THE INSULIN RECEPTOR: STRUCTURE AND FUNCTION

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I. INTRODUCTION

Insulin binding to specific membrane receptors on target cells initiates a wide spectrum of biological effects. These include enhanced transport of sugars and amino acids, stimulation of anabolic pathways through the modulation and induction of specific enzymes, and enhancement of cell growth by triggering RNA and DNA synthesis (for recent reviews on insulin action see References 1 to 5). These effects of insulin vary in their time of onset, from very rapid effects of enhanced transport, which occur within a few seconds, to up to enhanced synthesis of nucleic acids, which initiates only several hours following stimulation of cells with the hormone.

At the molecular level, insulin action appears to be mediated through translocation, phosphorylation, and dephosphorylation of several hormone receptors, transporters, and enzymes located at key regulatory positions of fat and carbohydrate metabolic pathways. Examples are the stimulatory effects of insulin on the rate of internalization of its own receptor[6,7] and on the redistribution between the plasma membrane and intracellular organelles of glucose transporters,[8,8a] the receptors for insulin-like growth factor-II (IGF-II),[9,9a] and transferrin.[10]

Many of the insulin's actions are mediated through phosphorylation/dephosphorylation reactions. Phosphorylation, in general, is the most common type of reversible modification exercised to regulate biological functions. More than 70 enzymes and numerous other proteins are modified in such a manner (for recent reviews see References 11 to 14a). Aside from inducing phosphorylation of its own receptor, insulin enhances phosphorylation of other proteins such as acetyl-CoA carboxylase, ATP citrate lyase, and ribosomal protein S6.[3] Insulin also enhances dephosphorylation of glycogen synthase, pyruvate dehydrogenase, triglyceride lipase, and phosphorylase kinase.[3] Dephosphorylation of glycogen synthase and phosphorylase b increases the activity of the former and decreases the activity of the latter. This leads to enhanced glycogen synthesis and reduced glycogen breakdown.

There is no evidence to suggest an effect of insulin on other types of chemical modifications such as nucleotidilation, methylation, acetylation, tyrosylation, or sulfation, but there are reports that insulin might inhibit cholera toxin-catalyzed ADP-ribosylation.[15]

Insulin action is initiated upon binding of the hormone to specific receptors on the cell surface. The information required to initiate insulin's bioeffects resides within the receptor itself. Such a notion is supported by findings that lectins[16] and certain antibodies to the receptor[17] act as insulinomimetic agents capable of inducing most biological effects of the hormone. Unraveling the molecular basis of insulin action therefore involves a detailed analysis of structure-function relationships within the receptor complex itself.

Progress in understanding the molecular basis of the receptor function has been achieved

by the demonstration that the insulin receptor is an insulin-sensitive tyrosine kinase able to phosphorylate itself as well as other protein substrates (for recent reviews see References 2 to 5, 18 and 19). The role of the kinase activity in hormone-signal transduction is not fully elucidated, but rapidly accumulating data support the notion that phosphorylation of the insulin receptor, which activates its tyrosine kinase, plays a key role in mediating insulin action. According to this hypothesis activation of the insulin receptor kinase may set into motion a cascade of reactions leading eventually to the expression of some or all of the effects of insulin. Experimental evidence to support such a notion is briefly summarized below: (1) dissociation of insulin from its receptor, which results in a loss of insulin's response,[20] is paralleled by a reduced phosphorylation of the insulin receptor in intact cells;[21] (2) severe reduction in receptor autophosphorylation and tyrosine kinase activity is noted in insulin-resistant patients;[22-28] streptozotocin-induced diabetic rats[29] and insulin-resistant mice rendered obese by injection of gold thioglucose;[30] (3) insulinomimetic agents such as H_2O_2,[30a] vanadate,[31-33] trypsin,[34-36] lectins,[16] and antireceptor antibodies[17,37,38] lead to increased receptor phosphorylation; (4) injection of antibodies that block receptor kinase activity, into oocytes, inhibits their insulin-induced maturation;[39] (5) injection of the same antibodies into Chinese hamster ovary cells or rat adipocytes[40] blocks several of the rapid effects of insulin; (6) tumor-promoting phorbol esters[41] and catecholamines,[42] added to intact cells, decrease in parallel, receptor kinase activity and insulin's bioeffects; (7) cells transfected with insulin receptors mutated at their ATP binding sites[43-46] or autophosphorylation sites[47] lose most or all of their insulin-mediated biological effects.

Taken together, these findings clearly establish a crucial role for the insulin receptor kinase in mediating insulin action. The purpose of this review is therefore to summarize the recent knowledge about the structure-function relations of the insulin receptor with a special emphasis on the receptor function as a tyrosine kinase.

II. THE SUBUNIT STRUCTURE OF THE INSULIN RECEPTOR

The insulin receptor is a 350,000-kDa transmembrane glycoprotein made of two α (125 to 135 kDa on SDS gels) and two β (95 kDa) subunits, linked through disulfide bonds. Mammalian cells express 100 to 250,000 receptors on their cell surface. Cells expressing the highest number of receptors, i.e., adipose, muscle, and liver cells, are the more responsive to insulin and indeed function as mediators of most of insulin's metabolic effects.[1-5] The model for the insulin receptor structure is based upon biochemical and immunological studies, and was recently confirmed by analyzing the amino acid sequence of the human insulin receptor precursor deduced from human placental cDNA clones.[48,49] Structural studies required prior purification of the receptor.[50,50a] It involves three basic steps: solubilization of the receptor in Triton® X-100; isolation of the glycoprotein fractions through binding to and elution from a column of wheat germ agglutinin coupled to agarose; and affinity chromatography of the receptor on a column of insulin-agarose. Early studies revealed only the presence of the α subunits. This was evident when affinity-purified receptors were run under reducing conditions on SDS gels. The only stained protein appeared as having a molecular mass of 135 kDa.[51] The presence of the 95-kDa β subunits was revealed on stained SDS gels only a few years later,[52] when a receptor preparation was purified 2400-fold with an overall yield of 40%. Failure of earlier attempts to detect the β subunits presumably reflects the resistance of the β subunits to staining, combined with their higher susceptibility to proteolytic cleavage.[53]

Elucidation of the insulin receptor structure was largely facilitated upon introduction of antiinsulin receptor antibodies that specifically immunoprecipitate intact receptors. Four sources of such antibodies are now available: (1) naturally occurring antibodies in sera from patients (type B) with extreme insulin resistance and acanthosis nigricans;[54-57] (2) antibodies

prepared in rabbits by injecting them with purified receptor preparations;[58] (3) monoclonal antibodies to the purified receptor;[59,60] and (4) polyclonal antibodies to various synthetic peptides deduced from the amino acid sequences of both α and β receptor subunits.[61]

Such antibodies quantitatively immunoprecipitate insulin receptors from cells that are either surface labeled with ^{125}I [62] or metabolically labeled with [^{35}S] methionine, [^{3}H] leucine, [1,6-^{3}H] glucosamine, [^{3}H] fucose, [^{3}H] mannose, or [^{3}H] galactose.[63,64]

A. The Insulin Receptor α Subunit — The Insulin Binding Domain

Assigning the insulin binding domain to the α subunit of the receptor is based upon experiments that demonstrated that this α subunit is mainly labeled when receptors present on intact cells,[65] membranes,[65-67] or solubilized preparations[68] are first incubated in the presence of ^{125}I-insulin, which is then cross-linked to the protein by cross-linking agents such as disuccinimidyl suberimidate. Cross-linking of labeled insulin to the β subunit is much weaker[53] or does not occur at all.[51,69,70] In these experiments, inclusion of unlabeled insulin at nanomolar concentrations abolishes binding of the labeled hormone, while addition of irrelevant hormones[65,70] has no inhibitory effect. Furthermore, immunoprecipitation of affinity-labeled insulin receptors with antireceptor antibodies reveals only the presence of an affinity cross-linked α subunit.[71] These and similar studies lend support to the hypothesis that insulin binds specifically and with a high affinity to the α subunit.

B. The Insulin Receptor β Subunit — Protein Tyrosine Kinase

The structure and function of the receptor β subunits were elaborated with a somewhat greater difficulty. This is due to several reasons: (1) the β subunit is poorly affinity labeled by insulin;[53] (2) it stains weakly by Coomassie® blue or silver staining compared with the α subunit;[67] (3) it is more sensitive to proteolytic cleavage;[53] and (4) it is more heat labile.[72] For example, incubation of partially purified receptors at 45°C for 10 min completely abolishes the kinase activity associated with the β subunit, while only 20% of the insulin binding activity is lost.[72] In spite of these difficulties, a protein that migrates on reducing SDS gels as a 95-kDa protein was identified as the β subunit of the receptor based on affinity chromatography,[68] immunoprecipitation of surface or biosynthetically labeled receptors,[63,74,75] and affinity labeling with photoactive insulin analogs.[66,68,71,73] Since the β subunit of the receptor functions as a tyrosine kinase capable of autophosphorylation, this subunit is uniquely labeled when insulin receptors are immunoprecipitated from [^{32}P]-labeled cells.[76]

III. GENERAL PROPERTIES OF THE INSULIN RECEPTOR

A. The Oligomeric Structure of the Insulin Receptor

The native insulin receptor has an apparent molecular mass of 350,000 Da. This value is based upon sizing experiments involving gel filtration, ultracentrifugation, and resolving labeled receptors on nonreducing SDS gels.[1] Under such nonreducing conditions, a major fraction of the receptor (isolated from adipocyte and placental membranes[77]) migrates as an oligomer with an estimated molecular mass of 350 kDa. This suggests a stoichiometry of an $\alpha_2\beta_2$ for the native form of the receptor. Larger and smaller oligomers also exist in receptors isolated from lymphocytes or rat hepatoma cells.[78] These findings point to the possibility that the receptor maintains several different oligomeric forms that could result due to sequential proteolysis of the β subunit. It was shown,[18] for example, that an apparent 320-kDa receptor species is formed when one β subunit is cleaved, and an apparent 290-kDa receptor species is observed upon cleavage of both β subunits by elastase or elastase-like proteases.[18] Such treatment results in the appearance of a cleaved β subunit, with a molecular mass of 49 kDa, denoted β_1.[53]

B. Disulfide Linking of the Insulin Receptor Subunits

Reduction of native receptors with mercaptoethanol or dithiothreitol prior to separation on SDS gels reveals the presence of only free α and β subunits, which suggests that the oligomeric structure of the receptor is maintained through disulfide bonds. Two classes of such disulfide bonds are found: those denoted class I exist between homologous subunits of the receptor and are more susceptible to reduction with dithiothreitol. Reduction of these disulfide bonds generates "receptor halves" made of α-β dimers with an apparent molecular mass of 210 kDa.[77,79] Class II disulfides, which are more resistant to reduction, exist between the α and β subunits, and their reductions yield free α and β subunits.[79] It is of interest to note that even after reduction the mature receptor maintains its $\alpha_2\beta_2$ conformation unless SDS is added to dissociate the free receptor subunits.[79]

C. Structural Topology of the Insulin Receptor within the Plasma Membrane

The transmembrane orientation of the α and β subunits was initially determined by iodination and specific immunoprecipitation of the receptor from adipocyte plasma membranes.[80] The β subunit is iodinated in both right-side-out and inside-out plasma membrane vesicles, whereas the α subunit is iodinated on right-side-out vesicles only. These experiments suggest that the α-subunit, or at least all of its tyrosine residues available for iodination, are located at the extracellular side of the plasma membrane. By contrast, the β subunits appear as having extracellular, intracellular, and transmembrane domains. Such a model is confirmed by the deduced amino acid sequence of the insulin receptor,[48,49] which predicts the presence of a single stretch, 23 amino acids long, of a hydrophobic-rich transmembrane domain, only in the β subunit. These findings are compatible with the idea that the α subunits of the receptor, which are localized at the extracellular side of the plasma membrane, serve as ligand binding domains, whereas the β subunits are the catalytic units, which function as tyrosine kinases whose active sites are localized intracellularly at the inner face of the plasma membrane.

D. The Insulin Receptors as a Glycoprotein

The insulin receptor is a glycoprotein, since the extracellular domains of both receptor subunits contain N- and O-linked sugar moieties, attached to the protein moiety through aspargine or serine residues. The first notion that the receptor contains carbohydrate moieties is based upon studies of Jacobs et al.,[81] who demonstrated enhanced mobility of the receptor on SDS gels following treatment with neuroaminidase. Direct evidence that sugar moieties are attached to the receptor came from experiments where intact cells were biosynthetically labeled with different sugars (e.g., [1,6-{^{3}H}] glucosamine, [^{3}H] fucose, [^{3}H] mannose, or [^{3}H] galactose), and the receptor was subsequently immunoprecipitated with a variety of antireceptor antibodies.[63,64,74,82]

Based on the receptor cDNA clones, the α subunit contains 735 amino acids with a predicted M_r of 84,214, while the β subunit contains 620 amino acids with a predicted M_r of 69,703.[48] The difference between these molecular weights and the apparent molecular weights of the α and β subunits on SDS gels (i.e., 135 and 95 kDa, respectively) is likely to reflect sugar moieties with apparent molecular weights of 50 and 25 kDa, attached to the α and β subunits, respectively.[48,49] Thus, a substantial fraction of the mass of the receptor must be carbohydrates. There are 13 potential glycosylation sites on the α subunit and an additional 4 in the β subunit's extracellular domain.[48,49] Peptides containing 2 of the 17 predicted glycosylation sites, 1 in the C-terminal portion of the α subunit and 1 at the N terminus of the β subunit, were sequenced and found, indeed, to be glycosylated. These findings suggest that many of the potential glycosylation sites are indeed occupied *in vivo*.[48,49]

E. Biosynthesis and Degradation of the Insulin Receptor

Biochemical studies as well as cloning of the receptor precursor cDNA suggest that the

α and β subunits are synthesized as a single polypeptide precursor that is processed to the mature cell-surface receptor by proteolytic cleavage and glycosylation. The biosynthesis of the insulin receptor has been studied in detail, both in lymphocytes[64] and adipocytes.[82] Pulse-chase experiments with [^{35}S] methionine and subsequent immunoprecipitation of labeled receptors (and precursors) by antireceptor antibodies revealed that the first polypeptide, immunoprecipitated during biosynthetic labeling, is a 180-kDa protein.[64,82] This prereceptor protein is synthesized in the endoplasmic reticulum as a result of mRNA translation by membrane-bound ribosomes. The first glycosylation step is the addition of N-linked high mannose-type core oligosaccharides to the protein. These are added cotranslationally and change the mobility of the receptor precursor from 180 to 190 kDa on reducing SDS gels. Treatment of the 190-kDa precursor with endoglycosidase H restores the 180-kDa precursor,[82] whereas treatment of cells with tunicomycin,[83] which blocks core (N-linked) glycosylation, results in the appearance of a single prereceptor translation product of 180 kDa.[84] The 180 kDa is not transferred into the plasma membrane or into the Golgi, which suggests that core glycosylation is essential for these processes to take place. Although made as a single chain, the 190-kDa receptor precursor maintains a conformation that is very similar to the mature receptor. This conformation enables it to bind insulin and to function as an insulin-stimulated tyrosine kinase.[85,86] This suggests that similar mechanisms for information transfer exist within the single chain receptor precursor and the heterodimeric structures of the mature α and β receptor subunits. The 190-kDa receptor precursor moves to the Golgi apparatus where it undergoes further glycosylation. Complex-type oligosaccharides, which contain N-linked acetylglucosamine, are added to it[64] and result in change in its apparent mobility from 190 to 210 kDa. The 210-kDa precursor has several additional properties: (1) a partial resistance to endoglycosidase H;[82] (2) capability to be bound to and eluted from columns of wheat-germ agglutinin coupled to agarose;[64] (3) insensitivity to neuraminidase, which suggests that the 210-kDa precursor still does not contain sialic residues.[82]

The deduced amino acid sequence of the receptor precursor reveals that it contains only a single basic proteolytic cleavage site, Arg-Lys-Arg-Arg. Cleavage at this site yields the two mature receptor subunits α′ and β′, having molecular weights of 125 and 83 kDa, respectively. It is believed that at this stage oligomerization of the receptor subunits and formation of the S-S bridges take place. The receptor subunits undergo terminal glycosylation and acquire sialic acids before their final insertion into the plasma membrane. The half-life of the receptor is estimated to be between 9 and 12 h in different cell types.[75,87,88] When the cells are exposed to insulin, the half-life of the receptor is dramatically reduced.[75] This phenomenon, known as down-regulation, plays a key role in enabling cells to regulate their sensitivity and thus their responsiveness to circulating insulin.[89] Occupied receptors are internalized both via coated pits[90] and by coated pit-independent[91] processes. Internalized receptor-ligand complexes dissociate in endosomes probably due to acidification of the vesicle.[92] The receptors largely recycle intact to the cell surface,[93] whereas most of the internalized insulin is degradated, while a portion is released intact in a process termed retroendocytosis.[94] The receptor itinerary is still incompletely understood:[44] the specific biochemical signals that control the intracellular routing of receptors are not known, nor is the biological function of the itinerary clear. Endocytosis may function primarily to mediate hormone degradation. Alternatively, the presence of insulin and receptors inside the cell raises the possibility that internalization of receptors or hormone may be necessary for some aspects of insulin action.[95]

IV. STRUCTURAL HOMOLOGY BETWEEN THE INSULIN RECEPTOR, OTHER GROWTH FACTOR RECEPTORS, AND RELATED ONCOGENES

The insulin receptor is a member of a growing family of protein kinases that phosphorylate their substrates on tyrosine residues (for recent reviews see References 14 and 96 to

99). These kinases are divided into two major groups. The first includes receptors for growth factors such as epidermal growth factor (EGF),[100-103] platelet-derived growth factor (PDGF),[104-107] insulin-like growth factor-I (IGF-1),[108-110] and colony-stimulating factor-I (CSF-1).[111,112] The proteins belonging to the second group consist of viral oncogene products and their related cellular homologous. These include V-erb B, V-fms, V-abl, V-fes/fps, V-src, V-bgr, V-ros, and V-yes (for detailed reviews see References 96 to 99). The functional similarities between the two groups of tyrosine kinases led to the suggestion that there is a structural similarity between the catalytic domains of growth factor receptors and oncogene products. This hypothesis gained major support when it was shown that the amino acid sequences of the intracellular domains of the EGF receptor are nearly identical to those of the transforming protein V-erb B.[113,114] Similarly, there is a high degree of sequence homology between the catalytic domains of the insulin and IGF-1 receptors and viral oncogene product of V-ros.[48,49,115] These findings led the foundation for our current view that suggests that certain viral oncogene products are in fact altered unregulated forms of otherwise normal genes that code for growth factor receptors. These oncogene products lack the ligand-binding domains of normal receptors and have, in addition, a truncated or mutated and therefore altered tyrosine kinase catalytic domain. The combination of these alternations in structure, and possibly localization, of these oncogene products generates uncontrolled tyrosine kinase activities that transmit continuous signals, equivalent to these produced only transiently by native growth factor receptors. These unregulated signals cause rapid cell proliferation, abnormal growth control, and neoplasia.

