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After Insulin Binds

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Three recent advances pertinent to the mechanism of insulin action include (i) the discovery that the insulin receptor is an insulin-dependent protein tyrosine kinase, functionally related to certain growth factor receptors and oncogene-encoded proteins, (ii) the molecular cloning of the insulin proreceptor complementary DNA, and (iii) evidence that the protein tyrosine kinase activity of the receptor is essential for insulin action. Efforts are now focusing on the physiological substrates for the receptor kinase. Experience to date suggests that they will be rare proteins whose phosphorylation in intact cells may be transient. The advantages of attempting to dissect the initial biochemical pathway of insulin action include the wealth of information about the metabolic consequences of insulin action and the potential for genetic analysis in *Drosophila* and in man.

INSULIN IS ONE OF THE BEST STUDIED VERTEBRATE PROTEINS. The first protein to be completely sequenced (*1*) and chemically synthesized (*2*), it has been the protein used in seminal analyses of hormone processing (*3*) and quantitation (*4*). Structures of the crystal forms of insulin have been elucidated in remarkable detail (*5*), the genes for insulin have been cloned (*6*), and structural mutations have been discovered (*7*). In 1980, human insulin became the first recombinant protein to be made available for clinical use (*8*). This impressive history is matched by a half-century of fundamental discoveries pertinent to the physiological effects of insulin on glucose homeostasis and intermediary metabolism (*9*).

In this article, I summarize some of the current approaches to the study of the biochemical mechanism of insulin action. The review is limited to recent advances in our understanding of the structure and function of the insulin receptor and the view that protein phosphorylation and dephosphorylation of seryl, threonyl, and tyrosyl residues are the central mechanisms by which insulin affects cell function.

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Insulin

Insulin is considered a vertebrate hormone although insulin-like polypeptides have also been identified in invertebrates [(*10*) and this review]. The physiological effects of insulin in mammalian systems include stimulation of hexose, ion, and amino acid uptake (*11*); modification of the activities of rate-limiting enzymes such as glycogen synthase, hormone-sensitive lipase, and pyruvate dehydrogenase by net dephosphorylation (*9*); phosphorylation of seryl residues in proteins such as ribosomal S6, acetyl coenzyme A carboxylase, and adenosine triphosphate (ATP) citrate lyase (*9*); regulation of gene expression for a small number of regulatory enzymes (thus far) (*12*); redistribution of membrane proteins such as the glucose transporter and the insulin-like growth factor II (IGF-II) and transferrin receptors (*13*); and promotion of cell growth (*14*). Many of these effects are tissue- or cell-specific and involve only a discrete subset of proteins. The chronology varies. Transcription of the gene encoding phosphoenolpyruvate carboxykinase is inhibited within seconds of the addition of insulin, whereas growth promotion requires hours of exposure (Table 1). Many of the rapid actions of insulin, such as stimulation of hexose transport and alterations of enzyme activities, do not depend on synthesis of new proteins or nucleic acids. Even this incomplete summary of the actions of insulin, however, invokes seryl and threonyl phosphorylations and dephosphorylations of cytosolic and mitochondrial proteins, membrane translocations with the likelihood of cytoskeletal protein involvement, and nuclear action. If a simplifying assumption is made that a single mechanism is involved in initiating all of these biological effects, and certainly there is precedent in the multihormone-sensitive adenylate cyclase system for a reductionist approach, then the analysis should begin with the first essential and common step in insulin action, interaction with the insulin receptor.

