Banting Lecture 1989

Structure and Function of Insulin Receptors

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The actions of insulin are mediated by an integral plasma membrane protein, the insulin receptor. The processed receptor is a tetramer composed of two α-subunits that bind insulin and two β-subunits that traverse the plasma membrane and are, in their cytosolic domains, protein tyrosine kinases. The insulin proreceptor cDNA has been cloned and its complete amino acid sequence deduced. The availability of cDNA permitted an analysis of both the role of protein tyrosine kinase activity in insulin action and the autophosphorylation sites that regulate kinase activity. The human cDNA probe has also been used to identify a putative *Drosophila* insulin receptor. This work is reviewed, and approaches that may be used to identify physiological substrates for the receptor kinase are suggested. *Diabetes* 38:1508–11, 1989

Insulin is one of the best-characterized mammalian proteins. It has been the model used in landmark studies of protein sequencing, X-ray diffraction, biosynthesis and processing, and radioimmunoassay (1). However, 65 yr after its purification and lifesaving administration to insulin-dependent diabetic patients—the results of heroic efforts by Banting, Best, and Collip at the University of Toronto—the mechanism of action of insulin is still not understood (2). However, new tools have been developed that should help unravel the molecular pathways by which the insulin signal is transmitted.

I began to study insulin action after years of studying cyclic nucleotide metabolism and cyclic AMP (cAMP)–dependent protein kinases. In the early 1980s, I decided that, although much remained to be learned about cAMP-mediated protein phosphorylation, I wanted to study the action of a polypeptide hormone that was clearly not protein kinase mediated. The ligand that had the most puzzling mechanism of action and that was most clinically critical was insulin. Ironically, the more things change, the more they remain the same. My fate was to continue to study protein kinases.

Insulin, like many hormones and growth factors, has multiple effects in mammalian cells. It stimulates hexose transport, alters the seryl phosphorylation state and activity of enzymes involved in the regulation of metabolism, functions as a growth factor, and alters gene transcription (1). The common denominator for the initiation of all of these actions is the insulin receptor (IR). This molecule, which interacts specifically with insulin, also functions to transmit the physiological response system from cell surface to the intracellular milieu. I will summarize some of our findings on the structure and function of this protein.

PROCESSED IR

The work of several laboratories, including those of P. Cuatrecasas (Johns Hopkins Univ., Baltimore, MD; Burroughs Wellcome, Research Triangle Park, NC), J. Roth (National Institutes of Health, Bethesda, MD), S. Jacobs (Burroughs Wellcome), and M. Czech (Univ. of Massachusetts, Boston), established a probable IR structure. The best guess at the time we began our work was that the IR was an integral membrane glycoprotein, the extrafacial portion of which bound insulin reversibly and with high affinity. There was no known function for the intracellular domain. We purified the IR from what we considered the best source, the human placenta (3). Purification depended on detergent extractions of membranes followed by affinity chromatography on insulin-Sepharose, a step pioneered by Cuatrecasas (4). The protein had a M_r of ~350,000 and consisted of 135,000-M_r α-subunits that bound insulin and two other kinds of subunits of M_r 90,000 and 40,000. The latter turned out to be a proteolytic product of the 90,000-M_r or β-subunit. We concluded,

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as did others, that the processed receptor expressed on the plasma membrane of mammalian cells was probably a disulfide-linked tetramer composed of two α- and two β-subunits.

Thanks to a National Institutes of Health group (headed by J. Roth) and the new methodologies for purifying the IR to near homogeneity, polyclonal antibodies (human and rabbit) to the IR became available. Using the differentiated adipocyte form of the 3T3-L1 cell line (5), we found, by metabolic labeling of the IR with [^{35}S]methionine followed by specific immunoprecipitation, that the form of the IR synthesized first (*M*, 180,000–200,000) was larger than either of the identified subunits. Using pulse-chase experiments, we observed that this high–molecular weight polypeptide disappeared coincident with the appearance of radiolabeled bands consistent with the sizes of the α- and β-subunits. Thus, the IR, like insulin, appeared to be processed proteolytically from a precursor; two identical proreceptors yielding the tetramer expressed on cell surfaces.