All growth factor receptors that function as tyrosine kinases reveal striking similarities in their overall organization and primary sequence. They are generally composed of three major domains, an extracellular domain, with attached sugar moieties that comprises the ligand binding domain; a hydrophobic transmembrane region; and an intracellular domain that contains the tyrosine kinase active site.

The receptor tyrosine kinases can be subdivided into three major classes. Class IA, represented by the EGF receptor, is a single polypeptide chain, having two cysteine-rich domains on the extracellular site and an intracellular tyrosine kinase domain.[113,114] Another protein with a similar overall structure is the Neu/HER2,[116-118] which probably functions as a membrane receptor for an as yet unknown growth factor. Class IB tyrosine kinases, represented by the insulin receptor, are heterotetramers formed by S-S linking of two heterodimers, each made of an extracellular α subunit (with a single cysteine-rich domain) linked through disulfide bridges to a β subunit made of an extracellular, transmembrane, and an intracellular domain. Two α-β heterodimers form tetramers with the structure $\alpha_2\beta_2$, where disulfide bridges between adjacent α subunits form the tetrameric structure. Other members of this family are C-ros, met, and trk proteins,[14] as well as the IGF-1 receptor,[108-110] which shares extensive similarity with the insulin receptor at the level of precursor and subunit size, as well as at the level of structural topology and primary sequence.[115] The receptors for insulin and IGF-1 differ, however, from each other, both in the extracellular ligand binding domains as well as in intracellular regions that confer upon them their unique physiological functions (for recent reviews see References 119 and 120). For example, although both receptors function as tyrosine kinases, they manifest a similar, yet distinct, substrate specificity (to be discussed below). Class IC tyrosine kinases represented by the PDGF receptor are single polypeptides that do not have a cysteine-rich domain at their extracellular portion. Instead, they are characterized as having a split tyrosine kinase domain where a highly heterogeneous sequence is localized within an otherwise highly conserved sequence of the tyrosine kinase domain. Other members of this family are the C-fms/CSF-1 receptors and C-kit, C-sea, and ret proteins.[14]

V. THE INSULIN RECEPTOR cDNA AND PROTEIN SEQUENCE

Based on the cDNA structure it appears that all of the insulin receptor-derived protein sequences are contained in a single open reading frame. Ebina et al. reported that this cDNA codes for a protein of 1355 amino acids, predicting a M_r of 153,917.[48] Ullrich et al.[49] reported the presence of a shorter variant of the insulin receptor, which lacks 12 amino acids at the C-terminal end of the α subunit. Since the cloning strategy of both groups involved the use of oligonucleotides corresponding to insulin receptor purified from human placental membranes, and since both groups used total poly(A)-containing RNA from human placenta to construct their cDNA library, it is difficult to speculate on the reason for such a discrepancy in the predicted receptor structure.

Northern blot analysis of human placental, liver, hepatoma, and IM-9 lymphocytes[48] revealed that at least four mRNA transcripts exist in these tissues. The four transcripts are in the size of 7.0, 8.5, 9.6, and 11.0 kb. These transcripts could be derived from the same gene by variation in initiation or termination codons, but it is unclear whether all these mRNA code for the same insulin receptor precursor sequence or whether the different mRNAs code for structural variants. Amino acid numbers used throughout this article will be those of Ebina et al.[48]

A. The Extracellular Domain of the Insulin Receptor

The extracellular portion of the insulin receptor consists of the entire α subunit and about one third of the β subunit. These portions of the receptor form the insulin binding domain. The α subunit is comprised of several domains: the most interesting one, close to the N terminus, is a single 158-amino-acid-long (residues 155 to 312) cysteine-rich domain. It is made of 16.5% cysteine residues and 72% hydrophilic residues, which suggests that this region is embedded in a hydrophilic environment. Out of the total 37 cysteine residues present in the α subunit, 26 are localized within this region. This region is probably rigid, as some of these residues may form intramolecular or intermolecular S-S bridges. There is only 48% homology between the cysteine-rich domains of insulin and IGF-1 receptors, but there is a complete conservation of the cysteine residue spacing between these two receptors. Furthermore, of the 27 cysteine residues of the insulin receptor, 18 occupy homologous positions in the EGF receptor and the neu oncogene product.[5] This indicates a common evolutionary origin of these growth factor receptors and must be indicative of some shared structural and mechanistic themes.[48] Divergence of sequences flanking the conserved cysteine residues in this ligand binding domain suggests that they may define ligand specificity within a common, compact, structural unit formed by a network of disulfide bridges.[115] Support to this hypothesis are findings that suggest that mutations introduced into a cysteine-rich domain of the low-density lipoprotein (LDL) receptor abolish its ligand binding capability.[121] It should be noted, however, that there is no sequence homology between the cysteine-rich domains of the LDL and insulin receptors. Most cysteine residues outside this cysteine-rich cluster are also conserved between the insulin and IGF-1 receptors, including three cysteine residues located at the extracellular domain of the β subunit. This suggests that these cysteines are the ones that form S-S bridges between the α and β subunits to form the α-β heterodimers, which upon further cross-linking through yet undefined S-S bridges generate the biologically active α_2-β_2 heterotetrameric receptor complexes.

B. The Transmembrane Domain

Since the α subunit of the insulin receptor is completely extracellular, the single transmembrane domain is part of the β subunits. It contains 23 hydrophobic amino acids (22 in the IGF-1 receptor), flanked at the C-terminal end by a short sequence of basic residues (Arg-Lys-Arg), a feature of the primary sequence of the cytoplasmic region in many mem-

brane proteins, including the EGF (Arg-Arg-Arg),[114] IGF-1 (Arg-Lys-Arg),[115] and LDL (Lys-Asn-Trp-Arg-Leu-Lys)[121] receptors. In addition to their potential interactions with the head groups of negatively charged phospholipids, these basic residues may represent a stop-transfer signal anchoring the insulin receptor in the membrane during biosynthesis.[122]

C. The Intracellular Cytoplasmic Domain

The cytoplasmic domain of the insulin receptor is generated by the carboxyl-terminal 403 amino acids of the β subunit. This domain shows a high degree of homology with other growth factor receptors[48,49] (84% with IGF-1 receptor and 40% with the EGF receptor) as well as the src-related oncogene products, especially V-ros and onc D.[123,124] The highest homology is within the tyrosine kinase catalytic domain that includes the ATP binding site.

The ATP binding domain of the insulin receptor, as well as of other tyrosine kinases, begins exactly 50 amino acids downstream from the end of the transmembrane domain. It starts with a "consensus" sequence Leu-Gly-X-Gly-X-X-Gly, which is the suggested ATP binding domain of both tyrosine as well as serine/threonine kinases.[125] Another part of the ATP binding site is a lysine residue (Lys 1030) localized 19 amino acids downstream the consensus sequence. A lysine at a similar position (13 to 20 amino acids following the consensus sequence) is conserved among all tyrosine and serine kinases studied so far. It is thought to provide a salt bridge to β/α phosphates of ATP in the ATP binding site.[126,127] This lysine residue is affinity labeled with the ATP analog fluorosulfonyl-benzoyl adenosine, when added to the cAMP-dependent protein kinase[128] or to tyrosine kinases such as $pp60^{src}$[127] and the EGF receptor.[129] Although the insulin receptor is affinity labeled with ATP analogs,[130-132] no evidence is yet available to prove that it is indeed Lys 1030 that is being labeled. It has been shown, however, that mutated insulin[43-46] or EGF receptors,[133] expressed in Chinese hamster ovary (CHO) cells in which Lys 1030 (insulin receptor) or Lys 721 (EGF receptor) was replaced by arginine, alanine, or methionine residues, are devoid of tyrosine kinase activity. This is true whether hormone-dependent autophosphorylation of the receptors is studied in intact cells, or whether the hormone-dependent tyrosine kinase activity is assayed in cell-free systems. Furthermore, cells expressing mutated receptors, unlike cells expressing wild-type receptors, fail to mediate the postreceptor effects of insulin or EGF, respectively (*vide infra*). The only reservation regarding experiments in which mutated receptors are used relates to the assumption that introduction of point mutation obliterates exclusively the function of the amino acid under study (in this case Lys 1030 or Lys 721). It still remains possible that certain point mutations cause gross or local alterations in receptor structure, which accounts for loss in receptor function. Rigorous proof that such alterations do not take place is still missing in many studies.

One hundred amino acids, downstream from Lys 1030, start a third region containing the conserved sequences Arg-Asp-Leu (1131 to 1133), Asp-Phe-Gly (1150 to 1152), and Ala-Pro-Glu (1177 to 1179). These triplets appear to be characteristic to all known protein kinases[97] and are therefore considered to form part of the active site.

Since the insulin receptor undergoes autophosphorylation on tyrosine residues (*vide infra*), it became of interest to identify the autophosphorylation sites and determine the sequences surrounding them. The basis for such analysis is the sequence surrounding the major autophosphorylation site (Tyr 416) of the viral oncogene $pp60^{src}$.[134,135] It appears that Tyr 1162 of the insulin receptor, like Tyr 416 or $pp60^{src}$, is placed within a sequence having the form of Asp(Glu)-X-(Asp)(Glu)-Tyr-X-X-X-Gly. The main features of this tyrosine are the acidic amino acids (Glutamic or Aspartic residues) localized adjacent to it and up to three amino acids away. A glycine residue is usually found three to four amino acids away from this tyrosine, toward the C terminus. Tyrosine residues surrounded by this type of sequence were found in the receptors for EGF and IGF-1 as well as in the oncogene products of V-fms, V-fes, V-erb B, V-fgs, V-yes, and V-fgr. Six additional tyrosine residues, found in the cytoplasmic portion of the insulin receptor, are localized adjacent to acidic amino acids:

972
–Glu–Tyr–X

1011
X–Tyr–Glu–

1158
Asp–X–Tyr–Glu–X–Asp

1163
Glu–X–Asp–X–Tyr–X–

1227
Asp–X–X–Tyr–X–Asp–

1328
–X–Tyr–Glu–Glu

This acidic environment turns these tyrosines into potential candidates for autophosphorylation.

Despite its overall homology to other tyrosine kinase domains the insulin receptor also includes at least three discrete regions of sequence divergence, following residues 1001, 1088, and 1223. The nonapeptide, which begins at residue 1088 (1073 of the IGF-1 receptor), is present at the exact position where 70- and 100-residue-long heterologous insertion sequences are found in the C-fms/CSF-1 receptor and PDGF receptor structures.[111,118,136] The presence of such highly heterogeneous sequences within otherwise highly conserved tyrosine kinase domains of gene family members was suggested[115] to be an indication that these subdomains define the specific functions associated with each receptor and could as well be the subdomains that determine substrate specificity.

The carboxy-terminal domain of the insulin receptor is relatively hydrophilic and presents a low degree of sequence homology to other tyrosine kinases. It has therefore been proposed that the carboxy-terminal domain, a membrane-proximal region with sequence divergence, and the three heterologous peptides within the tyrosine kinase domain are all part of a domain through which the receptor interacts with its substrates or other effector systems.[115]

VI. THE INSULIN RECEPTOR AS A TYROSINE KINASE

Originally described by Kasuga et al.,[76] phosphorylation of the insulin receptor is now a well-documented phenomenon taking place both in intact cells[21,108,137-144] and various cell-free systems.[72,131,132,145-153] In intact cells insulin enhances phosphorylation of the receptor on serine, threonine, and tyrosine residues,[139] whereas in cell-free systems, purified[152,154] or semipurified[146] receptor preparations were shown to undergo an insulin-stimulated autophosphorylation exclusively on tyrosine residues. It was further demonstrated that the insulin-stimulated receptor kinase is capable of phosphorylating tyrosine residues of exogenously added substrates (*vide infra*). These findings set the foundations for the well-accepted notion that the insulin receptor itself act as a tyrosine kinase, while it serves as a substrate for at least two types of protein kinases: the receptor itself, and another serine/threonine kinase(s), which is not an integral part of the receptor (*vide infra*). Multisite phosphorylation is a known mechanism for regulation of enzyme function and is thought to be of common occurrence.[11,14] Phosphorylation at one site may amplify or antagonize the effects of phosphorylation at other sites or alter the rates at which other sites are phosphorylated or dephosphorylated.