Insulin Receptor

The insulin receptor, identified initially as a cell-surface component capable of selectively binding insulin with high affinity and only later as a complex integral membrane glycoprotein with intrinsic enzymatic activity, is the necessary first step in insulin action. The receptor is ubiquitously distributed in mammalian organisms, with cells expressing different numbers of receptors (from 10^2 to 10^5 per cell), but rarely none. Although some tissue-specific differences have been reported, the basic oligomeric structure of the receptor is the same in all vertebrates examined and in the one invertebrate thus far analyzed (*15*). The capacity of the detergent-solubilized insulin receptor to bind insulin with the same affinity and specificity as it exhibits in an intact cell paved the way for the first purification of the receptor (from rat liver) by affinity chromatography (*16*). Subsequent analyses of the purified receptor, particularly of the human placental receptor (*17*), coupled with the application of procedures for selectively radiolabeling the ligand-binding component of the receptor (*18*) led to the conclusion that the receptor is a heterodimer. Its four subunits are glycosylated; the α subunits, with relative molecular mass (M_r) of 125,000 to 135,000 (on SDS–polyacrylamide gel electrophoresis), bind insulin and are linked, both to each other and to the β subunits, of M_r 95,000, by disulfide bonds. The disulfides linking the two α-β dimers are more sensitive to reduction than those linking the α and β subunits to each other. The proposal that there are only two kinds of subunits in the receptor tetramer was substantiated by biosynthetic studies in cultured 3T3-L1 preadipocytes and IM9 lymphoblasts (*19*). A proreceptor of M_r 180,000 to 210,000 was identified as the earliest biosynthetic product immunoprecipitable with antibodies to the receptor. With time, it generated the α and β subunits found in the processed cell-surface receptor. The half-life of the receptor, estimated to be between 7 and 12 hours in different cell types, is shortened by exposure of the cell to ligand. The latter phenomenon, a component of down regulation or net loss of cell-surface receptors on exposure to the homologous ligand, may play a role in enabling cells to regulate their sensitivity to ambient concentrations of hormone (*20*).

In 1982, insulin was reported to stimulate the phosphorylation of the β subunit of the insulin receptor in intact cells on seryl, threonyl, and tyrosyl residues (*21*). Within the same year an insulin-dependent protein kinase activity was described that catalyzed the phosphorylation of the β subunit of the receptor as well as the phosphorylation of other proteins and peptides (*22*). A striking difference between phosphorylation of the receptor in vitro and in intact cells was that the former reaction was absolutely specific for tyrosyl residues. It became apparent, when the insulin-dependent protein tyrosine kinase activity was found to copurify precisely with the insulin-binding activity of the receptor, that the insulin receptor itself was probably a ligand-dependent protein tyrosine kinase (*23*). A corollary of this conclusion is that the insulin-dependent seryl-threonyl phosphorylations of the β subunit are catalyzed by an enzyme or enzymes other than the receptor (see below). The discovery that the insulin receptor is an insulin-dependent protein kinase suggested a functional and structural relationship to the epidermal growth factor (EGF) receptor, the only other receptor that had been identified as a protein tyrosine kinase at that time (*24*). It cast the insulin receptor and, by inference, insulin with the oncogene-encoded protein tyrosine kinases and activators of protein tyrosine kinases (like EGF), a category of molecules that affect cell growth and differentiation. It seemed fairly straightforward then to propose that the signal elicited by the interaction of insulin with its receptor was mediated by one or more protein tyrosyl phosphorylations. The difficulty, not totally unanticipated given the preceding experience with the protein tyrosine kinase oncogene product pp60src, was finding the meaningful substrates.

Activation of the receptor kinase by autophosphorylation was first reported in 1983. Although the degree to which receptor tyrosine kinase activity depends on prior receptor autophosphorylation varies in different kinds of experiments, a consensus has emerged that autophosphorylation of one or more tyrosyl residues activates the receptor kinase activity toward exogenous substrates without affecting the insulin-binding properties of the oligomer (*25*). Furthermore, receptor isolated from insulin-treated cells under conditions that minimize dephosphorylation is more active and less insulin-dependent than receptor isolated from control cells. There is precedent for activation of protein kinases by autophosphorylation. The cyclic adenosine monophosphate (cAMP)–dependent and Ca^{2+}-calmodulin–dependent protein kinases are examples of protein serine kinases so regulated, and several of the oncogene-encoded protein tyrosine kinases also appear to be activated by tyrosine phosphorylation [(*26*) and this review]. A generalization cannot yet be made, however, about the role of autophosphorylation in the function of the growth factor receptor protein tyrosine kinases.

In contrast to autophosphorylation, seryl and threonyl phosphorylation of the β subunit of the receptor, induced by treating intact cells with the phorbol ester phorbol myristate acetate (PMA) (*27*) or agents that raise intracellular cAMP (*28*) or by incubating purified receptor in vitro with protein kinase C (*29*) or cAMP-dependent protein kinase (*30*), decreases receptor kinase activity. This decrease renders the receptor less susceptible to activation by autophosphorylation (*28*). It is thus possible that some of the physiological actions of cAMP or activators of protein kinase C that oppose those initiated by insulin may result from multisite phosphorylation of the receptor. The generalization can be advanced that tyrosyl phosphorylation activates the insulin receptor kinase and seryl-threonyl phosphorylation reverses that activation and inhibits the kinase. However, until all of the sites whose phosphorylation affects receptor kinase activity are identified and the function of each of the phosphoforms of the receptor is evaluated, it is not possible to fully estimate the functional complexities of receptor phosphorylation. In the case of pp60src, for example, biological activity is enhanced by tyrosyl dephosphorylation at the carboxyl terminus and by tyrosyl phosphorylation at position 416 (Tyr-416), a conserved autophosphorylation site (*31*). Although there is some information about autophosphorylation sites of the insulin receptor (see below), it is not known where the seryl and threonyl phosphorylations occur; there is also no reason to believe that phosphorylation of the β subunit on these residues in response to insulin is catalyzed by either protein kinase C or the cAMP-dependent protein kinase.