. The next significant step in understanding IR function was made by Kasuga et al. (6). They found that the addition of insulin to cells bearing IRs led to the rapid phosphorylation of the β-subunit on seryl and threonyl residues. This was perhaps not surprising for a hormone that somehow changed the seryl phosphorylation state of various known metabolic enzymes. The big news was that they also found tyrosyl phosphorylation of the β-subunit. This immediately suggested a number of possibilities that had not been seriously considered before. Foremost was the possibility that the IR, like the epidermal growth factor (EGF) receptor, the prototypical growth factor receptor, might possess intrinsic tyrosine kinase activity. This would cast insulin with certain oncogenes, whose effects on cell growth and differentiation are mediated by phosphorylation of proteins on specific tyrosyl residues. Support for the proposal that the IR contained intrinsic protein tyrosine kinase activity came from our studies documenting copurification of insulin binding and insulin-dependent protein tyrosine kinase activity to near homogeneity (7). The phosphorylation catalyzed by the receptor (in retrospect, not surprisingly) was exclusively on tyrosyl residues of substrate proteins. As far as we know, there are no enzymes that catalyze the phosphorylation of proteins on both seryl and tyrosyl residues.

Because the only identified protein phosphorylated on tyrosyl residues in response to insulin in intact cells at the time was the IR β-subunit, we elected to compare the properties of the IR that had been autophosphorylated in vitro with receptor that had not. Using purified receptor from 3T3-L1 cells and human placenta, we found that autophosphorylation of the IR led to an activated, ligand-independent tyrosine kinase (8). Enzymatic dephosphorylation was required to reverse the activation and restore the insulin-activable form of the kinase. The ability of the α-subunits to bind insulin remained unaffected by the state of β-subunit phosphorylation. This observation presaged what is now acknowledged to be true in general for this class of growth factor receptors, namely, that each domain can function independently in the presence of alterations in other parts of the molecule. In an example of this, Herrera et al. (9) described the results of overexpressing the active, soluble tyrosine kinase domain with a baculovirus expression system.

CLONING THE PRORECEPTOR cDNA

Because autophosphorylation of the IR altered its function as a kinase, we were interested in localizing the specific sites on the β-subunit that were covalently modified. For this, it would have been helpful to know the amino acid sequence of the β-subunit. For many obvious reasons, it also would have been useful to have the complete DNA sequence of the IR cDNA and the deduced amino acid sequence of the insulin proreceptor. To this end, two superb graduate students (L.M. Petruzelli and R. Herrera) sloshed through buckets of placenta to purify sufficient IR to obtain NH_2-terminal amino acid sequence for both α- and β-subunits. In collaboration with a group of scientists at Genentech (headed by J. Ramachandran and A. Ullrich), we used amino acid sequences to generate high–specific activity double-stranded oligonucleotide probes. In a relatively short time, a 5.2–kilobase pair (kb) insert in a human placental cDNA library was identified that hybridized with both α- and β-subunit–specific probes (10). The organization and sequence of the cloned DNA were consistent with all of the predictions from the extensive biochemical characterization of this molecule. The deduced 1370–amino acid sequence of the insulin proreceptor contained (from NH_2- to COOH-terminals) a signal sequence, the α-subunit, and a processing site for cleaving the precursor protein into α- and β-subunits followed by the β-subunit. The latter contained a single transmembrane domain, placing the α-subunit entirely on the extracellular surface of the plasma membrane. The α-subunit had a cysteine-rich domain, similar in organization to the two cysteine-rich domains previously described for the EGF receptor. Most strikingly, the intracellular domain of the β-receptor contains a 260–amino acid domain homologous to all of the other protein tyrosine kinases known at the time, i.e., the EGF receptor and the v-*src* family of oncogenes (10). The sequence confirmed the conclusion of studies of purified IR—it looked like a protein tyrosine kinase. Unlike the EGF receptor, however, there was no evidence (then or now) that the IR can serve as a proto-oncogene (Fig. 1).

AUTOPHOSPHORYLATION SITES

Looking at the 13 tyrosyl residues in the cytoplasmic domain of the IR, we wondered which of them might be important for autophosphorylation and the subsequent activation of the kinase. There were three regions of the β-subunit in which tyrosines were close enough to acidic amino acid residues to suggest that they might be substrates for the IR kinase. At the NH_2-terminal, there was Tyr 960 (and possibly Tyr 953); in the kinase domain there were Tyr residues 1146, 1150, and 1151; and at the COOH-terminal of the protein were Tyr residues 1316 and 1322. We proceeded to make anti-peptide antibodies to some of the amino acid sequences of interest. We then autophosphorylated purified IR in vitro and cleaved the phosphorylated β-subunit by enzymatic and chemical means (11). Using site-specific antibodies, we identified regions of β-subunit autophosphorylation. Approximately equivalent amounts of ^{32}P (from [γ-^{32}P]ATP) were incorporated into the kinase domain (containing Tyr 1146, 1150, and 1151) and into the COOH-terminal portion of the β-subunit. Phosphorylation of the former domain correlated well with activation, a result substantiated by in vitro mutagenesis of the IR cDNA performed by Ellis et al. (12). Ad-