VII. RECEPTOR PHOSPHORYLATION IN INTACT CELLS

A. Effects of Insulin

Kasuga et al.[76,139] first demonstrated that insulin stimulates receptor phosphorylation in ^{32}P-labeled cultured human lymphocytes and rat hepatoma cells. In these experiments, cells were preincubated with [^{32}P]Pi, solubilized in 1% Triton® X-100, and their glycoproteins purified on columns of wheat-germ agglutinin coupled to Sepharose. Immunoprecipitation of the proteins eluted from these columns with antibodies directed against the insulin receptor revealed a labeled 95-kDa protein, whose phosphorylation was markedly enhanced when the cells were pretreated with insulin. This 95-kDa protein was identified as the β subunit of the insulin receptor based on the following criteria: (1) it could be specifically immunoprecipitated by a panel of antibodies directed against the insulin receptor;[139] (2) ^{32}P in the immunoprecipitates decreased in the presence of excess unlabeled insulin (as expected from studies showing that insulin bound to the receptor reduces the affinity of the receptor for antireceptor antibodies;[1] (3) the insulin-stimulated 95-kDa phosphoprotein migrated in the same position (under reducing and nonreducing conditions) as the β subunit of the receptor labeled with [^{35}S]-methionine or [^{3}H]-sugars.[139] Pretreatment of intact cells with insulin enhances four- to sevenfold ^{32}P incorporation into the 95-kDa β subunit. Half-maximal phosphorylation in Fao cells occurs at 10^{-8} *M* insulin with a maximal effect obtained with 10^{-7} *M*. This is consistent with an equilibrium constant of 4.9 n*M* for dissociation of insulin from its receptor in these cells.[155] Studies in freshly isolated rat hepatocytes[156] reveal that half-maximal effect of insulin on receptor phosphorylation is somewhat lower and occurs at 2 n*M*. This concentration is comparable with that causing half-maximal insulin-stimulated amino acid transport (1.3 n*M*) and is also consistent with the equilibrium constant for dissociation of insulin from its receptor on hepatocytes (0.6 n*M*).[157] Phosphorylation *in vivo* is rapid and can be detected after 1 min exposure of intact cells to physiological concentrations (10^{-9} *M*) of the hormone.[76,139] Guinea pig insulin, which is about 2% as potent as porcine insulin in stimulating glucose uptake,[158] is about 2% as potent in stimulating receptor phosphorylation.[76] EGF, ACTH, and hGH are ineffective.[76,137] It appears, therefore, that insulin-stimulated phosphorylation of its receptor occurs in a rapid and specific manner, and that the effect is triggered by physiological concentrations of the hormone. This is consistent with the notion that insulin receptor phosphorylation is an early event mediating insulin bioeffects. In early experiments[139] it appeared that insulin treatment of intact cells mainly enhanced receptor phosphorylation on serine and threonine residues, with relatively little effect on tyrosine residues. More recent experiments revealed, however, much more pronounced effects of insulin on tyrosine phosphorylation.[159] This occurs when precautions are taken to avoid dephosphorylation of tyrosine residues by rapid freezing of the cells in liquid nitrogen at the end of the experiment.[159] In addition, the homogenization of the cells is done in buffers containing sodium orthovanadate, fluoride, and pyrophosphate, which inhibit the action of phosphotyrosine phosphatases.[160] Under these conditions, and with the aid of antibodies directed against phosphotyrosine residues,[161] Kahn et al. studied in more detail effects of insulin on receptor phosphorylation in Fao rat hepatoma cells.[159,161] These studies, which were subsequently carried out in freshly isolated hepatocytes,[156] revealed that in its basal state the receptor contains phosphoserine and phosphothreonine residues, but virtually no phosphotyrosine residues. Pretreatment of the cells with insulin results in an abrupt (within 5 s) rise in phosphotyrosine content of the receptor that reaches steady state within 20 s and remains constant for at least 60 min. This is followed by a slower increase in phosphoserine content that gets to its maximal levels by 10 min.[160] These results suggest that insulin-stimulated tyrosine phosphorylation precedes insulin-stimulated serine phosphorylation of the β subunits. Furthermore, sequential immunoprecipitation of receptors with antiphosphotyrosine antibodies, followed by precipitation of the remaining proteins with

antireceptor antibodies, suggests that insulin receptors, which contain phosphoserine in their basal state, are tyrosine phosphorylated more slowly, or even not at all, when compared with receptors that are dephosphorylated in the basal state.[161] Moreover, receptors that fail to precipitate with antiphosphotyrosine antibodies contain exclusively phosphoserine residues.[156] These results suggest that serine phosphorylation may act as a feedback regulatory control mechanism to inhibit insulin-stimulated tyrosine phosphorylation.

The presence of serine/threonine-phosphorylated and nonphosphorylated receptors in untreated cells suggests that these could be localized in different parts of the cells or are functionally distinct subsets of the receptor.[156,161]

B. *In Vivo* Phosphorylation Sites

The receptor sites that undergo phosphorylation in intact cells are currently only partially characterized. Characterization is carried out on ^{32}P-labeled receptors obtained from cells treated with or without insulin. The analysis involves enzymatic or chemical digestion of the receptor, separation of phosphorylated peptides by reverse-phase high-performance liquid chromatography and analysis of phosphoamino acid content of each fragment. In several studies, antipeptide[162] and antiphosphotyrosine antibodies[163,164] were employed as well. Such analysis revealed that in the basal state the serine and threonine phosphorylation sites are localized each at a single peptide whose phosphate content increases upon treatment of the cells with insulin. By contrast, tyrosine phosphorylation sites, which are absent in the basal state, appear to be localized on different peptides.[159] It appears that receptor phosphorylation *in vivo* correlates with phosphorylation of the kinase domain (tyrosines 1158, 1162, and 1163) rather than with phosphorylation at the carboxyl-terminus domain.[165]

Tyrosines that are localized at the kinase domain,[162] as well as three additional ones, two at the C-terminal end (1328, 1334) and one at a yet undefined site,[162] are also phosphorylated by the purified receptor in cell-free systems,[163,164] supporting the notion that tyrosine phosphorylation in intact cells could indeed take place on more than a single site. Interestingly, only less than 10% of the total β subunit tyrosine phosphorylation *in vivo* takes place at the amino-terminus portion of the cytoplasmic domain of the β subunit (Tyr 965, 972). This was taken as evidence to suggest[166] that this region has some other important functions in insulin-dependent receptor kinase activation that do not necessarily involve tyrosine phosphorylation of this domain. This conclusion is supported by the findings that antibodies to that domain specifically and completely inhibit the kinase activity of unphosphorylated receptors.[166] It is still unclear why more sites are phosphorylated *in vitro* than *in vivo*. One possibility is that the lower intensity of ^{32}P-labeling and lower amounts of receptors available in *in vivo* studies preclude detailed analysis of all *in vivo* phosphorylation sites. Alternatively, one cannot rule out the possibility that some of the *in vitro* phosphorylation sites are created only in cell-free systems and are nonphysiological.

VIII. CHARACTERIZATION OF THE INSULIN RECEPTOR KINASE ACTIVITY IN CELL-FREE SYSTEMS

Insulin stimulates phosphorylation of its own receptor in a variety of cell-free systems. When added to solubilized, lectin-purified preparations, insulin, in the presence of [γ-^{32}P] ATP and divalent cations, stimulates severalfold ^{32}P incorporation into the β(95 kDa) subunit of the receptor.[131,132,145-153,167,168] In contrast to intact cells, phosphorylation in the cell-free systems results in ^{32}P incorporation exclusively into tyrosine residues.[146] Four lines of evidence now support the notion that the kinase that phosphorylates the receptor on tyrosine residues is part of the β subunit itself: (1) the β subunit contains a consensus sequence for an ATP binding site;[48,49] (2) the β subunit is affinity labeled with oxidized [α-^{32}P] ATP[131] or photoreactive azido-[α-^{32}P] ATP;[132] (3) highly purified human placental receptor retains

the kinase activity that phosphorylates the receptor on tyrosine residues;[148,152] and (4) the insulin-binding activity and the insulin-stimulated tyrosine kinase activity of the receptor copurify at a constant stoichiometric ratio to homogeneity.[130,148,152,154,169]

Phosphorylation in cell-free systems has a similar time course and specificity as that observed in intact cells.[72,146] EGF, ACTH, glucagon, or cAMP do not alter phosphorylation of the receptor in the cell-free systems.[151] Insulin analogs, like guinea pig insulin or IGF-1, stimulate receptor phosphorylation in proportion to their potency to inhibit insulin binding, which suggests that activation of the receptor kinase is regulated by its interaction with a highly specific ligand. This conclusion is supported by the fact that other growth factor receptors, like those for IGF-1,[108-110,170] EGF,[100-103,171] and PDGF,[104-107] also display a high degree of ligand specificity.

A. Kinetic Properties

Within 30 s at 22°C insulin-stimulated autophosphorylation reaches 50% of maximum and attains steady-state levels after 10 min.[172] At 4°C, autophosphorylation, as expected, is less rapid. It is detected within 1 min, reaches half-maximum levels in 5 min, and maximal levels by 20 min.[151] These findings are compatible with the notion that receptor phosphorylation is the earliest event following insulin binding. Insulin-stimulated receptor phosphorylation follows occupancy of the receptor by the hormone, thus, half-maximal effects are observed at approximately 10 *nM* insulin.[151,154,166] Insulin activates the autophosphorylation reaction severalfold by changing the apparent V_{max} rather than the K_m for ATP.[172] The receptor-catalyzed autophosphorylation appears to be an intramolecular reaction, since the reaction rate is linear with receptor concentration, namely, the specific activity of the reaction is held constant upon dilution.[37,154,172,173] Intramolecular autophosphorylation appears to be a general phenomenon, since the tyrosine kinases associated with the receptors for EGF,[174] IGF-1,[175] as well as the type II cAMP-dependent protein serine kinase[176] are all known to undergo intramolecular autophosphorylation.

B. Kinase Activity in Different Receptor Preparations

In order to assay the receptor kinase activity *in vitro*, membranes or tissues first need to be solubilized in detergents and to be further purified by affinity chromatography on columns of wheat germ agglutinin coupled to agarose.[146] This procedure results in a 20-fold purification of the insulin receptor,[177] which now comprises about 1% of the total protein in the lectin column eluate. The need to perform partial purification is evident from the fact that insulin-stimulated receptor autophosphorylation performed *in vitro* in membranes[147] or Triton extracts is barely detected, while it is readily observed in lectin-purified receptor preparations. This could be explained by the presence of active protein tyrosine phosphatases that obliterate the insulin-dependent phosphorylation of the receptor in membranes. Alternatively, insulin receptor phosphorylation in membranes or Triton extracts could be masked due to the low abundance of the receptors in these preparations. Indeed, when the phosphorylation reaction is carried out in Triton extracts, an insulin-dependent receptor phosphorylation can be readily detected when the labeled receptor is immunoprecipitated either with antireceptor[72] or with antiphosphotyrosine antibodies. In several studies, the lectin-purified receptor preparations were further purified by chromatography on insulin-agarose columns.[148,152,178] This preparation manifests similar characteristics as the lectin-purified receptor, but this additional purification step is somewhat disadvantageous as it subjects the receptor to rough elution conditions from the column (e.g., pH 5.5) that cause some receptor denaturation.

C. Nucleotide and Metal Ion Requirements

The insulin receptor kinase, like the IGF-1 receptor kinase,[175] utilizes ATP but not GTP as the substrate.[151] This is in contrast to the EGF-mediated phosphorylation of EGF receptors

where GTP can substitute for ATP.[103] K_m values for ATP of the insulin-stimulated phosphorylation reaction were found to be 30 to 150 μM,[154,166,172,179] but, as discussed below, these depend on assay conditions, especially on the type and concentration of metal ions present.

When assayed at 4°C and with micromolar concentrations of ATP (5 μM), phosphorylation of the receptor requires the presence of Mn^{2+} or Co^{2+};[151] other cations such as Mg^{2+}, Cr^{3+}, or Ca^{2+} are ineffective. When assays are performed at 22°C with 50 μM ATP the cationic requirements are different. Under such conditions Mg^{2+} appears to be more potent than Mn^{2+}, with maximal phosphorylation rates at 40 mM Mg^{2+}, or 20 mM Mn^{2+}. These concentrations exceed by far those required to generate metal-ATP complexes, which suggests that the metal cations have an additional regulatory role. The effects of Mg^{2+} and Mn^{2+} synergize with each other and give a maximal phosphorylation rate with a combination of 2 mM Mn^{2+} and 12 mM Mg^{2+}.[179] Similar observations were reported for the EGF receptor, where Mg^{2+} at high concentrations could substitute for Mn^{2+}, and a combination of Mg^{2+} and Mn^{2} gives a higher activity than either metal alone.[179] It appears, therefore, that the insulin receptor possesses specific Mg^{2+}/Mn^{2+} binding sites that alter the enzyme activity when occupied. Binding of Mn^{2+}/Mg^{2+} to their regulatory sites increases the kinase affinity for ATP[172] and thus increases the rate of autophosphorylation and phosphorylation of exogenously added substrates. On the other hand, increasing ATP concentrations decreases the K_m for Mn^{2+}, indicating that at physiological ATP concentrations (i.e., 1 mM), phosphorylation may proceed even at a low concentration of divalent cations.[172] The alterations in the receptor kinase activity induced upon binding of Mn^{2+} or Mg^{2+} to their regulatory sites presumably reflect conformational changes in the soluble receptor kinase. Indeed, receptors complexed with Mg^{2+} or Mn^{2+} are more susceptible to heat inactivation when compared to native enzymes. Furthermore, based on kinetic studies, it has been suggested[172] that Mn^{2+} (or Mg^{2+}) binding and the resulting changes in receptor conformation may precede [Mn/Mg-ATP] binding, which is required for catalyzing the autophosphorylation reaction. The physiological significance of these findings is still unclear.

In contrast to Mg^{2+} or Mn^{2+}, other cations such as Cu^{2+}, Ca^{2+}, Ni^{2+}, as well as Zn^{2+} which are the transition metals second in abundance in man,[180] inhibit the insulin[181,182] and EGF[183] receptor kinase activities. Inhibition by Zn^{2+} is readily detected at physiological concentrations of this cation (10 μM) with a half-maximal effect at 40 μM. Inhibition takes place whether the kinase activity has been stimulated by preincubation with insulin or other insulinomimetic agents such as antireceptor antibodies.[182] The inhibitory effect of Zn^{2+} is fully reversible upon addition of EGTA.[182] Inhibition by Zn^{2+} occurs independent of the presence of Mg^{2+} (5 to 50 mM) or ATP concentrations of 50 to 200 μM,[182] which suggests that Zn^{2+} does not inhibit the formation of [Mg-ATP] complexes. Whether inhibition of the receptor kinase activity by micromolar Zn^{2+} concentrations has any physiological relevance still remains to be determined.

IX. MECHANISM OF TRANSMEMBRANE ACTIVATION OF THE INSULIN RECEPTOR KINASE

Activation of the insulin receptor kinase is the last step in a process initiated upon insulin binding to the outer surface of its membrane receptor. To date, only limited information is available regarding the nature of the transmembrane signal itself. The primary structure of the receptor[48,49] reveals that only a single 23-amino-acid stretch of the β subunit transverses the membrane, but this apparent simple communication between the receptor subunits across the membrane gives no clues as to the nature of the coupling process that leads to kinase activation.

Inspection of the similar structural features of the transmembrane domains of insulin,

IGF-1, and EGF receptors leads to the conclusion that the mechanism utilized by all three receptors to transmit a signal across the membrane should be very common if not identical. This is in contrast to some other transmembrane proteins involved in signal transduction that transverse the lipid bilayer more than once.[184,185]

In principle, two potential transmembrane signaling mechanisms could be considered: a vectorial or a lateral signal propagation pathway. In the first, ligand binding induces a conformational change in the extracellular domain, which changes the proximity of the subunits from the lipid bilayer and thus alters the conformation of the intracellular domains. Such a mechanism is less favorable in view of the fact that the insulin, EGF, and IGF-1 receptors each have three basic amino acid residues on the cytoplasmic side of the transmembrane domain and a Pro-Ser dipeptide flanking the membrane anchor sequence on the extracellular side. The density of such charged residues may impose a high-energy barrier for a putative vertical conformational change from the extracellular domain to the cytoplasmic region.[186] An alternative mechanism could be a lateral signal-transduction pathway where ligand binding induces microaggregation or oligomerization of the receptor's extracellular domains, which changes the lateral distance between the intracellular domains, thus leading to a change in their conformation and to receptor kinase activation. Several observations in a variety of systems[187-189] support the notion that such a mechanism indeed plays a role in transmembrane signaling.

Studies employing human polyclonal antireceptor antibodies[190] and mouse monoclonal antibodies[191] were carried out[37,191] in order to determine whether receptor aggregation is essential for activation of the insulin receptor kinase in solution. These antibodies are appropriate tools for such a study since (1) they bind specifically to the receptor and mimic most biological effects of the hormone and (2) they can be presented to the receptor either as bivalent [IgG, $F(ab')_2$] or monovalent (Fab') ligands. The receptors used were derived from rat liver membranes[37] or human placenta[191] and were solubilized and partially purified by affinity chromatography over lectin columns. In these studies it could be demonstrated[37,191] that (1) purified antireceptor IgG and their $F(ab')_2$ fragments act as partial agonists of insulin and partially activate the receptor kinase; (2) monovalent Fab' fragments that inhibit insulin bindings are devoid of such activity; (3) monovalent Fab' fragments regain capacity to stimulate the receptor kinase when cross-linked with goat antihuman Fab'. These data suggest that receptor occupancy per se (e.g., by monovalent Fab') is necessary but insufficient for activation of the receptor kinase, whereas microaggregation or clustering of the receptor's extracellular domains is the necessary trigger for kinase activation. Microaggregation of the receptor, induced upon binding of antireceptor antibodies, could, in principle, take place between subunits within the same receptor tetramer or between subunits of two adjacent receptors. Activation of the receptor kinase by the antibodies is independent of receptor concentration,[37] suggesting an intramolecular activation process that is similar to the mechanism of receptor activation by insulin. Such a conclusion is supported by the findings[191] that antibody-receptor complexes generate mainly intramolecular cross-links as judged by sucrose gradient centrifugation experiments.

The need for a dimer-dimer ($\alpha\beta$-$\alpha\beta$) interaction in order to maintain an insulin-dependent receptor kinase activity was also established in an independent study.[192] There it was shown that reduction of heterotetrameric receptors ($\alpha_2\beta_2$) with dithiothreitol into $\alpha\beta$ dimers results in a complete loss of insulin-dependent autophosphorylation of the isolated dimers. It could be further demonstrated that when insulin receptors are first phosphorylated and then reduced, receptor kinase activity toward exogenous substrates is not affected. These observations could be interpreted to suggest that maintenance of a tetrameric structure and the need for subunit-subunit interactions between receptor halves are indeed critical for transmembrane signal transmission. Once the receptor undergoes autophosphorylation and it is present in the active state, there is no further need to keep its tetrameric integrity.