In 1985, the complementary DNA (cDNA) for the human insulin proreceptor was cloned and its complete amino acid sequence was

Table 1. Chronology of insulin action.

Seconds
Binding to receptor
Activation of receptor protein tyrosine kinase
Receptor autophosphorylation
Seconds to minutes
Changes in gene transcription
Stimulation of hexose and ion transport
Ligand-mediated receptor internalization
Alterations in intracellular enzyme activities
Seryl and threonyl phosphorylation of the receptor
Hours
Synthesis of protein, lipid, and nucleic acid
Maximal down regulation of the receptor
Cell growth

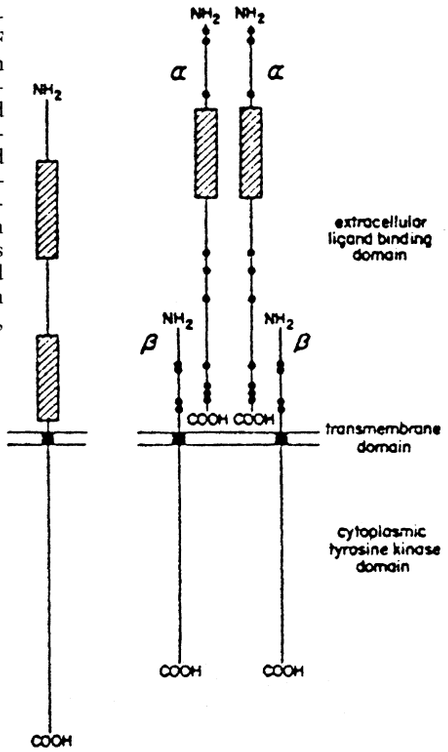

Fig. 1. Schematic comparison of insulin and EGF receptors. Regions of high cysteine residue concentration are shown as hatched boxes, transmembrane domains as black boxes, and single cysteine residues—possibly involved in formation of the α_2-β_2 insulin receptor complex—as black circles. [Reprinted from (*32*) with permission © Macmillan Journals, Ltd.]

deduced (*32*) (Fig. 1). Shortly after the first report, an independent analysis revealed a nearly identical sequence (*33*). In both cases, the cloning strategy involved the use of oligonucleotides corresponding to partial amino acid sequences of both α and β subunits to probe human placental cDNA libraries. Proof that the cloned cDNA encoded the insulin receptor and not the IGF-I receptor (which has a similar subunit composition and is also a ligand-dependent protein tyrosine kinase) was obtained with the use of antipeptide antibodies to deduced sequences of the insulin receptor that were not conserved in the human placental IGF-I receptor (*34*), by analysis of the ligand specificity of the receptor expressed in cells transfected with the insulin receptor cDNA (*35*) and, recently, by the cloning of the IGF-I receptor cDNA (*36*).

The cloning of the insulin proreceptor cDNA confirmed the predictions that the receptor precursor is an integral membrane protein with a protease-sensitive site separating α and β subunit domains. The α subunit is on the amino-terminal side of the β subunit, and the cytoplasmic domain of the latter contains a sequence homologous but not identical to other known protein tyrosine kinases. The α subunit has a cysteine-rich domain and is predicted to reside in its entirety on the external surface of the plasma membrane. It is anchored to and through the membrane by the β subunit, which is predicted to traverse the membrane once. The sequence of the cytoplasmic kinase domain of the receptor is most homologous to a subfamily of protein tyrosine kinases that includes the kinase domains of the oncogenes *ros* and *onc* D (*37*) and the IGF-I receptor. The protein kinase domain of an insulin receptor homolog in *Drosophila melanogaster*, the only insulin receptor kinase other than the human whose partial amino acid sequence has been reported (*38*), is 53 percent homologous to the human insulin and IGF-I receptor kinases and slightly less so to *ros*. The ligand-binding subunits of the human insulin, human IGF-I, and *Drosophila* insulin receptors are more different from each other than are their corresponding kinase domains. It will be of interest to compare the sequences of these three receptors to the sequence of the IGF-II receptor, which is not thought to be a protein kinase and might, therefore, be homologous only to the ligand-binding portions of the insulin and IGF-I receptors.