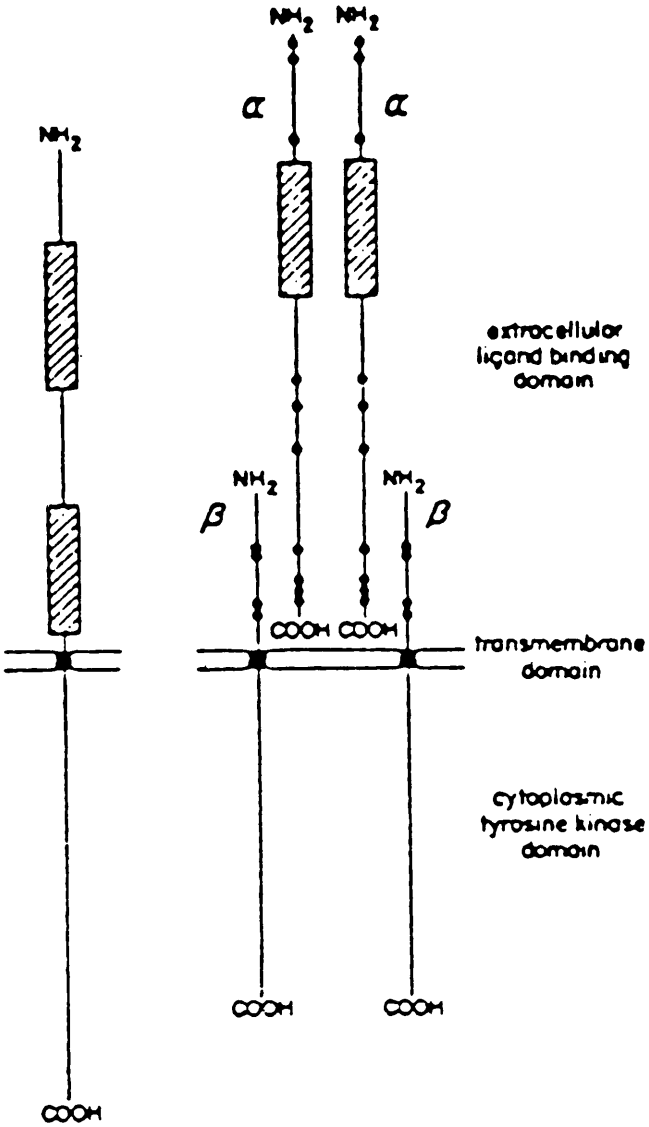

FIG. 1. Schematic comparison of insulin and epidermal growth factor receptors. Regions of high cysteine residue concentration (*hatched bars*); transmembrane domains (■); and single cysteine residues (●) possibly involved in formation of α_2-β_2 insulin-receptor complex. (Reprinted by permission from Ullrich et al. [10]. © 1985 by Macmillan Magazines Ltd.)

ditionally, we now know that truncation of the COOH-terminal domain of the IR kinase does not abrogate its ability to autophosphorylate and autoactivate. Therefore, although this portion of the receptor may be necessary for some aspect of insulin action, its tyrosyl residues are not essential for autoactivation of the IR tyrosine kinase. Tornqvist et al. (13) and White et al. (14) subsequently extended these observations by actually sequencing the phosphopeptides derived from the autophosphorylated receptor. Both studies showed that the phosphorylated sites included Tyr 1146, 1150, 1151, 1316, and 1322. The case for IRs autophosphorylated in intact cells appeared to be the same as that for IRs autophosphorylated in vitro.

DROSOPHILA IR HOMOLOGUE

Why think about, much less try to find a molecule like, the IR in fruit flies? I must admit that it did not happen as rationally as the way we usually present it. We were curious. How conserved were insulin, IR, and the IR protein tyrosine kinase? It would be exciting to be able to use the unparalleled genetics of *Drosophila* to analyze the functions of an IR. There was also literature substantiating the existence of an insulinlike molecule in insects that was bioactive in both insects and mammalian cells and immunologically cross-reactive with antiserums against vertebrate insulin (14). Since this work was initiated 5 yr ago, it has become patently clear that many of the mechanisms used by little eukaryotes are remarkably similar to those used by vertebrates. It is no longer surprising to find highly conserved proteins serving highly conserved functions in yeast, flies, worms, and mammals.