A similar clustering mechanism has been proposed[186] to be involved in the activation of the EGF receptor that consists of a single polypeptide chain. It therefore appears that receptor clustering could be a general mode for triggering the activity of the whole family of receptor tyrosine kinases. The generality of this process could be further inferred from studies where chimeric receptors were constructed from the extracellular portion of the insulin receptor joined to the transmembrane and intracellular domain of the EGF receptor.[193] Such chimeric receptors, when expressed in COS cells, demonstrate an insulin-stimulated EGF receptor kinase activity. Similarly, a hybrid receptor molecule composed of the extracellular ligand binding domain of the insulin receptor and the transmembrane and protein tyrosine kinase domains of $p68^{gag\text{-}ros}$ was generated and expressed in CHO cells.[194] Such a hybrid possesses an insulin binding activity and an insulin-stimulated phosphorylation on tyrosine residues of the hybrid β subunit, both *in vivo* and *in vitro*. These findings[193,194] therefore suggest that insulin and EGF receptors, as well as related protein tyrosine kinases, utilize a common transmembrane signal transfer mechanism.

X. ACTIVATION OF THE RECEPTOR KINASE BY INSULINOMIMETIC AGENTS AND ANTIRECEPTOR ANTIBODIES

Several insulinomimetic agents have been tested for their ability to stimulate the insulin receptor kinase activity. Vanadate[31,32] or peroxide vanadate,[33] trypsin,[34,35] wheat germ agglutinin, ConA,[16] and certain antibodies against the insulin receptor[17,37-40,191,195-197] lead to increased receptor phosphorylation. Trypsin treatment stimulates phosphorylation of the receptor β subunit and of a proteolytic product of 72 kDa,[34] however, another report[198] questions these findings and suggests that proteolytic cleavage of a 2-kDa fragment from the C-terminus end of the β subunit inactivates, rather than stimulates, receptor kinase activity. Further studies will therefore be needed before the role of proteolysis in regulation of the receptor kinase activity is clearly elucidated.

Conflicting results were reported with respect to the potency of antireceptor antibodies to stimulate receptor kinase activity. Part of the problem originates from the fact that the antibodies employed in various studies vary in origin and antigenic specificity. Some are human polyclonal antibodies that develop in patients with extreme resistance to insulin,[190] while others are either mono-[38-40,191,196,197] or polyclonal[17,37,154,195] antibodies directed against the whole receptor or against synthetic peptides representing various parts of its primary amino acid sequence.[154] Four polyclonal human antisera directed against the insulin receptor were tested for their capability to activate the insulin receptor kinase.[17] All four antisera were shown to inhibit insulin binding and to stimulate lipogenesis in fat cells. Two antisera were shown, as expected, to stimulate the receptor kinase activity in cell-free systems, but other sera failed to do so when assayed either in intact cells[199] or in cell-free systems.[17,200] Similarly, mouse monoclonal antibodies that react with the α subunit of the receptor were reported to inhibit insulin binding and stimulate insulin's bioeffects without stimulation of the receptor kinase, either in intact cells or cell-free systems.[191,196,197] These findings are difficult to rationalize in terms of a model whereby the major physiological role of the receptor kinase is phosphorylation of a protein substrate. Because antireceptor antibodies would not be expected to bind directly to such substrates, it is hard to construct a hypothetical mechanism whereby the nonstimulatory antibodies might mimic insulin effects upon such putative substrates by a kinase-independent mechanism. However, in view of the large body of evidence in favor of the involvement of the receptor kinase in insulin action, it is reasonable to assume that more information is needed before a definitive conclusion may be reached. The possibility still exists that the so-called "nonstimulatory" antibodies do induce receptor phosphorylation, but only to a small extent,[201] which is below the current detection methods in certain experimental systems, but is sufficient to induce insulin's bioeffects. It appears,

however, that certain of insulin's effects, such as induction of negative cooperative binding and down regulation, both of which are mimicked by nonstimulatory monoclonal antibodies,[197] could occur independent of receptor autophosphorylation.

XI. REGULATION OF THE RECEPTOR KINASE ACTIVITY BY PHOSPHORYLATION ON TYROSINE RESIDUES

Multisite phosphorylation of the receptor kinase on serine, threonine, and tyrosine residues presumably acts to regulate its activity. Phosphorylation of the receptor on tyrosine residues activates the enzyme, whereas its phosphorylation on serine/threonine residues most likely acts to inhibit the tyrosine kinase activity (*vide infra*). Evidence to support such a notion is provided below.

A. Autophosphorylation on Tyrosine Residues

One of the earliest observations made in studying the insulin receptor kinase was that activation of the receptor kinase occurs by autophosphorylation.[72,178] In these experiments it could be demonstrated that both autophosphorylation[72] as well as phosphorylation of exogenous substrates[178] are enhanced following an insulin-dependent prephosphorylation of the receptor. For example,[72] partial prephosphorylation of the receptor with increasing ATP concentrations in the presence of insulin results in a dose-dependent increase in autophosphorylation rate.[72,202] This abolishes the lag period in phosphorylation of exogenous substrates usually observed when ATP is added to a preparation that contains both the receptor and the exogenous substrates.[178] It can therefore be concluded[163] that insulin binding transforms the receptor to a functional state capable of a rapid intramolecular autophosphorylation; autophosphorylation transforms the receptor to yet another state capable of phosphorylating exogenous substrates. The latter presumably corresponds to the active conformation of the receptor *in vitro*, where it is fully activated even in the absence of insulin. Dephosphorylation of the receptor's tyrosine residues by alkali phosphatase is accompanied by a marked decrease of its kinase activity that returns to basal levels.[202] These and similar studies[202-206] established the notion that reversible autophosphorylation of one or more tyrosine residues activates the receptor kinase without affecting the insulin binding properties of the enzyme.

B. The Correlation Between the *In Vitro* Autophosphorylation Sites and Activation of the Insulin Receptor Kinase

In order to identify the major tyrosine autophosphorylation sites, partially purified receptors were phosphorylated *in vitro* in the presence of insulin.[163] The ^{32}P-labeled receptors were further purified by adsorption to monoclonal antiphosphotyrosine antibodies covalently coupled to Sepharose 4B. This procedure adsorbed essentially all ^{32}P-labeled receptors, which could be eluted from the column with phenylphosphate. Enzymatic and chemical digestion of the receptors and separation of the peptides on HPLC columns and SDS/urea gels enabled the sequencing of purified peptides. Such analysis revealed the presence of five phosphorylation sites: tyrosines 1158, 1162, and 1163, which are localized within the receptor kinase domain, and tyrosines 1328 and 1334 which are localized at the C-terminus region.

Kinetic studies were carried out in order to determine which are the tyrosine residues whose phosphorylation correlates with activation of the receptor kinase. Studies involving antipeptide antibodies revealed that phosphorylation at the kinase domain (tyrosines 1158, 1162, 1163) correlates with activation of the receptor kinase *in vitro*.[162] These tyrosines are first phosphorylated (within 5 s) during insulin stimulation, whereas phosphorylation of the other tyrosine residues at the C-terminal end occurs after a lag period.[4] Removal of Tyr-1328 and Tyr-1334 by mild trypsinolysis has no effect on the activation of the receptor kinase, suggesting that these residues do not play a regulatory role in kinase activation.[207]

Furthermore, using antiphosphotyrosine antibodies to arrest the autophosphorylation cascade, it could be demonstrated[164,207] that the first two autophosphorylation events occur in Tyr-1158 and either Tyr-1162 or 1163; however, diphosphorylation of these sites does not activate the kinase. Activation does occur concomitantly with the formation of the triphosphorylated state of the receptor (i.e., Tyr-1158, 1162, and 1163).[164,207] This conclusion is further supported by studies showing that substitution of these tyrosine residues (Tyr 1162, 1163) to phenylalanine generates a mutated receptor that expresses markedly reduced insulin-stimulated receptor kinase activity.[47]

C. Tyrosine Phosphorylation of the Insulin Receptor by pp60^{src}

The tyrosine kinase associated with the oncogene product pp60^{src} catalyzes phosphorylation of lectin-purified insulin receptor preparations.[4,208] Phosphorylation by high concentrations of pp60^{src} is independent of insulin binding and occurs at sites that are the same or very similar to the receptor autophosphorylation sites.[208] Indeed, phosphorylation of the receptor β subunit by pp60^{src} activates the receptor kinase in a manner similar to the autophosphorylation reaction.[208] These findings suggest that cellular transformation by certain viral oncogenes could be associated with uncontrolled expression of the insulin receptor kinase activity. If autophosphorylation of growth factor receptors proves to be a general mechanism for receptor activation, then mimicking this effect via direct phosphorylation of growth factor receptors by a viral protein tyrosine kinase could provide a mechanism to explain one of the well-known characteristics of transformed cells, namely, their ability to grow in the presence of reduced amounts of growth factors.[209]

Activation of protein kinase activity by autophosphorylation is not a property unique to the insulin receptor. The cAMP-dependent protein kinase[176] and the Ca^{2+}-calmodulin protein kinase[210] are regulated in a similar manner. IGF-1 receptors are also activated through autophosphorylation,[174] whereas several studies,[211] but not all,[212] suggest that autophosphorylation of the EGF receptor activates its tyrosine kinase. Oncogene-encoded protein tyrosine kinases also appear to be activated by tyrosine phosphorylation,[20] but of most interest are findings[213-215] implicating tyrosine phosphorylation of specific residues of pp60^{src} as inhibitors of its kinase activity. There are no reports, so far, to indicate that a similar inhibitory feedback mechanism regulates the activity of the insulin receptor, although such a possibility is most appealing in view of the fact that tyrosine phosphorylation takes place at multiple sites.

D. Autophosphorylation of the Receptor *In Vivo* Activates the Insulin Receptor Kinase

Several findings indicate that activation of the receptor kinase, as a result of autophosphorylation, also occurs in intact cells.[204,216] These studies were performed by assaying *in vitro* insulin receptor kinases derived from control and insulin-treated cells. Such indirect approach was necessary, since no natural substrates for the insulin receptor kinase have yet been identified in intact cells and there are no means to directly measure the receptor kinase activity *in vivo*. Utilizing this approach it could be demonstrated that insulin, added to intact cells at physiological concentrations, stimulates 10- to 20-fold the tyrosine kinase activity of the receptor in a time- and a dose-dependent manner. This results in enhanced *in vitro* autophosphorylation of the receptor and enhanced phosphorylation of exogenously added substrates. A correlation between the elevated level of receptor kinase activity, assayed *in vitro*, and the insulin-stimulated *in vivo* tyrosine phosphorylation of the receptor was shown as well.[216] Furthermore, as with purified receptors,[72] *in vivo* activation of the receptor kinase could be reversed upon treatment of partially purified receptors with alkaline phosphatase. Activation by insulin added to intact cells increases the V_{max} of the phosphorylation reaction by decreasing the K_m for ATP,[217] with no change in K_m for exogenous substrates. It can therefore be concluded that insulin-stimulated autophosphorylation of the receptor in intact cells activates the receptor kinase.

XII. SUBSTRATE SPECIFICITY OF THE INSULIN RECEPTOR KINASE

Aside from autophosphorylation, the insulin receptor kinase is capable of phosphorylating *in vitro* a variety of exogenous substrates, including histones,[72,138,218] casein,[150,218] antibodies against pp60[src],[152] angiotensin II,[149,178] a synthetic peptide related to the site of tyrosine phosphorylation in pp60[src],[149,152] reduced and carboxamidomethylated-lysosyme,[203] and several synthetic copolymers containing tyrosine residues.[219,220] Most, if not all, of these substrates cannot be physiologically relevant cellular substrates because of their localization or nature; nevertheless, they were found most useful in attempts to elucidate the kinetic properties and substrate specificity of the insulin receptor kinase.

A. Casein and Histones

Casein[150,218] and histones[72,138,218] were the first exogenous substrates used to characterize the insulin receptor as a tyrosine kinase. Insulin enhances by two- to threefold tyrosine phosphorylation of casein[150] while having only a trivial effect on ^{32}P incorporation into serine residues.

Several histones (H1, H2a, H2b) were also applied as exogenous substrates for the receptor kinase. The best appears to be histone H2b.[72,150,152] Since both casein and histones serve as substrates for serine and threonine kinases, their use for determination of insulin receptor kinase activity, especially in crude preparations, is less favorable. Under such conditions it is often necessary to carry out a phosphoamino acid analysis in order to verify that insulin indeed enhances tyrosine phosphorylation of these substrates.

B. Antibodies Directed Against pp60[src]

Antisera obtained from rabbits bearing tumors induced by the Rous sarcoma virus (TBR sera) contain IgG molecules that react immunologically with a tyrosine kinase that is a viral gene product — pp60[src].[221] This IgG serves as a substrate for the insulin receptor kinase[152] as well as for other tyrosine kinases.[221-223] In contrast to the TBR sera, rabbit and human sera containing antibodies toward the insulin receptor are only poorly phosphorylated by the insulin receptor kinase. TBR sera do not immunoprecipitate the insulin receptor, which suggests that the affinity of the receptor kinase for TBR is relatively low; therefore, phosphorylation of TBR takes place, but immunoprecipitation does not.

C. Synthetic Peptides Containing the Tyrosine Phosphorylation Site of pp60[src]

A synthetic peptide that corresponds to amino acid residues 414 to 424 of pp60[src] [222-224] has been modified[225] to enhance its usefulness in kinetic assays of phosphorylation by tyrosine-specific kinases. The modified peptide, designated peptide A,[148] has the following sequence: Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly. The extension of the natural NH_2 terminus to include the leucine and arginine residues and the addition of a second arginine enhance adsorption of the peptide to phosphocellulose paper, which allows rapid separation of the phosphopeptide from a reaction mixture.[226] Peptide A serves as a substrate for the insulin receptor kinase[149,152,179] which phosphorylates it with a K_m of 1 to 2 *mM*. The addition of insulin enhances threefold the phosphorylation of peptide A. Using 350,000 as the M_r of the receptor the average turnover number for phosphorylation of peptide A is 4/min, at 22°C.[152] This turnover number is more than 100-fold lower than the value extrapolated from the kinetic data obtained from phosphorylation of peptide A by the EGF receptor kinase. Such a difference might reflect different specificities of the two kinases[152] or different requirements for optimal activation,[152] such as a need for a phospholipid environment.[227]

A different peptide, peptide B, was also assayed as a substrate for the insulin receptor kinase. Peptide B is a tyrosine analog of a serine-containing peptide used as a substrate for

Table 1
RANDOM COPOLYMERS OF AMINO ACIDS

Polymer	Molar ratio	Unit (M_r)	Amino acid concentration at 2 mg/ml polymer (m*M*)			
			Tyr	Glu	Ala	Lys
Ala/Glu/Lys/Tyr	6:2:5:1	1992	1.0	2.0	6.0	5.0
Glu/Tyr	4:1	789	2.5	10.2	—	—
Glu/Ala/Tyr	6:3:1	1306	1.5	9.1	4.6	—
Glu/Tyr	1:1	315	6.3	6.3	—	—
Glu/Ala/Tyr	1:1:1	386	5.2	5.2	5.2	—

the cAMP-dependent protein kinase.[228] It has the following sequence: Leu-Arg-Arg-Ala-Tyr-Leu-Gly. The receptor kinase catalyzes phosphorylation of peptide B with a K_m similar to peptide A, but with a V_{max} that is more than an order of magnitude lower than that for peptide A.[152] Similar differences in phosphorylation of peptides A vs. B are obtained using the EGF receptor tyrosine kinase.[223] The major difference between peptides A and B is in the amino acids surrounding the phosphorylated tyrosine. While those in peptide A are acidic in nature, those in peptide B are basic. These findings are compatible with the notion that the favorable sites for tyrosine phosphorylation are those adjacent to acidic residues.[224] Indeed, tyrosine kinases stimulated by insulin, IGF-1, and EGF (*vide infra*) best phosphorylate polymers that contain only tyrosine and glutamic residues. This suggests that certain combinations of glutamic and tyrosine residues are the minimal definitive chemical feature necessary to turn a polypeptide into a substrate for these enzymes.[229] As previously mentioned, these glutamic residues should be best localized four, three, or one amino acid away on the N-terminal side of the tyrosine to be phosphorylated.[230] This requirement is not obligatory as glutamate residues are located at the C-terminal end of the phosphorylated Tyr 1322 of the insulin receptor, while [Val^5] angiotensin II, which serves as a substrate for the insulin receptor kinase,[149,204] does not contain any glutamic or aspartic residues.