Knowledge of the primary sequence of the human insulin receptor made it possible to analyze autophosphorylation sites and domains critical for kinase activity. With the limited information available from in vitro analyses (*39*) to predict those tyrosyl residues that serve as targets of autophosphorylation, Tyr-1316 near the carboxyl terminus, Tyr-1146, Tyr-1150, and Tyr-1151 in the kinase domain, and Tyr-960 near the amino terminus of the cytoplasmic portion of the β subunit seemed reasonable possibilities (*40*). However, since the sequences surrounding the phosphorylation sites of physiological substrates of the receptor kinase (other than the receptor itself) are not known, and we have no notion of how multiple sites of phosphorylation might influence each other (within a single β subunit or between the two linked β subunits), it is probably necessary to consider all tyrosines in the cytoplasmic domain of the β subunit as potential autophosphorylation sites. Chemical and enzymatic digestion of the β subunit after autophosphorylation in vitro followed by immunoprecipitation of the fragments with antipeptide antibodies indicate two major regions of phosphorylation at the carboxyl terminus (including Tyr-1316 and Tyr-1322) and in the conserved kinase domain (Tyr-1146, Tyr-1150, and Tyr-1151) (*41*). Less than 10 percent of the total β subunit phosphorylation is precipitated by an antibody to the amino terminus of the cytoplasmic portion of the β subunit (amino acids 952 to 967 of the proreceptor), which includes Tyr-953 and Tyr-960. The latter region, between the transmembrane portion of the β subunit and the ATP-binding site, may be important, however, since the antibody to this sequence specifically and completely inhibits the kinase activity of the unphosphorylated receptor (*40*). Although the antibody also binds to the phosphoreceptor, it does not affect the activity of this form of the enzyme. Thus, this portion of the receptor may to play a role in insulin-dependent receptor kinase activation, but it is not clear that this is the result of autophosphorylation of its resident tyrosines (Tyr-953 and Tyr-960).

Receptor activation in vitro correlates temporally with phosphorylation of the kinase domain (tyrosines 1146, 1150, and 1151) rather than with phosphorylation at the carboxyl terminus (*41*). The same domain (including Tyr-1146, Tyr-1150, and Tyr-1151) is also phosphorylated in intact cells in response to insulin (*42*). Consistent with the proposal that phosphorylation of this region of the subunit is involved in the autophosphorylation-mediated kinase activation is an experiment in which mutant receptors were generated that contained phenylalanine residues in place of Tyr-1150 or both Tyr-1150 and Tyr-1151. These receptors, expressed in transfected Chinese hamster ovary (CHO) cells, exhibited greatly diminished insulin-stimulated receptor protein tyrosine kinase activity (*43*). Unquestionably receptor autophosphorylation occurs at multiple sites (*44*); the number of permutations increase further when seryl and threonyl phosphorylations are considered, to say nothing of the array of possibilities introduced by having two β subunits in each receptor oligomer. It may be that successive autophosphorylated reactions amplify the initial activation of the unphosphorylated receptor kinase by insulin. In fact, recent analyses in which the in vitro autophosphorylation sites were sequenced indicate that the tyrosines at positions 1146, 1150, 1151, 1316, and 1322 are all modified (*45*).

Signal Transduction

Insulin interacts with the extracellular α subunit of the receptor, activating the protein kinase of the cytoplasmic β subunit. How an interaction on the outside of the cell is transmitted through a single

transmembrane domain in the lipid bilayer is unknown. It has been suggested that ligand occupation induces receptor aggregation thereby altering the conformation of the β subunit or that the two α-β dimers of the receptor tetramer are essential for insulin-dependent activation of the receptor kinase (*46*). Ultrastructural analyses indicate that insulin can induce receptor clustering (*47*). Whatever the biophysical mechanism, the most rapid postbinding effect of insulin is receptor kinase activation. Is this, then, the second step in insulin action? Certainly the conclusion after extensive genetic manipulations of the protein tyrosine kinase oncogene products is that their kinase activity is essential (although not necessarily sufficient) for biological activity.