First, we identified and partially purified a molecule from adult flies that had some of the properties of the IR (15). It was a membrane-associated glycoprotein of the appropriate size capable of binding porcine insulin with high affinity and selectivity. An insulin-dependent protein tyrosine kinase activity was identified during midembryogenesis (16). With this information, Petruzzelli and, later, another graduate student, Fernandez, isolated genomic DNA that hybridized with probes derived from the human IR cDNA and then isolated cDNA that hybridized with *Drosophila* genomic clones (17). The deduced amino acid sequence of the kinase domain was remarkably similar in the two species. Interestingly, the autophosphorylation sites that we had suggested were important in the regulation of human IR protein tyrosine kinase were absolutely conserved in the *Drosophila* homologue, so much so that some of the anti-peptide antibodies made to the human IR immunoprecipitated an 85,000-*M*, phosphotyrosyl protein that was identified as the β-subunit of the *Drosophila* IR (17). The principal mRNA for the *Drosophila* receptor is large (11 kb) and most abundant during midembryogenesis. The gene has been mapped, and we are now in position to determine the phenotype of flies with deficient or mutant receptors. Garofalo and Rosen (18) studied the distribution of the *Drosophila* IR mRNA by in situ hybridization. Although we found that it was distributed rather generally during early stages of oocyte development, it was ultimately most concentrated in neural tissue, imaginal disks, and ovarian oocytes.

Curiously, we know much less about *Drosophila* insulin and how it compares to vertebrate insulins. Fortunately, until the *Drosophila* insulin is isolated, cloned, and sequenced, we can substitute mammalian insulins for study.

ROLE OF KINASE IN INSULIN ACTION

In organisms as different as flies and humans, the overall structure and enzymatic activity of the IR has been maintained. This suggests, but of course does not prove, that the insulin-dependent protein tyrosine kinase is an essential component of insulin signal transduction. Chou et al. (19) examined the wild type human IR cDNA and a mutation of it in which the codon for Lys 1018 (an essential amino acid at the active site of the kinase) had been mutated to encode alanine and transfected both cDNAs into CHO cells. Neomycin-resistant clones (of cells) were selected by rosetting erythrocytes coated with antibody to the IR. Two cell lines were selected for further study: WT, which expressed ~40,000 normal human IRs, and *Mut*, which expressed an

equivalent number of the putative kinase-defective receptors. After establishing that *Mut* (Lys 1018 → Ala) processed and bound insulin properly, we confirmed the prediction that it had no detectable kinase activity in intact cells and in vitro (19). We were then in a position to test various insulin actions in CHO cells with a total content of IRs that was 95% human—either WT or *Mut* (kinase deficient)—and 5% endogenous CHO cell IR. Of the five physiological activities of insulin studied—deoxyglucose uptake, S6 kinase activation, endogenous protein tyrosyl phosphorylation, stimulation of glycogen synthesis, and thymidine incorporation into DNA—all were mediated solely by IRs that possessed protein tyrosine kinase activity. Further analyses revealed that the protein tyrosine kinase activity of the IR was also essential for IR downregulation and for the insulin-dependent seryl phosphorylation that occurs in intact cells (20). Our analysis thus confirmed our assumption that the insulin-dependent protein tyrosine kinase is essential (but not necessarily sufficient) for most, if not all, of the actions of insulin.

PHOSPHORYLATION OF PROTEINS ON TYROSYL RESIDUES AND PATHWAYS PROMOTED BY INSULIN

Our conclusions at this point are: *1*) the IR protein tyrosine kinase is essential for many kinds of insulin actions, *2*) it is likely that phosphorylation of substrate proteins in addition to receptor autophosphorylation is important in initiating insulin action, and *3*) at least some of the actions of insulin probably involve activation (or inactivation) of seryl protein kinases and phosphatases with substrates that are more abundant (or stable) than the direct substrates of protein tyrosine kinases. Examples of such kinases are microtobule-associated protein II kinase (21,22) and S6 kinase (23,24). The former, but not the latter, may be directly modified in cells by protein tyrosine kinase (21). Thus, we must search for the initial phosphorylation events catalyzed by the IR protein tyrosine kinase.

There are numerous approaches that might be used to define direct substrates of the IR protein tyrosine kinase. The search for phosphotyrosyl-containing proteins in cell extracts resolved by various sodium dodecyl sulfate–polyacrylamide gel electrophoretic systems has not proven very successful. Another approach is to learn as much as possible about the biochemistry of proteins in insulin-stimulated pathways in the hope that some of them might be substrates. This process is more complicated and possibly more interesting now that we know that many proteins are members of families of isozymes that, although very similar in structure and function, may have differences sufficient to alter their mode of regulation. Recent examples of relevant proteins now known to be members of families include phospholipase C, protein kinase C, and the facilitative hexose transporter. With clones encoding specific isozymes and various molecular manipulations and expression systems, it should be possible to begin to reconstitute partial reactions and pathways of insulin action in intact cells and in vitro and to examine the proposition that, although all phospholipase Cs, protein kinase Cs, and hexose transporters may not be directly involved in growth factor action, a specific isoform—perhaps a nonabundant one—may be.

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