D. Tyrosine-Containing Polymers

Tyrosine-containing synthetic polypeptides (Table 1) have long been used as a simple high-molecular-mass model for biochemical and immunological studies.[231,232] These polymers have certain features that make them particularly suitable to serve as common substrates for the insulin receptor and other tyrosine kinases: (1) each polymer has a different, defined amino acid composition; (2) ^{32}P is incorporated exclusively into tyrosine residues (which are present in a high concentration, yet in a soluble form); (3) ^{32}P-labeled polymers are quantitatively precipitated by trichloroacetic acid, thus providing a simple and convenient means for assay of a large number of samples.

The receptor kinase best phosphorylates (highest V_{max}) a random copolymer composed of glutamic and tyrosine residues at a 4:1 ratio Poly Glu/Tyr (4:1).[219,220] Other tyrosine-containing polymers, Poly Ala/Glu/Lys/Tyr (6:2:5:1) and Poly Glu/Ala/Tyr (6:3:1), are also phosphorylated but to a lower extent. All three polymers, however, are better substrates than casein or histone. Two additional polymers, Poly Glu/Tyr (1:1) and Poly Gly/Ala/Tyr (1:1:1), do not serve as substrates even though they contain (on molar basis) 2.5 to 3.5 times more tyrosine residues (Table 1). Since introduction of tyrosine residues into polyglutamic regions disrupts the helical nature of the polymer[233] and since Poly Glu/Tyr (4:1) and Poly Glu/Tyr (1:1) differ in their glutamic/tyrosine ratio, it seems reasonable to assume that the two polymers also differ in their overall conformation, which determines their potency to serve as substrates.[234] Several other tyrosine-specific kinases (stimulated by EGF, IGF-1, and tumor-promoting phorbol ester [TPA]) phosphorylate these polymers with a similar,

yet distinct, specificity. The TPA-stimulated tyrosine kinase was in fact characterized as such based on its ability[235] to phosphorylate these polymers. This tyrosine kinase does not require the presence of calcium ions, phospholipids, or diolein for its activity, features that make it distinct from the TPA-stimulated protein kinase C which is a serine kinase.[236] Tyrosine-containing polymers are also substrates for other tyrosine kinases, for example, those coded by Fujinami and Rous sarcoma virus.[219] This feature makes the phosphorylation intensity of different polymers a potential tool to determine possible homologies between various kinase active sites. Indeed, the greatest similarity in substrate specificity is between insulin and IGF-1 stimulated kinases, which are also known to have a high homology in receptor structure.[115] Likewise, based on their polymer phosphorylation profile, the EGF- and insulin-stimulated tyrosine kinases have a similar, yet distinct, substrate specificity.[220] A similar conclusion was reached when the ability of the insulin and EGF receptor kinases to phosphorylate angiotensin II, histones, casein, and src-related peptides was compared.[179,237] The insulin and EGF-stimulated kinases exhibited nearly identical K_m values for each protein or peptide substrate examined; however, differences between the two kinases could be discerned when the V_{max} values for the different substrates were compared.

The similarity in *in vitro* substrate specificity of insulin and EGF receptor kinases suggests that the *in vivo* specificity of these two enzymes may overlap. A number of similarities in the effects of insulin and EGF on various cell functions have indeed been noted. These include stimulation of phospho fructokinase activity,[238] glycolysis,[239] increased ion fluxes,[18] and hexose transport.[240] If we assume that the receptor kinase activities play a key role in mediating the action of insulin and EGF, then the similarity in substrate specificity of these two enzymes could provide us with a reasonable explanation why these two hormones elicit similar biological responses.

Tyrosine-containing polymers are powerful tools in detection of tyrosine kinase activity. For example, in BRL cells, IGF-1 stimulated incorporation of 1×10^4, 4×10^4, and over 1×10^6 cpm of ^{32}P into endogenous proteins, casein, and Poly Glu/Tyr (4:1), respectively. Therefore, the use of Poly Glu/Tyr (4:1) resulted in a 100-fold amplification of the signal obtained with endogenous substrates. This feature is of particular importance when studying receptor functions in patients, where tissue availability is limited, or in studies of tyrosine kinases whose native substrates are unknown or present at too low concentrations. For example, tyrosine-containing polymers were employed in studying the correlation between insulin resistance and defects in receptor kinase activity in freshly isolated human monocytes,[22] where autophosphorylation of the receptor is difficult to detect.

XIII. NATURAL SUBSTRATES FOR THE INSULIN RECEPTOR IN INTACT CELLS AND CELL-FREE SYSTEMS

The insulin receptor kinase may, in principle, mediate some or all of insulin's biological actions. A possible mechanism could be phosphorylation of cellular proteins on tyrosine residues. Such phosphorylation is expected to alter the substrate's structure, which will result in changes in its localization or function. This may set into motion a cascade of reactions eventually leading to the expression of some or all of the effects of insulin. A major challenge for such contention is the demonstration of cellular targets for the insulin receptor kinase.

Different approaches were attempted to identify natural substrates for the insulin-receptor kinase. One has been to search for unknown proteins that undergo tyrosine phosphorylation in response to insulin, favorably in intact cells or, alternatively, in cell-free systems. A second approach has been to screen as substrates, in cell-free systems, various known proteins, localized at key positions in the major biochemical pathways leading to the metabolic and growth-promoting effects of insulin.[1] These proteins could be regarded as potential candidates to undergo phosphorylation by the insulin receptor kinase in a manner that could modulate their function and promote insulin action (see Table 2).

Table 2
POTENTIAL *IN VIVO* PROTEIN SUBSTRATES FOR THE INSULIN RECEPTOR KINASE

Protein	Tissues	Ref.
pp240	Human epidermal carcinoma cells	141
pp185	Rat hepatoma (Fao) cells	140, 144
	Human epidermal carcinoma cells	141
	3T3-L1 adipocytes	141, 299
	BALB/c 3T3 fibroblasts	141
	Rat and canine kidney cell lines	142
	Mouse neuroblastoma N18 cells	143
	L6 myocytes	144
	CHO cells	144
pp120	H-35 hepatoma cells	300
pp46	Rat adipocytes	304
pp15	3T3-L1 adipocytes	305, 306
Calmodulin	Rat adipocytes	308
Tyrosine-phosphorylated serine kinase	Rat adipocytes	324

A. Antibodies against Phosphotyrosine Residues as Potent Tools in Studies Aimed at Identification of Protein Substrates for the Insulin Receptor Kinase

A major problem encountered in studies aimed at identification of protein substrates for the insulin receptor kinase in intact cells is the very low abundance (less than 0.1%) of proteins phosphorylated on tyrosine residues.[241,242] This implies that powerful purification procedures need to be employed in order to detect the tyrosine-phosphorylated proteins and separate them from the bulk of irrelevant proteins phosphorylated on serine or threonine residues. Conventional methods for detection of tyrosine-phosphorylated proteins involve (1) separation of labeled extracts on two-dimensional gel electrophoresis; (2) treatment of the gels with strong alkaline solutions in order to hydrolize most phosphothreonine and phosphoserine bonds; and (3) isolation of individual proteins and performance of a phosphoamino acid analysis. These procedures are both tedious and have certain disadvantages: (1) certain phosphoproteins, especially those with pI values below 5.5 or phosphoproteins of high molecular weights, are poorly resolved on two-dimensional gels due to the background phosphorylation that occurs in these regions;[243] (2) not all phosphoserine or phosphothreonine bonds are alkaline labile; (3) phosphoamino acid analysis, which requires exposure of the proteins to strong acid conditions, results in low (25%)[244] and somewhat variable yields of individual phosphoamino acids, making it difficult to assess their actual original content.

An alternative approach was developed, which makes use of antibodies directed against phosphotyrosine residues in order to selectively immunoprecipitate proteins phosphorylated on tyrosine residues. Antiphosphotyrosine antibodies were originally developed by Ross et al.[245] and subsequently by others.[161,246-248] Some of these antibodies, when used in immunoblots,[46,249,250] were able to decorate specific proteins containing phosphotyrosine residues. As shown below such antibodies are now widely used to identify the substrates for the insulin receptor kinase. It should be noted, however, that methods using antiphosphotyrosine antibodies suffer from their own limitations: the antibodies may not be able to recognize sterically or conformationally hidden phosphotyrosines or to immunoprecipitate phosphotyrosine-containing proteins present at low concentrations.[141]

B. Protein Substrates for the Insulin Receptor Kinase

1. pp185

When incubated *in vitro* with [γ-^{32}P] ATP and insulin, purified insulin receptor kinase

phosphorylates several proteins on tyrosine residues (*vide infra*). However, so far only few proteins, other than the receptor itself, have been identified as undergoing tyrosine phosphorylation in response to insulin added to intact cells. The first that was identified is a phosphotyrosine-containing protein of M_r 185,000 (pp185) that was isolated from ^{32}P-labeled insulin-treated (Fao) cells using antiphosphotyrosine antibodies.[140,144] Phosphorylation of pp185 has since been reported to take place in other cell lines, including human epidermoid carcinoma KB cells,[141] 3T3-L1 adipocytes,[141,251] BALB/c 3T3 fibroblasts,[141] rat and canine kidney cell lines,[142] mouse neuroblastoma N18 cells,[143] L6 myocytes, and CHO cells, transfected with the expression vector containing the human insulin receptor cDNA.[144] Phosphorylation of pp185 has a "spike-like" pattern. P-Tyr content of pp180 increases at least tenfold within seconds after exposure of intact cells to insulin, remains at maximal levels for 1 to 2 min, and then rapidly declines. pp180 loses 50% of its phosphate content following a 10-min treatment with insulin.[141,143] Prolonged incubation of Fao cells with insulin leads to the disappearance of pp185 and to the appearance of two other phosphotyrosine-containing proteins with molecular masses of 210 and 170 kDa. It has therefore been suggested[144] that pp185 is, in fact, composed of two distinct phosphotyrosine-containing proteins, or, alternatively, that some of the pp185 undergoes a further covalent modification that alters its mobility. Phosphorylation of pp185 exhibits a dose-response curve similar to that of receptor autophosphorylation, which suggests that phosphorylation of pp185 is an early event that occurs following insulin binding. Insulin binding increases mainly phosphotyrosine content of pp185, although a significant increase in the amount of phosphoserine and phosphothreonine occurs as well.

It still remains unclear whether pp185 is an *in vivo* substrate of the insulin receptor kinase or whether its phosphorylation results due to insulin-mediated activation of some other tyrosine kinase(s). It is also unknown whether p185 serves as an *in vitro* substrate of the insulin receptor kinase, a property that could contribute to its assignment as a native substrate of the receptor kinase. Part of the difficulty in carrying out *in vitro* studies is the low cellular content of pp185.[140] Interestingly, pp185 undergoes enhanced tyrosine phosphorylation when N18 neuroblastoma,[143] KB, or MDCK cells[141,142] are treated with IGF-1. The kinetic characteristics of the IGF-1-mediated phosphorylation are indistinguishable from those observed in insulin-treated cells, and the effects of insulin and IGF-1 on pp185 phosphorylation are not additive. These findings suggest, but do not prove, that pp185 is a common substrate for both insulin and IGF-1 receptor kinases. Tyrosine kinases associated with the receptor for insulin and IGF-1 manifest a similar profile of substrate specificity *in vitro*.[220] The above findings indicate that the homologies between insulin and IGF-1 receptors can be extended to a similar substrate specificity of these receptor kinases *in vivo*. By contrast, pp185 does not serve as a substrate for the EGF or PDGF receptor kinases, since cells treated with these ligands express no elevated levels of phosphorylation of pp185.[144] The nature of pp185 has not yet been elucidated. It is a cytosolic protein[144] and is probably not the insulin receptor itself or any of its precursors for the following reasons: (1) unlike the insulin receptor, pp185 does not bind to wheat germ agglutinin and it is not immunoprecipitated by antibodies against the insulin receptor; (2) tryptic phosphopeptide map of pp185 does not correspond to the profile of the β subunit of the insulin receptor; (3) pp185 is not labeled by cross-linking with ^{125}I-insulin or surface iodination.[144] pp185 is probably unrelated to the receptors for EGF or PDGF. Such conclusion is based, at least in part, on the fact that these receptors are glycoproteins which bind to wheat germ agglutinin, whereas pp185 is a cytosolic protein.

Taken together, these findings suggest that pp185 is a cytosolic protein substrate for insulin-mediated tyrosine phosphorylation in intact cells. As such it could well represent a molecular link between the membrane-bound insulin receptor and the regulation of metabolic reactions in the cytoplasm of insulin-sensitive cells.

2. pp120

When added to intact ^{32}P-labeled H-35 hepatoma cells, insulin enhances twofold phosphorylation of a protein with a molecular mass of 120,000 present in lectin-purified preparations of detergent-solubilized cells.[252] Phosphorylation of pp120 in response to insulin treatment occurs in a similar time course as that of the receptor itself; furthermore, the ligand specificity of the reaction corresponds to that observed for enhanced phosphorylation of the receptor. EGF is ineffective, while IGF-1 is only 1% as potent as insulin in stimulating the phosphorylation of pp120.

pp120 is different from the insulin receptor itself, and is probably not a degradation product of pp180, the other known *in vivo* substrate of the insulin receptor. This conclusion is based upon the following findings: (1) antibodies against the insulin receptor fail to precipitate pp120, while antibodies raised against pp120 fail to precipitate the insulin receptor; (2) both the electrophoretic mobility of pp120 on nonreducing SDS gels and the phosphopeptides generated by partial proteolysis of pp120 differ from that of the insulin receptor;[253] (3) pp120, unlike the receptor or pp180, is not precipitated by several antiphosphotyrosine antibodies; (4) unlike pp180, which is a cytosolic protein, pp120 is either a membrane glycoprotein or it is a protein tightly bound to membrane proteins; (5) unlike pp180, pp120 was reported so far to serve as a substrate only for the insulin receptor, but not for the IGF-1 receptor kinase. *In vitro* phosphorylation of a protein having a similar molecular weight (120 kDa) by the insulin receptor kinase[253-255] strengthens the possibility that pp120 is a physiological substrate of the receptor in intact cells. The *in vitro* substrate, like the *in vivo* one, is present in partially purified receptor preparations, derived from hepatic microsomes by solubilization in Triton® X-100, followed by lectin affinity chromatography. Three lines of evidence support the notion that pp120 is phosphorylated *in vitro* directly by the insulin receptor kinase: (1) insulin and proinsulin stimulate phosphorylation of pp120 with potencies proportional to their binding affinities for the insulin receptor; (2) pp120 undergoes insulin-mediated phosphorylation on tyrosine residues, which is compatible with its being a substrate for the insulin receptor kinase; and (3) the optimal conditions needed to assay the insulin receptor kinase activity are identical to those required to obtain phosphorylation of pp120.

The physiological role of pp120 is yet undefined. However, Dexamethasone-treated rats demonstrate elevated levels of pp120 phosphorylation in partially purified receptor preparations, most likely due to an increase in the amount of pp120 present there.[253] No data are yet available, however, to better correlate phosphorylation of pp120 with any effect of glucocorticoids on insulin binding or action.

3. pp240

Human epidermoid carcinoma cells (KB cells), treated with insulin, IGF-1, or EGF for 30 s, were found to contain an elevated level of a tyrosine-containing protein of 240 kDa that could be immunoprecipitated by antiphosphotyrosine antibodies.[141] This protein differs from receptors for those growth factors as it does not immunoprecipitate with the appropriate antireceptor antibodies. Phosphorylation of pp240 is transient, peaks 1 min following hormonal stimulation, and rapidly decays thereafter. pp240 does not bind to lectin columns and in this respect it is similar to pp185. Further characterization of pp240 will be needed in order to identify its function.

4. pp46

Stimulation of ^{32}P-labeled rat adipocytes with insulin enhances tyrosine phosphorylation of 46-kDa protein localized in the membranal fractions of these cells.[256] This protein is immunoprecipitated by antiphosphotyrosine antibodies, but not by antibodies against different regions of the insulin receptor. Furthermore, its HPLC tryptic peptide map is different from the tryptic peptide map of the insulin receptor, suggesting that it is not derived from

the receptor β subunit. Insulin treatment of cells enhances phosphorylation of pp46 exclusively on tyrosine residues. This property is unique to pp46, since all other *in vivo* substrates (e.g., pp240, pp180, pp120) as well as the receptor itself undergo enhanced phosphorylation on serine, threonine, and tyrosine residues in response to insulin.

Insulin added to intact cells stimulates three to fourfold tyrosine phosphorylation of pp46 within 2 min. Phosphorylation declines to almost basal levels by 5 min. Insulin's effect is dose dependent and is detected even at 0.5 n*M* insulin.