Direct evidence that the protein kinase activity of the insulin receptor is essential for insulin action has recently been obtained with monoclonal antibodies to the insulin receptor kinase domain as well as site-directed mutagenesis of the insulin receptor cDNA. Stable CHO cell transfectants were isolated that express either normal human insulin receptors or human receptors whose lysine at position 1018 of the ATP-binding site has been replaced by alanine (*48*). In both cases the cell lines express ten times more human than CHO receptor. The functional analysis is based on the prediction that expression of an increased number of receptors will render the CHO cell more sensitive to insulin if the newly introduced receptors can transduce insulin's actions. The mutant and wild-type human insulin receptors are processed normally and equivalently and bind insulin with the same affinity. As predicted, the Lys → Ala-1018 mutant has no kinase activity; it neither autophosphorylates (in intact cells or in vitro) nor catalyzes exogenous substrate phosphorylation. Six post-receptor effects of insulin were studied: glycogen synthesis, thymidine incorporation into DNA, hexose (deoxyglucose) uptake, S6 kinase activation, cellular substrate phosphorylation on tyrosyl residues, and receptor seryl and threonyl phosphorylation. In the first five cases, cells expressing the normal human receptor were about ten times more sensitive to insulin than the parental CHO cell line. In the last case, the increase in receptor seryl and threonyl phosphorylation is commensurate with the cellular content of wild-type receptor. Cells expressing the mutant receptor show no increase in insulin sensitivity with respect to any function and, in most cases, exhibit the same insulin sensitivity as the untransfected CHO cells. Glycogen synthesis, thymidine incorporation into DNA, S6 kinase activation, nonreceptor tyrosine phosphorylation, and receptor seryl and threonyl phosphorylation are the same in cells bearing 20,000 mutant human receptors and 2,000 normal CHO receptors and in cells expressing only the 2,000 CHO receptors.

It is surprising that basal deoxyglucose uptake is depressed and far less sensitive to insulin in cells expressing the mutant receptor than in parental, untransfected cells. A similar result for transport was reported for cells transfected with cDNAs encoding mutant, kinase-deficient human receptors bearing substitutions of alanine, methionine, or arginine for the critical lysine in the active site of the enzyme (*49*). It was also observed in the mutant that had a defect in the Tyr-1150–Tyr-1151 autophosphorylation site and a consequent decrease in protein kinase activity (*43*).

The simplest interpretation of all of the mutant receptor experiments is that insulin action depends on the kinase activity of the receptor. This conclusion covers a range of insulin actions including transport, modifications of enzyme activities, and DNA synthesis. The possibility that the lysine substitution alters the conformation of the receptor, rendering it dysfunctional for reasons unrelated to its kinase activity, is unlikely. Receptor processing, turnover, subunit size, and insulin binding are unaffected by this mutation (*48*) and a set of conformation-sensitive monoclonal antibodies do not distinguish mutant and wild-type receptors (*49*). In addition, substitution of arginine for lysine at position 1018 yields a receptor with the same properties as the Lys → Ala-1018 mutant (*49*).

The deoxyglucose uptake studies are of interest. Possible explanations for the negative rather than the nugatory effect of the mutant receptors include a requirement for clustering of wild-type receptors for this function and not for the others, inability of hybrid receptors (human mutant–CHO wild type) to mediate transport effectively, and adsorption by the nonfunctional receptor of some rate-limiting cellular factor required for glucose transport. The perplexing observation that basal deoxyglucose uptake is depressed in cells expressing kinase-deficient receptors suggests that the wild-type receptor may somehow be involved in constitutive hexose transport even in the absence of insulin.

The lack of seryl and threonyl phosphorylation of the mutant receptor (*50*) suggests that either the receptor protein tyrosine kinase activity is necessary to activate the requisite protein serine kinase or that the autophosphorylated receptor is the substrate for this enzyme. If the latter turns out to be the explanation, the situation might be analogous to the β-adrenergic receptor kinase that recognizes only the agonist-occupied receptor (*51*).

