (23), a pathway utilized in at least one major insulin target tissue, the liver (128). For technical reasons, phosphopeptides from the juxtamembrane region were not identified in early HPLC profiles (184, 197), but, recently, Tricine-SDS-PAGE (43) analysis and modified HPLC conditions (85) revealed their presence. In vitro, phosphorylation of the juxtamembrane region accounts for 15% of total phosphorylation and occurs mainly on residue 960 (43). However, in the context of receptor endocytosis, it is the aromatic nature of this tyrosine that is important, not the phosphorylation (6). In cells, the juxtamembrane region accounts for ~30% of the total phosphate incorporation due to serine phosphorylation in this general region (43).

It had previously been shown that the insulin receptor is phosphorylated on serine and threonine residues in vivo (67, 180), possibly due to direct phosphorylation by adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (165), protein kinase C (68, 93, 175), and casein kinase I-like kinase (133). Also, serine kinase activity has been reported to be tightly associated with purified insulin receptor preparations (9) even after insulin affinity chromatography (94). Attempts to separate serine kinase from tyrosine kinase activity of the insulin receptor were unsuccessful, and recently it has been reported that insulin receptor itself has serine kinase activity (12). One of the phosphorylation sites is at Ser-1293/Ser-1294 in the carboxy-terminal (94), and, as noted above, another is in the juxtamembrane region (43). Because receptor phosphorylated at serine or threonine residues by protein kinase C shows decreased tyrosine kinase activity and phorbol ester-treated cells showed decreased insulin action (175), it has been suggested that this phosphorylation opposes the actions of insulin, i.e., serves as a counterregulatory effector. More recent studies of insulin action in cells overexpressing various isoforms of protein kinase C support this hypothesis, and it was suggested that chronically elevated protein kinase C activity may give rise to some of the insulin resistance seen in type II diabetes (25). However, for the acute effects of insulin and its counterregulatory hormones, one observes effects on each other's biological activities very rapidly before changes in insulin receptor serine/threonine phosphorylation are evident, and therefore the physiological importance of these events remains uncertain.

There have been several reports concluding that insulin action can be observed in the absence of receptor autophosphorylation and activated kinase activity. In view of the general evidence concerning the role of tyrosine kinase activity in biology, which must be considered firmly established, these reports are surprising. In the case of insulin action, a number of studies employed anti-receptor antibodies that mimicked the actions of insulin, for example, on glucose uptake with little or no observable receptor autophosphorylation (46, 58, 129, 163). These studies can be questioned on technical grounds because the amount of receptor may not be limiting for most biological responses and can become dephosphorylated during its isolation (51, 167). Thus if <5% of the receptors in a cell need to be occupied for full biological activity, as is the case for activation of fat cell glucose transport (86), it is technically very difficult to demonstrate insulin receptor autophosphorylation under these circumstances. More difficult or impossible to explain is the report that a totally kinase-inactive receptor can activate mitochondrial pyruvate dehydogenase in an insulin-dependent manner and therefore retain some signaling function (54).

POSTRECEPTOR SIGNALING PATHWAY(S)

For the insulin receptor as well as for the large family of receptor and nonreceptor tyrosine kinases, it had been widely assumed that this kinase activity would initiate a phosphorylation cascade analogous to that produced by G protein-linked receptors coupled to adenylate cyclase. Indeed, following stimulation of cells with a variety of agonists for receptors with intrinsic tyrosine kinase activity, a variety of cellular phosphotyrosine proteins (52) can be identified in addition to multiple changes in the cellular phosphoserine/threonine content. Kinase cascades activated by receptor tyrosine kinases have been identified (42, 147), but they do not appear to be amplified the way that cAMP production amplifies kinases involved in glycogen metabolism. Moreover, despite divergent biological activities, receptors such as those for insulin, EGF, and PDGF seem to engage and activate similar, if not identical, signaling pathways. Very recent reviews have summarized receptor/tyrosine kinase signaling pathways in general (42, 147), postreceptor insulin signaling (113), and kinase cascades (30). The reader should refer to these for more details, and we will integrate aspects of these reviews and provide our own perspective on these closely related topics.