5. pp15

A cytosolic protein with molecular mass of 15,000 is phosphorylated on tyrosine residues, when intact 3T3-L1 adipocytes are incubated in the presence of insulin and phenylarsine oxide (PAO).[257,258] This agent, which complexes vicinal dithiols, reversibly blocks activation of hexose uptake by insulin, without affecting hexose uptake per se.[259] The insulin-dependent phosphorylation of pp15 in PAO-treated cells occurs in a slower rate compared with receptor autophosphorylation, but is faster than the rate of glucose uptake into the cells (measured in the absence of PAO). This is consistent with a role of phosporylated pp15 in mediating insulin-activated hexose uptake.[257] Phosphorylation of pp15, like that of pp46,[256] occurs exclusively on tyrosine residues[257] and is specifically stimulated by insulin. IGF-1, IGF-II, EGF, or PDGF fail to induce phosphorylation of pp15. Failure of these growth factors, which are also potent stimulators of glucose uptake, to enhance phosphorylation of pp15 places this protein in a signal transmission pathway unique for insulin action. PAO has no effect on the insulin concentration dependence or the kinetic of β subunit phosphorylation,[257] nor does it affect insulin binding or phosphorylation of exogenous substrates by the receptor kinase.[258] By these criteria it appears that PAO inhibits a postreceptor step in insulin action that enables accumulation of otherwise undetected tyrosine-phosphorylated pp15. The accumulation of pp15 in the presence of PAO is reversible. Incubation of the cells with the vicinal dithiol 2,3-dimercapto propanol enables detection of insulin-stimulated glucose uptake, which is accompanied by a decrease in accumulation of pp15. Further studies will be needed in order to determine the role of pp15 in insulin-dependent glucose uptake, and to clarify how phosphorylation of pp15 modulates its function.

6. Calmodulin

Calmodulin undergoes enhanced tyrosine phosphorylation in response to insulin added to intact cells[260] and was shown as well to serve as an *in vitro*[261,262] substrate for the insulin receptor kinase.

Addition of insulin at physiological concentrations (0.3 n*M*) to ^{32}P-labeled rat adipocytes increases sixfold the phosphate content of calmodulin as detected by two-dimensional electrophoresis.[260] The concentration dependence for insulin-induced phosphorylation of calmodulin is similar to the concentration dependence for insulin-stimulated glucose incorporation into these cells. All phosphorylated calmodulin appears to be present in the cytosol. This may indicate that phosphorylation of calmodulin on tyrosine residues may alter its cellular distribution. Phosphorylation of calmodulin following insulin treatment is almost stoichiometric, with 0.3 to 0.7 mol Pi incorporated per mole of calmodulin. The phosphorylated calmodulin is alkali resistant, which suggests, though not proves, that tyrosine residues are phosphorylated. Calmodulin contains only two tyrosine residues (residues 99 and 138), both located within the third and forth Ca^{2+} binding sites of calmodulin.[263] Furthermore, Tyr-138 is localized adjacent to acidic amino acids (Glu-Val-Asn-Tyr-Glu-Glu), which turn it into a potential substrate for tyrosine phosphorylation.

The role of calmodulin in insulin action is still unknown, and the physiological significance of calmodulin phosphorylation following insulin binding needs to be determined. Calmodulin is a ubiquitous calcium-binding protein, known to regulate a number of Ca^{2+}-sensitive

enzymatic activities.[264] Insulin is known to increase Ca^{2+} [264] and calmodulin[265] binding to adipocyte plasma membranes and to inhibit the activity of a calmodulin-dependent membrane (Ca^{2+} + Mg^{2+})-ATPase.[266] Furthermore, the insulin receptor itself binds Ca^{2+} [267] and calmodulin,[268] which enhances receptor kinase activity in cell-free systems.[262] It is therefore tempting to speculate that calmodulin phosphorylation by the insulin receptor kinase could have a dual purpose: to modulate the activity of the receptor kinase itself and to mediate the effects of insulin on other intracellular components. This could be carried out either directly by the phosphorylated calmodulin or indirectly where phosphorylation of calmodulin serves as means to modulate interactions of the insulin receptor with calmodulin-binding proteins (e.g., GTP-binding proteins [*vide infra*]).

7. Tyrosine-Phosphorylated Serine Kinases

The regulatory action of insulin on cellular metabolism involves the modulation of serine and threonine phosphorylation states of key enzymes.[3] This action of the hormone is believed to be mediated by serine/threonine kinases or phosphatases. It is therefore possible to hypothesize that through tyrosine phosphorylation of serine kinases the insulin receptor directly or indirectly alters their activity. Amplification of serine kinase activities through a cascade of phosphorylation reactions is well established. The cAMP-mediated cascade provides a classical example where the cAMP-dependent protein kinase phosphorylates and activates phosphorylase kinase, which phosphorylates and activates phosphorylase b.[269] Such an example rationalizes efforts made to identify a serine kinase whose activity will be modulated upon tyrosine phosphorylation by the insulin receptor kinase. Since insulin action is known to affect glycogen metabolism, five serine-protein kinases known to phosphorylate glycogen synthase *in vitro*[270] were tested as putative *in vitro* substrates for the insulin receptor kinase.[261] These include phosphorylase kinase,[271] glycogen synthase kinase 3,[272] casein kinase I and II,[273] and Ca^{2+}/calmodulin-dependent protein kinase.[274] Of the five kinases tested, only the latter was found to serve as *in vitro* substrate for the receptor kinase. More recent studies have shown that insulin stimulates a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*.[275] No data were provided, however, to demonstrate that this enzyme is indeed an *in vivo* substrate for insulin receptor kinase. In another study it was shown[276] that Triton® X-100-solubilized high-density microsomes from insulin-treated rat adipocytes exhibit a marked increase in serine/threonine kinase activity toward exogenous histone when compared with controls. Stimulation of this serine kinase occurs at physiological insulin concentration and it is ligand specific. The substrate specificity of this enzyme distinguishes it from kinases that phosphorylate ribosomal protein S6, casein, phosvitin, ATP citrate lyase, and glycogen synthase. It also differs from Ca^{2+}/calmodulin, Ca^{2+}/phospholipid, and cAMP- and cGMP-dependent protein kinases. Activation of this kinase has been suggested[276] to be the result of an insulin-dependent tyrosine phosphorylation of the enzyme. This suggestion is based upon the observation that about 20% of the insulin-sensitive serine kinase is adsorbed by monoclonal antiphosphotyrosine antibodies immobilized on agarose. It should be noted that the stimulatory action of insulin on this serine kinase can only be detected in membranes from insulin-treated adipocytes, while direct addition of insulin to isolated membrane preparations activates the insulin receptor kinase present there, but fails to activate the serine kinase. These findings raise the possibility that activation of the serine kinase by the insulin receptor is either indirect or requires the presence of additional components missing from isolated membranes.

C. Exogenously Added Protein Substrates for the Insulin Receptor Kinase

1. Tubulin and Microtubule-Associated Proteins (MAPs)

Cytoskeletal proteins are potential substrates for the insulin receptor kinase, because they are located close to the plasma membrane and participate in many cellular functions. Inter-

action of other tyrosine kinases such as pp60^{src} and the EGF receptor kinase with cytoskeletal proteins like vinculin[277] and myosin light chain[278] have already been reported.

Evidence has been presented to suggest that purified native tubulin and MAPs serve as substrates for purified insulin receptor kinase.[279] Insulin (10^{-7} *M*) stimulates this phosphorylation four- to tenfold by increasing the V_{max} with little change in the K_m of the reaction. α-Tubulin is a better substrate compared with β-tubulin. Phosphorylation of tubulin by the insulin receptor is almost stoichiometric and up to 0.5 mol phosphate is incorporated per mole of tubulin. The K_m of the reaction is 40 μ*M*, which is comparable to the concentration of tubulin in cells.[280] The microtubule-associated proteins MAP2 and tau are even better *in vitro* substrates for the insulin receptor. This reaction has an apparent K_m of 5 μ*M* and approximately 1.7 mol phosphate is incorporated per mole of MAP2. Not all cytoskeletal elements are substrates for the insulin receptor kinase; for example, actin and tropomyosin are only poorly phosporylated,[279] while vinculin fails to serve as a substrate.[149]

The physiological significance of phosphorylation of cytoskeletal proteins like tubulin by the insulin receptor kinase still remains to be established. For example, it needs to be determined how the receptor, a plasma-membrane protein, encounters cytoskeletal elements that are anchored at some distance to the plasma membrane through specific proteins such as vinculin[281] or talin.[282] A possible encounter is likely to take place only between the receptors and the free cytosolic forms of MAPs or tubulin, or with the organized, polymerized forms of these cytoskeletal elements during receptor recycling.

Microtubules are likely to play a role in regulating cellular traffic induced upon insulin binding to this receptor. This includes translocation of cellular elements associated with endocytosis of insulin,[283] internalization and recycling of the receptor itself[6,7] and the insulin-stimulated redistribution between plasma membranes and intracellular organelles of the glucose transporter[8,8a] and IGF-II receptors[9,9a,284] (*vide supra*). Furthermore, insulin binding to intact cells induces changes in morphology and organization of microtubules.[285] This suggests that microtubular function is altered as a result of insulin binding and the subsequent activation of the insulin receptor kinase. Regulation of cytoskeletal protein function through phosphorylation is a known phenomenon. Both tubulin and MAPs serve as substrates for the cAMP-dependent protein kinase[286] and a Ca/calmodulin-dependent protein kinase.[287] This serine and threonine phosphorylation regulates microtubule assembly and inhibits MAP-actin association. It remains to be determined whether tyrosine phosphorylation of tubulin and MAPs by the insulin receptor kinase or other tyrosine kinases alters these functions as well.

Finally, tubulin is known to undergo a unique posttranslational modification in which a tyrosine residue is reversibly added to the C terminus of α-tubulin.[288] The tyrosinated and nontyrosinated forms of α-tubulin are predominantly present in different populations of microtubules[289] that may as well be functionally distinct. Since the C-terminal region of α-tubulin is rich in glutamic residues[290] and since a tyrosine located in the vicinity of glutamic residues is a preferred substrate for tyrosine kinase,[224] it is an intriguing question whether these particular tyrosine residues serve as substrates for the insulin and related receptor tyrosine kinases.

2. *The Progesterone Receptor*

Purified progesterone receptors serve as high-affinity substrates (K_m 0.1 μ*M*) for the insulin receptor kinase.[291] Tyrosine phosphorylation of the 80-kDa subunit of the progesterone receptor by the insulin receptor kinase occurs with stoichiometry that is about 0.5 mol/mol. Insulin decreases the K_m of the reaction but has no significant effect on V_{max}. The progesterone receptor is a substrate for other serine and tyrosine kinases as well. The EGF receptor kinase phosphorylates the progesterone receptor on tyrosine residues,[292] while the cAMP-dependent protein kinase phosphorylates this receptor on serine and threonine residues.[293] There is no

evidence, however, that relates phosphorylation of the progesterone receptor by the insulin receptor kinase (or any of the other kinases) with either modification of high-affinity steroid binding or steroid-mediated cellular activity. There is also no evidence that phosphorylation of the progesterone receptor affects the binding of the receptor to DNA[294] or to proteins bound to DNA,[295] actions that could influence sites controlling expression of different genes. Since insulin is known to affect the level of expression of several genes,[296] it will be of interest to determine whether phosphorylation of progesterone (or other steroid) receptors is involved in this process.

3. GTP-Binding Proteins (G-Proteins)

GTP-binding proteins (G-proteins) play a key role in transmembranal signal transmission. They act to couple receptors to their second messengers generating systems in many biochemical events, including hormone action, phototransduction, and cell growth.[297-299]

Isolation of multiple cDNAs that code for G-proteins[300-303] suggests that the family of G-proteins includes quite a large number of proteins that mediate multiple biological activities.[298] Signal transmission mediated by cAMP is dually regulated by G-proteins. Hormones that increase intracellular levels of cAMP interact with Gs, which stimulates adenylate cyclase activity.[304] Other hormones that lower cAMP interact with a distinct G-protein, Gi, which inhibits cyclase activity.[305] Another G-protein, termed Go, has been isolated from brain.[306] The outer segment of the rod in the retina contains substantial amounts of a specific G-protein named transducin (TD),[307] which plays a key role in phototransduction. Other functions such as regulation of ion channels and activation of phosphoinositide (PIP_2) metabolism are also regulated by G-proteins.[298]

G-proteins have been suggested as mediators of certain insulin actions. Both inhibition of adenylate cyclase[308] and activation by insulin of a cAMP-phosphodiesterase depend on the presence of GTP.[309] Moreover, insulin reduces cholera toxin-catalyzed ADP-ribosylation of a 25-kDa protein in liver membranes,[15] which is suggested to be a unique G-protein, termed G-ins,[15] based on its photoaffinity labeling with a GTP analog.

It has been suggested that by analogy with other hormone receptors,[310-312a] a unique G-protein, G-ins, coupled to the insulin receptor, mediates activation of a specific phospholipase upon insulin binding.[313] This specific phospholipase hydrolyzes a unique glycano phosphoinositide to generate two second messengers: inositol-phosphate glycan and diacylglycerol,[314-317] the natural activator of protein kinase C.[318,319] Involvement of G-proteins in insulin action has also been implicated from studies demonstrating that ob/ob mice that suffer from hyperglycemia, hyperinsulinemia, and extreme resistance to insulin manifest an abnormality in G-protein function.[320] Furthermore, hepatocytes isolated from streptozotocin-induced diabetic rats express reduced levels of Gi.[321] Finally, p21, the cellular homolog of the ras oncogene product, was shown to mediate insulin-induced maturation of *Xenopus* oocytes.[322]

Based on sequence similarities as well as immunological and functional cross-reactivity, the G-proteins isolated so far form a family of proteins that are structurally and functionally related. They are heterotrimeric with distinct α and γ subunits and a conserved homologous β subunit.[323] The mode of activation of G-proteins by receptors that are coupled to them is similar. Binding of a ligand to a specific receptor facilitates exchange of GDP, bound to the α subunit of the G-protein with GTP. Subsequently, the α subunit dissociates from the $\beta\gamma$ regulatory complex and activates the effector enzyme responsible for second messenger generation.[324] Termination of the signal is achieved by the intrinsic GTPase activity of the α subunit that hydrolyzes the bound GTP to form GDP-bound G-α. The latter then recombines with the $\beta\gamma$ subunits regenerating the intact inactive G-protein.[325] G-proteins are subjected to two types of posttranslational modifications: they undergo ADP-ribosylation when treated with bacterial toxins[326,327] and they serve as *in vitro* substrates for phosphorylation by both

serine and tyrosine kinases. TD,[170,328] Gi,[329] Go,[329] as well as the ras product p21[322] serve as *in vitro* substrates for the insulin and IGF-1 receptor kinases, which phosphorylate them on tyrosine residues. Phosphorylation of holo TD occurs to a lower extent than phosphorylation of its isolated GDP-bound α subunits. The latter are phosphorylated in a dose-dependent manner with a K_m of 1 μ*M*,[328] suggesting a high affinity of the insulin (and IGF-1) receptors to the G-proteins. This conclusion is further supported by recent findings demonstrating that phosphorylated TD-α and insulin receptors are tightly bound and coprecipitate with antibodies directed against the C-terminal end of certain G-proteins. Serine kinases such as protein kinase C[328,330] and a polypeptide-dependent kinase from baker's yeast[331] were also shown to phosphorylate the α subunits of G-proteins. Other kinases such as the cAMP-dependent protein kinase[328,330] or Ca/calmodulin-dependent myosin light chain kinase[330] failed to phosphorylate G-proteins. Protein kinase C phosphorylates Gi-α, present in human platelet membranes, in a reaction that is specifically blocked upon addition of purified β subunits.[330] These results were interpreted as an indication that only free α subunits, rather than whole trimeric G-proteins, are substrates of protein kinase C. Such conclusion is supported by the findings that free Gi-α is an excellent *in vitro* substrate for this kinase (0.5 mol phosphate incorporated per mole of subunit), but the Gi oligomer is not.[330] Similar observations were made when TD was tested as a substrate for protein kinase C.[328] While the free TD-α-GDP serves as a high-affinity substrate (K_m 1 μ*M*) for protein kinase C, holo TD does not undergo any significant phosphorylation.

Interestingly, *in vitro* phosphorylation of G-proteins by tyrosine and serine kinases depends upon the conformation of the G-protein. For example, TD-α-GDP but not TD-α-GTP(γ-S) serves as substrate for both the insulin receptor kinase and protein kinase C.[328] Furthermore, addition of GTP(γ-S) to a preparation containing the insulin receptor and TD-α-GDP inhibits phosphorylation of the latter with no significant effect on insulin receptor autophosphorylation.[328] These results are consistent with the notion that the α subunit undergoes a marked conformational change upon exchanging GDP for GTP during the G-protein cycle.[332] Phosphorylation of G-proteins could result in a marked alteration of G-proteins' structure and function. It could affect (1) the interactions between G-proteins and hormone receptors and alter binding of the receptor either to the G-proteins, to the ligand, or to both; (2) the efficiency of the G-cycle itself by modulating GTP binding, subunit-subunit interactions, or the intrinsic GTPase activity; (3) the interaction between the G-proteins and their effector systems, thereby modulating various cellular responses. However, it still remains to be determined whether G-protein phosphorylation occurs in intact cells and whether it indeed affects any of the G-protein functions mentioned above.