The data from the mutagenesis experiments lead to the conclusion that insulin action depends on receptor protein tyrosine kinase activity. The same conclusion was reached by Morgan and Roth (*52*), who found that monoclonal antibodies that inhibit receptor kinase activity interfere with insulin action in the cells into which they have been introduced. There are, however, some results with polyclonal antisera to the insulin receptor that challenge this proposal. The argument by analogy most difficult to understand concerns antisera that mimic insulin action on hexose transport and lipogenesis but do not appear to stimulate receptor kinase activity (*53*). We have recently reevaluated one of these antisera, however, and found that although it does mimic insulin action, it does not do so in cells expressing kinase-deficient receptors. Furthermore, the antiserum as well as an immunoglobulin fraction prepared from it activate the receptor kinase both in intact cells and in vitro (*54*).

It is now possible to determine whether the kinase activity of the insulin receptor can be activated by the ligand-binding domain of another receptor and, conversely, whether activation of a protein tyrosine kinase other than the insulin receptor can transduce the biological activities of insulin. When cells are transfected with a construction in which the DNA encoding the ligand-binding domain of the insulin receptor is fused to the DNA encoding the transmembrane and kinase domains of the EGF receptor, interaction of the expressed protein with insulin activates the protein tyrosine kinase activity derived from the EGF receptor (*55*). It would be interesting to know if EGF action is also mediated by such a chimeric receptor. In another analysis, substitution of the transmembrane and kinase domains of v-*ros* for the homologous β subunit sequences of the insulin receptor results in a hybrid receptor whose kinase is stimulated by insulin but fails to promote deoxyglucose uptake (*56*). This result emphasizes the point that although the primary amino acid sequences of the catalytic domains of the tyrosine kinases are similar, the differences in the enzymes are responsible for the distinctive biological effects of their stimulatory ligands. Transfection into CHO cells of a cDNA encoding solely the cytoplasmic domain of the β subunit results in expression of a highly active soluble kinase that does not increase the basal deoxyglucose uptake of the cell, whereas expression of the kinase as a membrane-bound enzyme does (*57*). Thus insulin action (or at least insulin-dependent stimulation of hexose transport) is mediated by a membrane-associated, specific protein tyrosine kinase.

Any consideration of insulin action must include some discussion of "insulin mediators," a group of low molecular weight compounds generated in response to insulin (*58*). When reported for the first time, before the kinase activity of the insulin receptor was elucidat-

ed, the concept of insulin mediators was modeled on the cAMP–second messenger system. The mediators, isolated either from insulin-treated cells or by direct addition of insulin alone to purified plasma membranes, have been reported to affect the activity of several cellular enzymes such as cyclic nucleotide phosphodiesterase, pyruvate dehydrogenase, and adenylate cyclase in the same manner that insulin does when added to intact cells. They were originally thought to be peptides, but recent studies indicate that one or more of them are, instead, inositol phosphate–glycans generated by hydrolysis of a glycanphosphoinositide in the plasma membrane (*59*). More work is needed to determine whether these molecules are, in fact, obligate mediators of some of the insulin's actions and, if so, how their generation is triggered. It will also be important to evaluate the effect of chemically defined mediators on purified enzymes. Mediators have not been invoked for other protein tyrosine kinases unless one considers the metabolic generation of inositol phosphates and diacylglycerol from phosphatidylinositol as a case in point. However, the conclusion that insulin action is initiated by a protein tyrosine kinase is not inconsistent with the possibility that small intracellular molecules generated as a result of receptor kinase activation may play a role in insulin physiology.

If insulin action is dependent on the protein kinase activity of the receptor, ligand-mediated internalization of the receptor might deliver an activated kinase to an intracellular site not accessible to the plasma membrane–bound enzyme. Insulin, linked to its receptor, has been detected in the nucleus (*60*), and activated insulin receptor kinase has been found in endosomal fractions after intact cells have been exposed to insulin (*61*). Thus ligand-mediated receptor internalization, a rapid process compatible with the kinetics of some early actions of insulin, may function not only to clear the cell surface of its insulin response system but also to deliver an activated insulin receptor kinase to intracellular substrates. In this connection, it is interesting that the kinase-deficient human insulin receptor previously discussed (*50*) does not internalize its receptors in response to ligand. Internalization of the receptor per se, however, is insufficient for insulin action or receptor phosphorylation (on tyrosyl, seryl, or threonyl residues), since neither occurs when receptors are internalized by monoclonal antibodies directed against the receptor that do not activate the receptor kinase (*50*). It would be interesting if one could engineer an insulin receptor that retains its insulin-dependent protein tyrosine kinase activity but does not undergo homologous downregulation.