Activation of the majority of receptor/tyrosine kinases by autophosphorylation permits high-affinity binding to receptor tyrosine kinases of soluble effector molecules that contain SH2 domains, and the effectors include PI-3-K, Ras Gap, and PLCγ (42), the last being capable of generating the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (134). This seems to be a quite different mechanism from adenylate cyclase-linked receptors where a soluble second messenger is produced at the cell surface via a series of membrane-associated proteins (176). The insulin receptor differs from other receptor/tyrosine kinases in that it does not directly bind effector molecules, but rather it phosphorylates its major substrate, IRS1, on tyrosine residues (170, 196), and IRS1, in turn, binds effector molecules. IRS1 has 20 possible tyrosines including 6 with YMXM sequence (159, 170) that appears important for binding the SH2 domain of the p85 regulatory subunit of PI-3-K (112). PI-3-K binds to IRS1 and is activated in an insulin-dependent fashion in several cell types (7, 118, 138) including fat cells (81), an important physiological target for insulin. Nevertheless, PI-3-K activation by

insulin has not been linked to physiological processes such as glucose transport that are insulin dependent.

Recent data have linked insulin receptor signaling to the Ras activation pathway (11, 162) via adapter molecules containing SH2 and SH3 domains but having no enzymatic activity (146). The mammalian adaptor is called Grb 2 and corresponds to the nonmammalian adaptors, Sem 5 and Drk, that have been genetically linked to tyrosine kinase-Ras pathways in *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively (146). Some of the same investigators have also described the possible involvement of Ras in insulin-stimulated glucose transport (88). It has long been known that Ras activation is necessary for mitogenesis induced by receptor/tyrosine kinases such as the EGF receptor (109), and, recently, the involvement of the adaptor molecules and GTP exchange proteins for Ras has been experimentally described. Thus the mechanism of Ras activation requires autophosphorylated receptor, e.g., PDGF or EGF receptors or phosphorylated IRS1 in the case of insulin, which bind the above-noted adapter, Grb 2, which binds in turn the Ras GTP exchange protein called mSos (for mammalian son of sevenless, Sos) (11, 41, 79, 112, 123, 146, 162). This then activates a serine/threonine kinase cascade (vide infra).

Why then do the PDGF and EGF receptors use at least two of the same effectors for cell proliferation, PI-3-K and Ras, that insulin may use for glucose transport stimulation? If PDGF, EGF, and insulin receptors use the same effectors, then they should have similar or identical biological effects, but they clearly do not, certainly in the context of mouse fibroblasts where PDGF and EGF act early and different steps in the cell cycle and insulin/IGF-I acts later (96). There are several possible explanations. First, PI-3-K activity (or IRS1 for that matter) has not yet been definitively implicated in glucose transport activation, whereas its involvement in mitogenesis seems clear. As for Ras, a recent study implicated its activity in glucose transport activation (88), but, on the other hand, a dominant interfering Ras mutant protein, when microinjected into fat cells, does not block insulin-stimulated GLUT-4 translocation (M. Birnbaum, personal communication). Thus it remains unclear whether the common pathways stimulated by the spectrum of receptor tyrosine kinases have relevance for metabolic regulation by insulin. Even if they do, the cellular context may give rise to the appropriate signal. Terminally differentiated rat fat cells are obviously unresponsive to mitogenic signals and express insulin receptors as the predominant tyrosine kinase. However, if the signal emanating from the small number of fat cell EGF receptors can be enhanced, as has recently been accomplished using putative inhibitors of glucose transporter endocytosis, an insulin-like glucose transport activation can be achieved by EGF (166). Conversely, when insulin receptors are transfected at high levels into fibroblastic cells, either through specific interactions and/or because of the promiscuous nature of the tyrosine kinase activity, a mitogenic effect can be manifested.

In summary, it is not yet clear if what is required for the metabolic action of insulin is simply the expression of ligand-activated tyrosine kinase activity in the context of a cell that has the appropriate insulin responsive genes IRS1 and GLUT-4 or if there is something unique about the enzymatic activity of the insulin receptor that allows its specific biological function in the regulation of metabolism. Of course, there are as yet unidentified genes, in addition to those just noted, whose expression is required for full insulin-responsive glucose transport. In any case, if all that is required is tyrosine kinase activity in the context of a cell expressing the target genes, it is still curious that insulin (and IGF-I) must use the intermediary role of IRS1, whereas other receptor/tyrosine kinases do not.