In summary, certain G-proteins undergo *in vitro* multisite phosphorylation, both on tyrosine and serine residues. Multisite phosphorylations could, in principle, act to synergize or antagonize each other, providing a cross-talk mechanism in controlling and regulating the various functions of G-proteins. As such, G-proteins could function as "programmable messengers"[333] mediating some of insulin's action. In particular, the findings that G-proteins serve as *in vitro* substrates for both the insulin receptor kinase and protein kinase C may implicate G-proteins in playing some role in mediating those actions of insulin associated with activation of protein kinase C. For example, it is possible to propose a model where the insulin receptor kinase, when coupled to a G-protein (in a process that might involve tyrosine phosphorylation of the latter), leads to activation of specific phospholipase and to the generation of diacyglycerol (*vide infra*). Diacylglycerol acts to activate protein kinase C which phosphorylates intracellular proteins as well as G-proteins. The latter, now phosphorylated on serine residues, synergize with insulin action or, alternatively, act as feedback regulators to terminate insulin's signals. This model is, at present, highly speculative and needs a lot of supportive data before found correct.

XIV. REGULATION OF THE RECEPTOR KINASE ACTIVITY THROUGH PHOSPHORYLATION ON SERINE RESIDUES

A. The Insulin-Stimulated Receptor Phosphorylation on Serine/Threonine Residues

Insulin added to intact cells causes a rapid phosphorylation of the receptor on tyrosine residues, which is followed by a slower phosphorylation on serine/threonine residues (*vide supra*). Since the receptor itself functions exclusively as a tyrosine kinase, there are at least two models that could account for the insulin-dependent increase in serine and threonine contents of the receptor. The (insulin-receptor) complex or tyrosine-phosphorylated receptors could serve as better substrates than native unoccupied receptors for an as yet undefined serine/threonine kinase, or, alternatively, the receptor tyrosine kinase, through direct or indirect tyrosine phosphorylation, could activate a serine kinase that then phosphorylates the receptor itself. Such phosphorylation could act as a feedback regulatory device to inhibit the receptor tyrosine kinase activity.

Studies with insulin receptors purified on lectin columns[334] or receptors bound to insulin agarose columns[202] indicate that addition of insulin causes a small but significant increase in phosphoserine[202,334] and phosphothreonine[202] contents of the receptor. This means that the receptor serine/threonine kinase is either itself a glycoprotein or it is tightly bound to the receptor. Furthermore, insulin added to the lectin-purified receptor preparations does not affect the rate of phosphorylation of serine/threonine residues in casein or histones, suggesting that histones or casein are not substrates for the receptor serine kinase, or, alternatively, that the receptor tyrosine kinase does not directly activate this enzyme. If the latter possibility is indeed the case, then insulin binding causes generation of tyrosine-phosphorylated (insulin-receptor) complexes that serve as substrates for an insulin-independent serine kinase. This conclusion is supported by the fact that when the insulin receptor is first phosphorylated with a high concentration of unlabeled ATP and then phosphorylated with [γ-^{32}P]-ATP, the labeling of the receptor is enhanced by about twofold, both on tyrosine and on serine residues.[202]

Although the insulin-stimulated serine kinase is not well characterized and the sites of serine/threonine phosphorylation of the receptor by this enzyme are yet undefined, the receptor appears to serve as an *in vitro* and *in vivo* substrate for at least two insulin-independent well-characterized serine/threonine kinases, the cAMP-dependent protein kinase and the Ca^{2+}- and phospholipid-dependent protein kinase C.

B. *In Vivo* and *In Vitro* Regulation of the Insulin Receptor Kinase by cAMP-Elevating Agents and by the cAMP-Dependent Protein Kinase

Catecholamine-induced insulin resistance is a well-established phenomenon, observed either in intact cells[335,336] or human patients.[337] Catecholamines, through binding to the β-adrenergic receptor, initiate a transmembranal signaling process that leads to activation of adenylate cyclase, increased intracellular cAMP levels, and activation of the cAMP-dependent protein kinase (for detailed reviews see References 12, 13, 184, and 338 to 340). Therefore, it has been suggested that the cAMP-dependent protein kinase directly phosphorylates the insulin receptor, inhibits its tyrosine kinase activity, and thus induces insulin resistance. This hypothesis is supported by the findings that elevation of intracellular cAMP levels increases the amounts of serine phosphorylation of the receptor in intact cells[42,341] and decreases its ability to function as an insulin-stimulated tyrosine kinase. Part of the impaired receptor kinase activity in catecholamine-treated rat adipocytes[42] (but not IM-9 cells)[341] is accounted for by a marked (50 to 75%) decrease in insulin binding; however, inhibition of the kinase activity occurs as well due to reduced affinity of the receptor kinase for ATP.[42] The K_m for ATP increases from 25 μM in control cells to 100 μM in catecholamine-treated cells. These findings suggest that the receptor kinase is an *in vivo* substrate

for the cAMP-dependent protein kinase. Such a conclusion is supported by recent *in vitro* studies[342] that demonstrate that phosphorylation of a highly purified receptor by the cAMP-dependent protein kinase results in incorporation of 1 mol phosphate per mole of receptor. This occurs concomitantly with a 25% decrease in the receptor kinase activity. Use of low concentrations of detergents during the phosphorylation reaction seems to be essential and could provide an appropriate explanation why in previous studies,[131,334,341,343] where somewhat different assay conditions were employed, this effect could not be detected. The low degree of inhibition of receptor kinase activity, observed in the *in vitro* studies,[342] compared with the *in vivo* results, raises the possibility that catecholamines modulate the activity of other protein kinases, phosphatases, or regulatory proteins (e.g., G-proteins)[344] that synergize with the cAMP-dependent protein kinase in inhibition of the receptor kinase activity.

C. The Insulin Receptor and Protein Kinase C

An increasing body of evidence suggests that the tumor-promoting phorbol 12-myristate 13 acetate (TPA) elicits many of its effects by mimicking or antagonizing the action of hormones such as insulin, IGF-1, or EGF. TPA potentiates the growth-promoting and metabolic action of insulin,[345] as well as EGF.[346] TPA, like insulin, increases glucose transport and oxidation,[347] stimulates lipogenesis in fat cells,[348] raises intracellular pH,[349] and stimulates phosphorylation of the ribosomal protein S6.[350,351] TPA has been shown as well to inhibit insulin-stimulated activation of glycogen synthase and aminotransferase.[41] Furthermore, TPA inhibits binding of both insulin[352,353] and EGF[354,355] to their receptors in some, but not all,[41,239] cell lines. Inhibition occurs by reducing the receptor affinity for the hormone without affecting receptor number. Both insulinomimetic and inhibitory effects of TPA on insulin action are likely to be mediated by the cellular receptor for TPA, protein kinase C, which is a Ca^{2+}- and diacylglycerol-activated, phospholipid-dependent, serine/threonine kinase.[318,319] Phorbol esters bind to the diacylglycerol binding site and replace it as an activator of protein kinase C.[204,236,356] Indeed, TPA treatment stimulates serine/threonine phosphorylation of insulin receptors in intact IM-9 lymphocytes, Hep G-2 rat hepatoma cells,[41] and bovine endothelial cells.[357] TPA also enhances serine/threonine phosphorylation of the IGF-1 receptor.[358] TPA-stimulated phosphorylation inhibits the insulin-stimulated receptor autophosphorylation in the rat hepatoma cells,[41] but not in the IM-9 cells,[359] which could be due to the low stoichiometry of IM-9 receptor phosphorylation in response to TPA. Phosphorylation of the insulin receptor by TPA occurs in the absence of insulin; furthermore, the effects of insulin and TPA are additive.[359] Peptide map analysis reveals that at least some of the residues phosphorylated by these two agents are distinct.[41,359] These findings suggest that protein kinase C differs from the extrinsic insulin-stimulated serine kinase that phosphorylates the receptor, but suggest as well a key role for protein kinase C in regulating the insulin receptor tyrosine kinase activity.

Support for this notion is provided by the fact that the purified insulin receptor serves as an *in vitro* substrate for protein kinase C.[360] Phosphorylation of the receptor on serine residues by protein kinase C is independent of the presence of insulin and results in incorporation of 0.5 to 1.5 mol phosphates per mole of receptor. Phosphorylation does not affect insulin binding, but results in a 65% decrease in the rate of receptor autophosphorylation. By contrast, phosphorylation by protein kinase C does not inhibit the receptors' ability to phosphorylate exogenously added substrates. No attempts were made to correlate between the sites that undergo *in vitro* phosphorylation by protein kinase C and those phosphorylated in intact cells in response to TPA.

The inhibitory effect of TPA on insulin receptor kinase activity is not a unique phenomenon. TPA also enhances phosphorylation of threonine residues of the EGF receptor, which is accompanied by reduced EGF-dependent tyrosine phosphorylation in A431 cells.[361] The same effect is observed when EGF receptors, purified from A431 cells, are incubated *in*

vitro with EGF and purified protein kinase C.[362] These findings imply that by activation of protein kinase C, which phosphorylates the growth factor receptors, TPA inhibits their tyrosine autophosphorylating activity. This may relate to TPA-induced inhibition of certain insulin, and EGF-mediated biological effects. Two questions remain unresolved: first, why does TPA play a dual role, both as an insulinomimetic agent as well as an inhibitor of insulin action, and, second, is there any evidence for the possible involvement of diacylglycerol, the physiological analog of TPA in insulin action?

A clue to the answers for these questions is provided by studies demonstrating that insulin treatment of intact cells or plasma membranes generates "insulin mediators" — low molecular weight compounds, originally thought to be peptides.[363] These mediators were proposed to act like classical second messengers (i.e., cAMP or cGMP) and to mimic insulin effects on the activity of several enzymes such as cAMP-phosphodiesterase, pyruvate dehydrogenase, and adenylate cyclase.[363] More recent studies in a search for second messengers adopted the concept that insulin acts similarly to hormones that generate such messengers through the hydrolysis of phosphoinositides.[364] These hormones (e.g., bombesin, vassopresin, and α-adrenergic) bind to specific cell-surface receptors and activate a specific phospholipase (PLC) that catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2).[310-312] As with hormonal regulation of adenylate cyclase and phototransduction in the retina, a specific G-protein, Gp,[365-367] which is coupled to these hormone receptors, has been implicated as mediating the stimulatory effect of these hormones on PLC activity. The hydrolytic reation catalyzed by PLC generates two second messengers: inositol tris phosphate (IP_3), which releases Ca^{2+} from intracellular stores, and diacylglycerol, which is the physiological analog of TPA and acts to activate protein kinase C *in vivo*.[310-312,364]

As already mentioned, there are suggestions that insulin might act in a similar manner. These were based upon the observation that insulin binding to intact cells (or membrane preparations) activates a specific phospholipase that hydrolyzes a unique glycano phosphoinositide to generate two second messengers: inositol-phosphate glycan (instead of IP_3) and diacylglycerol.[314-317]

Taken together, these findings enable one to present a unifying hypothesis according to which the insulinomimetic actions of TPA are analogous to the effects of insulin on diacylglycerol production, activation of protein kinase C, and phosphorylation of cellular proteins, whereas the inhibitory effects of TPA are due to serine phosphorylation of the insulin receptor by the now-activated protein kinase C. This hypothesis predicts that protein kinase C inhibits receptor kinase activity in a similar, if not an identical manner, to that of the insulin-stimulated serine kinase and cAMP-dependent protein kinase. This model is at present speculative and needs supportive evidence. While there are reports that demonstrate activation of protein kinase C following insulin binding,[368,369] others provide opposite findings.[370-371a] For example, prolonged treatment of intact cells with TPA, which results in abolishment of protein kinase C activity, fails to obliterate insulin-mediated biological effects. We must therefore at present be open minded to the possibility that insulin actions are not necessarily mediated by protein kinase C.

XV. ROLE OF INSULIN RECEPTOR KINASE IN MEDIATING INSULIN ACTION

As mentioned earlier (see Section I), there are at least seven independent lines of evidence that implicate a crucial role for the insulin receptor kinase activity in mediating insulin action. In this section several of these observations are discussed in more detail.

A. Studies Utilizing Site-Directed Mutagenesis

Site-directed mutagenesis of the human insulin receptor cDNA was found most instrumental in studies aimed at correlating receptor kinase activity and insulin action. CHO cells

transfected either with native or mutated receptor cDNA usually served as the model system. In the first set of experiments,[47] one or both of the twin tyrosines (residues 1162 and 1163) were replaced with phenylalanine. The CHO cells expressing these mutated receptors were designated CHO YF-1 and CHO YF-3, respectively. Alternatively, the last 112 amino acids at the C-terminal end of the receptor were deleted, creating a mutant expressed in CHO cells designated CHO-T-t. The receptors mutated on tyrosine residues maintained a conformation very similar, if not identical, to that of native receptors in terms of receptor processing, subunit size, insulin affinity, and binding capacity, as well as the capability to be precipitated by a panel of conformation-sensitive antireceptor antibodies.[47] That such kinase-mutated receptors express normal insulin binding is consistent with earlier observations indicating that activation[178] or inhibition[72,166] of the receptor kinase activity does not affect insulin binding. All three mutated receptors failed to express an *in vitro* insulin receptor kinase activity toward exogenous substrates, although the CHO-YF-1 and CHO-YF-3, but not CHO-T-t, demonstrated an *in vitro* autophosphorylation activity. These results are compatible with the view that in the absence of tyrosines 1162 and 1163, there is another site(s) for receptor autophosphorylation.[47] When intact cells were treated with insulin it appeared that CHO YF-1 and CHO YF-3 cells displayed markedly reduced insulin-dependent receptor autophosphorylation when compared with cells transfected with wild-type receptors. Cells expressing the truncated receptor failed to undergo any autophosphorylation. Interestingly, receptors that failed to undergo insulin-stimulated tyrosine phosphorylation also failed to undergo phosphorylation on serine/threonine residues. This suggests that either the receptor tyrosine kinase activity is essential for activation of the extrinsic insulin-stimulated serine kinase or, alternatively, that the autophosphorylated receptor is the main substrate for this serine kinase (see also Section XIV).

The insulin-stimulated uptake of 2-deoxyglucose in wild-type and mutated receptors was assessed in order to correlate between the reduced receptor kinase activity and an insulin's biological effect. Since CHO cells transfected with wild-type receptors express 250- to 1000-fold higher receptor numbers than native CHO cells, it was anticipated that such overexpression of normal receptors will render the CHO cells more sensitive to insulin if the transfected receptors are capable of transducing insulin's bioeffects such as glucose uptake. Indeed, this was the case and a 30-fold increase in insulin sensitivity occurred in cells expressing wild-type human insulin receptors. However, introduction of one or two tyrosine-to-phenylalanine point mutations into the receptor β subunit caused a progressive loss of this enhanced sensitivity to insulin action as assessed by monitoring glucose uptake. These results are consistent with the hypothesis that receptor kinase activity and insulin action are tightly linked. Of particular interest are the findings that cells transfected with the truncated receptor mutant expressed glucose uptake rates that were significantly reduced and far less sensitive to insulin, even when compared with untransfected native CHO cells. Such apparent inhibition of glucose uptake could be the result of extensive formation of inactive hybrids between the human mutant and CHO wild-type receptors. Furthermore, the high concentrations of human receptors could inhibit formation of homologous clusters of the endogenous CHO receptor molecules. Subsequent studies[43,46] made use of mutants where lysine 1030, which makes part of the receptor's ATP binding site, was replaced with either methionine,[43,44] arginine,[43] or alanine[43,46] residues. The mutated receptors, transfected into CHO cells, were normally processed and expressed normal insulin binding characteristics. Nevertheless, they failed to express any basal or insulin-stimulated receptor kinase activity either *in vivo* or in cell-free systems. When cells transfected with wild-type receptors were assayed for insulin's bioeffects, it could be demonstrated that such transfection increased the cells' sensitivity to insulin 10- to 100-fold. When the mutants were tested for insulin-mediated bioeffects it could be demonstrated that 2-deoxyglucose uptake, S6 kinase activity, glycogen synthesis, and thymidine uptake were markedly less insulin sensitive compared with CHO cells trans-

fected with wild-type receptors. In fact, these bioeffects, assayed in the mutants, were not different from those of native untransfected CHO cells. Again, glucose uptake was even lower than that observed in untransfected CHO cells. These studies, like the previous ones,[47] strongly support the contention that the functional insulin-activated tyrosine kinase plays a direct role in mediating most, if not all, of insulin's bioeffects. Such a conclusion implicates the phosphorylation of specific protein substrates by the receptor kinase as part of the signal transmission pathway. Indeed, when insulin-stimulated protein phosphorylation was examined in untransfected CHO cells it could be demonstrated (using immunoblotting with antiphosphotyrosine antibodies) that insulin enhances tyrosine phosphorylation of two proteins having molecular mass of 150 and 35 kDa. Cells transfected with wild-type receptors were tenfold more sensitive to this insulin effect. By contrast, cells transfected with the mutated receptor were not different from untransfected parental CHO cells. It can therefore be concluded that at least those insulin-mediated functions mentioned above are dependent upon the presence of an active receptor tyrosine kinase and a proper phosphorylation of at least some of its endogenous substrates.