Substrates for the Insulin Receptor Kinase

One can imagine at least two (nonexclusive) mechanisms by which the insulin-dependent receptor kinase transduces the insulin signal. The most obvious is by catalyzing the phosphorylation of cellular protein substrates. Alternatively, the receptor itself may be the principal substrate for its own kinase activity. Autophosphorylation would allow the receptor to interact with or dissociate from one or more cellular constituents, thereby initiating biochemical processes involved in insulin action.

Considerable effort has been spent in search of natural substrates for the insulin receptor kinase. The standard protocol for such studies, insulin-treatment of ^{32}P-labeled cells followed by two-dimensional SDS–polyacrylamide gel electrophoresis and autoradiography, has not been rewarding. In fact in some of the early searches for substrates for other protein tyrosine kinases, exposure of cells containing insulin receptors to insulin was considered a control (*62*). Most of the proteins phosphorylated in response to insulin are modified not on tyrosyl residues but on seryl and threonyl residues and therefore cannot be direct substrates for the receptor kinase. Examples include ribosomal protein S6, ATP citrate lyase, and acetyl coenzyme A carboxylase. A general problem frustrating the search for proteins phosphorylated on tyrosyl residues is the lability of the tyrosine phosphoester bond which makes it difficult to detect in cells and to preserve during protein isolation.

A major substrate for tyrosyl phosphorylation in essentially all mammalian cells is the insulin receptor itself. Although it is a minor cell constituent, it is the most prominent substrate evident after SDS–polyacrylamide gel electrophoresis. Perhaps this is because it is autophosphorylated, multiply phosphorylated, and ubiquitous. To qualify as a bona fide substrate, a protein should be phosphorylated in intact cells as well as in vitro, its activity should be demonstrably altered by its state of phosphorylation, and it must be modified specifically by physiological concentrations of insulin at a rate appropriate to its function. Thus far the insulin receptor itself is the only protein to fulfill these criteria. However, several proteins that may ultimately qualify have been recently described. Two of these are a 180-kilodalton protein (*63*) and a 15-kilodalton protein (*64*) that may play a role in insulin-mediated hexose transport. The former, but not the latter, is also phosphorylated in response to IGF-I. The identity and physiological roles of these proteins remain to be elucidated; both are rare and fleetingly phosphorylated in intact cells.

The fact that so little is known about substrates despite intensive investigation suggests that they are labile, nonabundant, or both. Until one or more physiologically significant substrates are identified, there remains the formal, albeit unlikely, possibility that there are none (other than the receptor itself). Clearly there does not appear to be a cascade of protein tyrosyl phosphorylations analogous to the classic reactions of glycogen breakdown. Each step in glycogenolysis involves phosphorylation of a protein present in greater abundance than the one preceding it (*65*). If rare molecules like brain polyphosphoinositide phospholipase C and hepatic S6 kinase [both of which comprise less than 0.001 percent of cell protein (*66*)] are examples of enzymes eligible for direct modulation by tyrosyl phosphorylation, the only rational approach to finding substrates is to know fairly precisely where to look.

A Role for Protein Seryl Phosphorylation and Dephosphorylation in Insulin Action

Since the substrates for insulin-mediated protein tyrosine kinase activity may be as rare or rarer than the insulin receptor itself, is the initial activation of the receptor kinase subsequently amplified? One possibility is that insulin ultimately uses reversible seryl and threonyl phosphorylation like the many hormones whose actions do not proceed through an obligatory tyrosine kinase step. Phosphorylation and dephosphorylation of specific seryl and threonyl residues in proteins comprise a central regulatory mechanism in eukaryotic biology, and the metabolic processes influenced by insulin include those whose rate-limiting enzymes are regulated directly by seryl and threonyl phosphorylation. It may, therefore, be profitable to consider insulin action within the framework of other pathways used by polypeptides to signal across membranes. The adenylate cyclase system utilizes a number of different gene products—hormones, receptors, cyclase, G proteins—to generate an intracellular ligand, cAMP, that activates a protein serine kinase, cAMP-dependent protein kinase (*67*). One form of guanylate cyclase may serve as a combined receptor-enzyme to generate a ligand, cyclic guanosine monophosphate (cGMP), that activates a cGMP-dependent protein serine kinase (*68*). Ligands interacting with certain growth factor receptors activate a membrane-associated phospholipase C that, coupled to a G protein, generates phosphorylated inositols and

diacylglycerol. These products of phosphatidylinositol turnover activate protein kinase C and, indirectly, other Ca^{2+}-dependent protein serine kinases (*69*). Perhaps features of these pathways for altering protein seryl phosphorylation are incorporated into a function of the insulin receptor. In such a model, the receptor, possibly coupled to a G protein (*70*) will catalyze the phosphorylation (on tyrosyl residues) of a small subset of proteins that may include one or more of the known insulin-activated serine kinases (*71*). These, in turn, activate or inactivate the more prevalent protein serine kinases and phosphoprotein phosphatases that are directly involved in some of the final steps of insulin action.