The Ras pathway activated by tyrosine phosphorylation leads to activation of serine/threonine kinases or "switch kinases" such as the MAP kinases or extracellular regulated kinases (Erks) in a cascade of kinase activity leading to alterations in gene transcription and other cellular changes (reviewed in Refs. 76, 115). The mechanism of conversion from tyrosine kinase to serine/threonine kinase specificity seems to be at the level of Ras activation of the serine/threonine protein kinase and protooncogene product, c-Raf (211). Raf activates additional serine/threonine kinases including MAP kinase kinase/MEK (MAP kinase/Erk kinase) (89), although the latter protein possesses dual specificity, i.e., it will phosphorylate tyrosine and threonine/serine residues (152). The study of insulin-dependent activation of MAP kinase was instrumental in uncovering the kinase cascade (169) leading to transcriptional activation, but there is no direct evidence for involvement of the MAP kinase cascade in glucose transport activation. Moreover, the same problem of specificity discussed in the previous paragraph continues to apply, since all receptor/tyrosine kinases are able to activate this cascade. Indirect evidence for serine/threonine kinase involvement in glucose transport translocation has been reported based on the use of kinase inhibitors (66). However, these are rarely as specific as one would like. On the other hand, based on its specific inhibition, the 70-kDa S6 kinase can be ruled out as activating glucose transport (44). In muscle, insulin-dependent regulation of glycogen metabolism probably involves a kinase cascade (34, 171) similar to the MAP kinase pathway described in cultured cells, although many of the details need to be elucidated.

FUTURE DIRECTIONS

Aside from the importance of determining the pathway(s) and specificity of tyrosine kinase-mediated signaling related to the actions of insulin, there are many aspects of insulin receptor structure and/or function that need further scrutiny. Obviously, structure determination by physical methods would be highly desirable, but X-ray crystallography appears beyond reach at present for unknown technical reasons. Nevertheless, images of the purified insulin holoreceptor (26) and ectodomain (142) have been obtained in the electron microscope by negative staining, and the holoreceptor

has been visualized in the frozen hydrated state (61). The receptor has a Y-shaped or slightly concave T-shaped structure (Fig. 3), and, in view of our discussion above that there is only one high-affinity binding site, it is interesting to speculate that this site may be in the fork of the Y. Our ability to covalently label the receptor with high efficiency using L-Bpa^{B25},B29^{e} biotin insulin (BBpa insulin) (160) should allow us to determine where in this relatively low-resolution picture insulin binds to its receptor. We may also be able to orient phosphorylation sites using anti-phosphotyrosine antibodies visualized in the electron microscope as frozen hydrated antibody-receptor complexes.

A relatively neglected but seemingly important aspect of receptor/tyrosine kinase biochemistry concerns the stoichiometry and symmetry of receptor autophosphorylation. As discussed above, all such receptors studied to date need to dimerize to become activated, and, based on studies of chimeric dimers with one-half inactivated, at least the initial autophosphorylation occurs in the *trans* direction (47, 49). We have verified that this mechanism applies to wild-type insulin receptors where, at early times of autophosphorylation (2–4 min), phosphotyrosine can be detected on only 50% of the αβ-halves of anti-phosphotyrosine antibody-precipitated receptor (J. Lee, S. E. Shoelson, and P. F. Pilch, unpublished observations). Thus the initial phosphorylation event is one-half phosphorylating the other. If the half being phosphorylated is kinase inactive, the resultant receptor cannot signal, and this mechanism can account for the *trans*-dominant negative phenotype seen for signaling for a number of receptor/tyrosine kinases (188) including the insulin receptor (182). When the ligand is monomeric, there is the possibility that if only one molecule of ligand binds, there will be asymmetry in binding and in autophosphorylation sites. This asymmetry might imply physiologically relevant functional differences in each receptor half. We have examined the issue of autophosphorylation asymmetry for insulin receptors covalently coupled to BBpa insulin where we can distinguish between the two halves of the receptor, one coupled to hormone and the other not. We had envisioned the possibility that one-half might be preferentially phosphorylated in the tri-tyrosine region and the other in the carboxy-terminal, but, to our surprise, all sites (we did not look at the juxtamembrane region) were phosphorylated on both halves of the receptor; however, there was 50% more incorporation on the half that did not bind insulin (91). The time of autophosphorylation and various ATP concentrations did not change this pattern. We are in the process of determining whether this asymmetry correlates with the relative amount of exogenous kinase activity. Taken together, the published data suggest that, within a single receptor, the initial phosphorylation event occurs as *trans*-phosphorylation in the tri-tyrosine region of only one-half of the receptor, but what happens next is still unclear. The stoichiometry of autophosphorylation is also unclear, although it has been reported that phosphate incorporation in vitro was ~4–8 mol phosphate/mol insulin holoreceptor (5, 84). These numbers are not necessarily precise (see next paragraph) and, in any case, represent a somewhat curious result since the expected maximum stoichiometry should be 14, if each tyrosine on each receptor half was phosphorylated. This does not seem to be the case, and one recent study concluded that only two tyrosine residues were phosphorylated in an insulin-dependent manner, and only these two phosphotyrosines could be reversibly dephosphorylated by transferring phosphates to ADP (5).