In another set of experiments, CHO cells were transfected with a construct of the insulin receptor where the intracellular portion of the β subunit was replaced with the protein tyrosine kinase encoded by the oncogene V-ros, which shares a 50% homology with the protein kinase domain of the receptor. The resulting hybrid undergoes an insulin-dependent *in vivo* autophosphorylation; however, it is unable to mediate major physiological responses to insulin such as glucose uptake or DNA synthesis.[194] These findings again suggest that specific postreceptor mechanisms, possibly involving the specificity of substrates for the membrane-bound insulin receptor kinase, are required to mediate cellular responses to this hormone.[194] The latter conclusion is further supported by the findings that transfection into CHO cells of a DNA encoding, solely for the cytoplasmic domain of the β subunit, results in expression of highly active soluble kinase that, unlike its membrane-bound form, does not increase the basal deoxyglucose uptake of the cells.[372]

The need for a functional tyrosine kinase in order to mediate biological activities is not a property unique for the insulin receptor. Abolishing the tyrosine kinase activity of the EGF receptor by mutating Lys-721 (present in the ATP binding site) with methionine obliterates EGF-mediated alterations in intracellular Ca^{2+}, activation of gene transcription, receptor down regulation, and stimulation of cellular proliferation.[133] Similarly, several reports have suggested that abolishing the tyrosine kinase activity of several oncogenes like V-src,[373] V-mos,[374] and V-fps[375] eliminates their capacity to induce cellular transformation.

Studies in rat 1 fibroblasts[44] or CHO cells[45] transfected with wild-type receptors or with receptor where Lys 1030 was replaced by alanine[44,45] or arginine[45] revealed that the mutated receptors not only fail to mediate insulin's biological actions, but also have a markedly reduced ability to enter the endocytotic pathway.[44,45,376] Unlike cells transfected with wild-type receptors, cells transfected with mutated receptors also fail to undergo down regulation after long (24 h) exposures to high concentrations of insulin. It therefore appears that phosphorylated insulin receptor may assume a more favorable conformation, allowing endocytosis to occur by facilitating physical interaction of the receptor with other components of the endocytotic process, or, alternatively, the activated receptor kinase may itself lead to phosphorylation of other cellular components which then mediate receptor internalization. It is of interest to note that while insulin-induced internalization of receptor mutants is largely impaired,[44,376] the constitutive rate of internalization of the mutated receptors appears to be the same as that of the wild-type receptor.[376] These results indicate that the signaling mechanisms for these two processes must be distinct.[376]

B. Defects in Receptor Kinase Activity and Insulin Resistance

The most physiologically relevant evidence implicating an important role for the insulin receptor kinase activity in mediating insulin action is gained from studies carried out on

human patients, animal models, and cultured cell lines where defects in insulin receptor kinase activity were found associated with insulin-resistant states.

Insulin resistance is a prominent feature of both human obesity[377,378] and noninsulin-dependent diabetes mellitus (NIDDM).[379-381] Several genetic syndromes of extreme insulin resistance have also been recognized. These include the type A syndrome of insulin resistance and acanthosis nigricans,[54,382] leprechanism,[383-385] and the Rabson-Mendenhall syndrome.[386] In some cases insulin resistance could be accounted for by a reduction in insulin binding to its receptor; however, most often insulin resistance is attributed to a postbinding defect in insulin action.

Shortly after the findings that the insulin receptor functions as a tyrosine kinase, studies were initiated in order to determine whether severe insulin-resistant states were associated with a defect in receptor kinase activity. Indeed, Grunberger et al.[22] described a marked reduction in the receptor kinase activity in a patient (type A) with extreme resistance to insulin but with a normal insulin binding. The receptor kinase isolated from the patient's monocytes failed to undergo insulin-stimulated autophosphorylation and its insulin-stimulated kinase activity toward exogenous substrates was decreased by about 90% when compared with normal controls. Such severe reduction in kinase activity occurred in spite of the fact that insulin's affinity and binding capacity to the patient's receptors were perfectly normal.[22] Similar defects in kinase activity, albeit less severe (~50% reduction), were found in erythrocytes and fibroblasts from other type A patients.[23,24] Lipodystrophy is another syndrome associated with extreme resistance to insulin.[387] Indeed, it could be demonstrated that receptor kinase activity in fibroblast, hepatocytes, and lymphocytes of a lipodystrophic patient is decreased by 50% when compared with normal controls. The above findings support the notion that several congenital syndromes of extreme insulin resistance are associated with a defect in receptor kinase activity. It should be emphasized, however, that extreme insulin resistance is not always associated with a kinase defect.[388-390] For example, a study carried out in cultured lymphocyte cell lines transformed with Epstein-Barr virus revealed that while the magnitude of *in vivo* insulin-stimulated receptor phosphorylation varied widely among the individual cell lines, no significant differences were found between cell lines from normal subjects and those from nine patients with extreme insulin resistance.[389] These findings point to the possibility that certain insulin-resistant syndromes are due to postkinase defect and could result, for example, from defects in proteins with which the receptor interacts (e.g., G-proteins).

Recent studies[25-28] evaluated the structure and function of the insulin receptor in less severe forms of insulin resistance, manifested by obese patients with and without NIDDM. It could be demonstrated, for example, that insulin-stimulated kinase activity in adipocytes from normal controls and obese patients is comparable, but it is reduced by 50% in the NIDDM group.[25] These findings indicate that the decrease in kinase activity in NIDDM results from a reduction in coupling efficiency between insulin binding and activation of the receptor kinase.

In addition to the overall diminished ability of insulin to activate the receptor kinase in the NIDDM patients, it appears that insulin bound at subsaturating concentrations activates the kinase less efficiently than insulin bound at maximally stimulated concentrations.[25] These results could indicate that different receptor subpopulations are uncoupled to different extents. Thus, a more extensive degree of uncoupling in receptors occupied at low concentrations of insulin (high-affinity receptor) than in those occupied at high concentrations of insulin (low-affinity receptors) could explain these findings. Defects in coupling efficiency of insulin binding and kinase activity are not unique to adipocyte. Similar defects were found in erythrocytes[27] and hepatocytes[26] from NIDDM, but not obese[24,27] patients.

The situation in the skeletal muscle appears to be different.[28] This is of particular interest since quantitatively, the most important site of insulin action resides in the muscle, which

plays the predominant role in insulin-mediated glucose utilization.[379] In skeletal muscle, a defect in insulin-stimulated receptor kinase activity appears to be related to obesity per se and it is characterized by a reduced (50%) ability of the receptor kinase from obese subjects, with or without NIDDM, to phosphorylate exogenously added substrates. By contrast, autophosphorylation of the insulin receptor kinase in muscle extracts is the same in nonobese control, obese controls, and obese patients with NIDDM.[28] The reasons for the selective defect in the kinase's ability to phosphorylate exogenously added substrates are currently unknown. Defects in kinase activity of skeletal muscle were also found in insulin-resistant db/db mice[391] and in mice rendered obese and insulin resistant by injection of gold thioglucose.[30] However, there is a striking difference in muscular kinase activity between obese (nonresistant) rat and human. While the kinase activity in muscles of obese humans appears defective, that of obese rats is normal. The reason for these differences remains to be clarified.

An interesting outcome of these studies is the observation that at least in certain tissues there is a different molecular basis for the insulin resistance observed in obese and NIDDM patients. In adipocytes,[25] hepatocytes,[26] and erythrocytes[27] a defect in receptor kinase activity is specifically associated with NIDDM, but not with obesity. In contrast, in muscle cells a defective kinase is found both in obese and NIDDM patients with insulin resistance. Thus, the molecular basis for insulin resistance depends not only on the state of obesity, but it is a tissue-specific defect as well. Such a conclusion is supported by studies in animal models where it was shown that the kinase activity of skeletal muscle,[30] but not of brown adipose tissue,[392] is defective in mice rendered obese and insulin resistant by injection of gold thioglucose. Since activation of the receptor kinase is the earliest known postbinding step in insulin action, the potential relevance of such a defect to the etiology of insulin resistance in the NIDDM state is obvious. Whether this kinase defect is primary or secondary needs further clarification. Obviously, insulin receptor kinase defects can be acquired, as shown by the development of altered receptor kinase activity secondary to streptozotocin-induced diabetes,[29] insulin resistance induced by catecholamine treatment,[42] dietary manipulations in rats[393] or chickens,[394] as well as during induction of progressive obesity with hyperglycemia in mice.[30] Reduction in insulin receptor kinase activity (not accounted for by reduction in receptor number or its affinity for insulin) could also be demonstrated in fat cells made insulin resistant by *in vitro* hyperinsulinemia.[195] The cause of these acquired defects is unknown, but potential factors are the prevailing levels of insulin and glucose in the NIDDM patients,[395] animal models, or cell cultures under study. Thus, the role of hyperinsulinemia and hyperglycemia in the development of altered kinase activity deserved further studies. An attractive possibility that accounts for a reduced coupling efficiency could be a defect in the process of receptor microaggregation, which is a prerequisite for kinase activation. In such a case it could be envisaged that changes in lipid composition and in membrane fluidity could affect the content of lipids present in close proximity to the receptor and, thus, alter the efficiency of the microaggregation process. Finally, we cannot rule out the possibility that the kinase defect in NIDDM patients reflects a subtle alteration in protein structure of the insulin receptor, at least in some cases. In this case a kinase defect could be genetic rather than acquired. The tendency toward NIDDM is a familial trait whose expression is influenced by a variety of dietary and environmental factors.[25] Thus, it is possible that a defect in kinase activity may be related to a primary genetic defect in the insulin receptor genes.

XVI. FUTURE PERSPECTIVES

Following 6 years of intensive studies, it is now generally accepted that the tyrosine kinase activity associated with the insulin receptor plays a key role in mediating most, if not all, of insulin's bioeffects. Although such knowledge provides crucial hints as to the mode of

the insulin signal transduction system, many important questions are still unresolved and should be addressed in the near future.

A major problem currently under study in many laboratories is the search for protein substrates for the insulin receptor kinase, whose phosphorylation is likely to be the next step in insulin's signaling pathway. To date only few such proteins (e.g., pp240, 185, 120, 46, 15) have been identified and their characterization is only in its primary stages. Attempts are now being made to determine whether these substrate proteins are *in vivo* substrates for the receptor kinases itself and, if so, what their major characteristics are. Cells transfected with receptors mutated at their ATP binding sites, antibodies against phosphotyrosine residues, or antibodies generated against the substrate proteins themselves are likely to be among the tools used in addressing these questions. Purification of the protein substrates will enable study of their properties, whereas determination of their primary amino acid sequence will provide us with better knowledge of their structure and potential functions. Transfection of cells with cDNA-encoding mutated substrates is likely to be most instrumental in future studies.

Data accumulated from a number of laboratories studying human patients seem to indicate that defects in insulin receptor kinase activity are associated with certain forms of severe and moderate insulin-resistant states. Furthermore, studies in animal models tend to suggest that defects in kinase activity are manifested in cases where insulin resistance is induced experimentally. Taken together these findings strongly suggest that defects in kinase activity could be acquired as well as congenital. The molecular basis for the acquired defects is unknown, but their relevance to the pathogenesis of type I and type II diabetes is obvious. Further studies will therefore be needed in order to determine whether these acquired defects are due to posttranslational modifications of the receptor itself or whether they are related to changes in the receptor environment, such as changes in membrane lipid composition or membrane fluidity. Studies related to the reversibility of the defects and their tissue specificity are likely to be performed in the near future.

Studies aimed at identifying substrate proteins for the insulin receptor kinase could be extended in order to investigate the role of postkinase defects in the development of insulin resistance. Using anti-P-Tyr antibodies and immunoblotting, it is now feasible to screen for impaired phosphorylation of receptor substrates in tissues and cell lines obtained from patients or animal models with insulin resistance and diabetes where there are no obvious defects in insulin binding or kinase activity. Reduction in content, altered localization, or structural modification of the receptor's substrates could turn them inaccessible for phosphorylation, thereby providing a molecular basis for the insulin-resistant state.

Finally, the mode of signal amplification, initiated upon insulin binding, deserves a few more comments. To date, there are two models that seem to offer two independent solutions to this question. One widely discussed in this article considers the receptor tyrosine kinase as mediator of most of insulin's bioeffects, but accumulating pieces of information suggest that the insulin receptor could act as well by generating two types of second messengers — diacylglycerol and inositol glycans. While the precise molecular events involved in generation of these mediators are unknown, they are likely to be produced by an insulin-sensitive phospholipase. Since the activity of certain phospholipases is regulated by G-proteins, it is tempting to speculate that the insulin-sensitive phospholipase is also regulated through interaction with G-proteins. Data recently accumulated indicate that some G-proteins appear to be relatively good *in vitro* substrates for the insulin receptor kinase, suggesting a possible high-affinity interaction between the receptor and G-proteins.

These observations enable one to provide a unifying hypothesis (Figure 1) according to which G-proteins are the coupling elements between the insulin receptor kinase and "second messenger" generating systems. The mode of coupling between the receptor β subunit and G-proteins may, but need not, involve phosphorylation of the latter, although the model

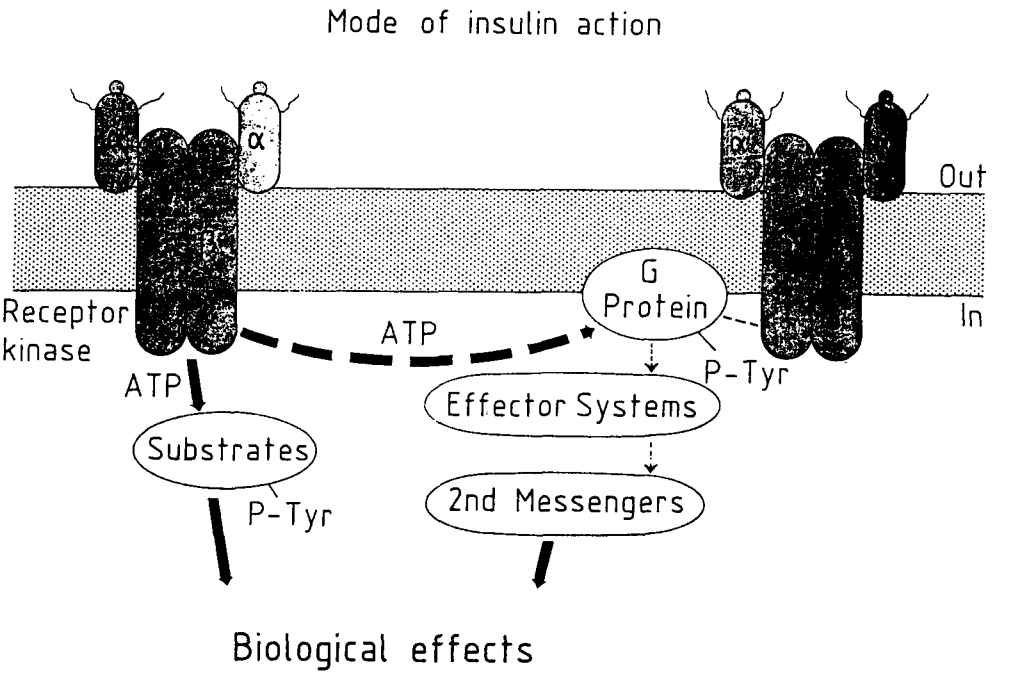

FIGURE 1. Mode of insulin action.

definitely predicts that the G-protein has some "sensing" mechanism to identify formation of an insulin-receptor complex. It still remains to be determined whether a tight coupling between the insulin receptor and G-proteins takes place in intact cells; furthermore, the effects of insulin on phosphorylation of G-proteins under physiological conditions also remain to be established.

In summary, a major breakthrough in our understanding of the mode of insulin action was carried out in the last few years, still there is much more to unravel in the years to come.

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