Future Directions

The promise of the insulin–insulin receptor system resides in the extensive repertoire of information about the biochemical, physiological, and pathophysiological consequences of insulin action. This, it is hoped, can be used to trace the distal actions of insulin (such as dephosphorylation of glycogen synthase or phosphorylation of ribosomal protein S6) back to the earliest reactions initiated by the binding of insulin to its receptor. Pathways may be analyzed in normal cells and contrasted with those in insulin-resistant cells derived from patients with diabetes mellitus or from animal models of obesity and diabetes.

The ability to modify the insulin receptor by manipulating its cDNA and introducing it into mammalian cells that contain few endogenous receptors makes it possible to map those regions of the protein that are critical for transduction of the insulin-specific signal. This should be particularly helpful in designing affinity-purification procedures for proteins with which the receptor interacts. Similarly the availability of cDNAs for some of the proximate targets of insulin action like the glucose transporter (*72*) should make it possible to design modifications of these molecules that will facilitate the identification of proteins with which they interact and help to detect their subcellular locations.

Two kinds of genetic systems are available for studying insulin action. The first are those diabetics (or animal models of diabetes) whose disease has a heritable component. The term diabetes mellitus embraces a complex group of diseases. Some forms of the disease result from deficiencies of insulin secretion or aberrancies in insulin structure and processing. In other forms of diabetes there are abnormalities in insulin receptor content, insulin binding, or receptor protein tyrosine kinase activity (*73*). It is likely that some diabetics have defects in proteins with which the receptor interacts. It is now feasible to delineate the molecular alterations in the receptor gene that result in abnormal receptor content or functional defects in receptor structure. Furthermore, since many, if not all, of these defects ultimately manifest themselves as cellular deficiencies in insulin-dependent protein tyrosine kinase activity, new approaches to therapy may evolve from an understanding of the critical differences between this enzyme and other types of protein kinases.

Another genetically amenable system for studying insulin action is *Drosophila melanogaster*. *Drosophila* and other insects synthesize an insulin-like polypeptide that has biological activity both in standard mammalian bioassays and in insects. The putative hormone is present in specific neurosecretory cells located in ganglia (*10*). An insulin receptor homolog with a subunit structure very similar to that of the mammalian receptor has been identified in *Drosophila* embryos and adults, as well as in cultured *Drosophila* cells (*74*). A genomic sequence corresponding to the kinase domain of the putative receptor was identified by using the human insulin receptor cDNA. The amino acid sequence of the *Drosophila* receptor is homologous to the human receptor. Antibodies to two independent sequences in the β subunit of the human receptor and to a sequence in the α subunit of the human receptor are able to immunoprecipitate the corresponding subunits of the fly receptor. The receptor in *Drosophila*, like the mammalian protein, is an insulin-dependent protein kinase; it is not activated by unrelated peptides like EGF or even by peptides with homology to insulin such as IGF-I or silkworm prothoracicotropic hormone. It is represented as a single-copy gene. The abundance of its messenger RNA changes during development and is markedly increased during mid-embryogenesis. This raises the possibility that there is a role during early development for an insulin-like molecule and the protein tyrosine kinase that it activates. Activation of the receptor kinase might also be mediated by specific cell-cell interactions. For example, an insulin-like molecule could be expressed on the surface of one cell and interact with an insulin receptor expressed on another cell. Such a mechanism may pertain in homeotic genes like "sevenless" that encode a transmembrane receptor-like protein (*75*).

The observation that *Drosophila* has an insulin response system like that found in mammalian organisms offers a unique opportunity to generate mutations and deletions that could then be rescued by reintroduction of wild-type or mutated genes. It might be possible to generate disease models in flies and, perhaps even more interesting, to devise schemes for selecting second-site mutations that would reveal unknown components involved in insulin action.

In conclusion, much has been learned about the early actions of insulin during the past 5 years. However, the story of what happens after insulin binds remains unfinished.

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