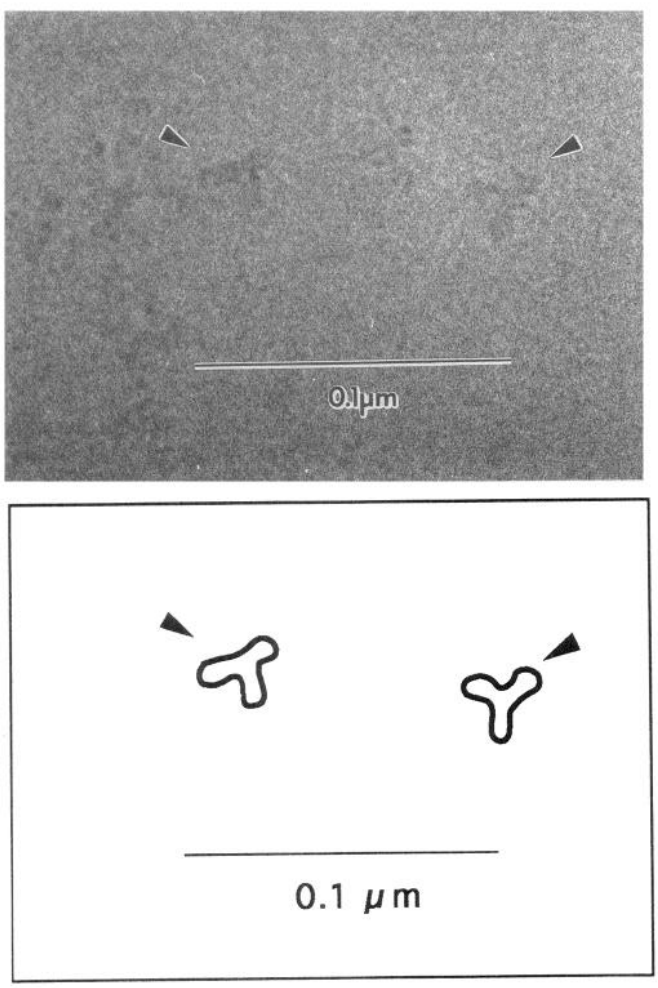

Fig. 3. The insulin receptor visualized by electron microscopy (×60,000) in the frozen hydrated states (*top*) and its illustration (*bottom*).

Part of the problem in ascertaining phosphorylation stoichiometry is that one is often dealing with mixed populations of receptor, some of which may not be phosphorylation competent due to partial proteolysis of the β-subunit (117) or denaturation. Thus normalizing to insulin binding is inadequate. Second, although many studies have determined that under the usual assay conditions only intramolecular receptor phosphorylation occurs, in cells, intermolecular mechanisms can clearly take place (10, 177), although the physiological significance of this has not been established. We are in the process of using BBpa insulin to address questions of phosphorylation stoichiometry and intermolecular phosphorylation because this reagent has particularly useful properties. We have shown that, in a population of

insulin receptors, only those covalently coupled to this insulin analogue (80–90%) become autophosphorylated, and thus we can normalize to fully functional receptor, not just to insulin binding (91) . Furthermore, we have used BBpa-insulin-activated insulin receptor, immobilized on avidin-agarose via the biotin group of Lys-B29, to phosphorylate soluble nonactivated receptor. This intermolecular phosphorylation proceeds to one-third the level of maximal autophosphorylation and occurs in both the tri-tyrosine and carboxy-terminal regions but gives no activation of exogenous kinase activity (J. Lee, S. E. Shoelson, and P. F. Pilch, unpublished observations). Addition of ligand, however, activates this receptor as if it had not been previously phosphorylated in an intermolecular manner. Thus we feel that this type of receptor cross talk, at least in the context of a homologous receptor system, is likely to be of no physiological significance.

In summary, the insulin receptor remains the focus of considerable experimental effort, both as a biochemical entity and in its physiological context in target tissues such as muscle and fat. The near future should see additional progress in understanding receptor structure, function, and physiology. Although there have been many recent breakthroughs in understanding the actions of insulin, history tells us that obtaining a complete understanding is almost certain to be more difficult than we anticipate.

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