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Structural Dynamics of Insulin Receptor and Transmembrane Signaling

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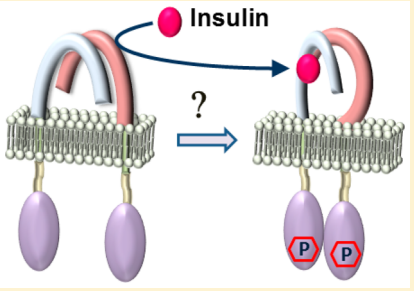
ABSTRACT: The insulin receptor (IR) is a $(\alpha\beta)_2$-type transmembrane tyrosine kinase that plays a central role in cell metabolism. Each $\alpha\beta$ heterodimer consists of an extracellular ligand-binding α-subunit and a membrane-spanning β-subunit that comprises the cytoplasmic tyrosine kinase (TK) domain and the phosphorylation sites. The α- and β-subunits are linked via a single disulfide bridge, and the $(\alpha\beta)_2$ tetramer is formed by disulfide bonds between the α-chains. Insulin binding induces conformational changes in IR that reach the intracellular β-subunit followed by a protein phosphorylation and activation cascade. Defects in this signaling process, including IR dysfunction caused by mutations, result in type 2 diabetes. Rational drug design aimed at treatment of diabetes relies on knowledge of the detailed structure of IR and the dynamic structural transformations during transmembrane signaling. Recent X-ray crystallographic studies have provided important clues about the mode of binding of insulin to IR, the resulting structural changes and their transmission to the TK domain, but a complete understanding of the structural basis underlying insulin signaling has not been achieved. This review presents a critical analysis of the current status of the structure−function relationship of IR, with a comparative assessment of the other IR family receptors, and discusses potential advancements that may provide insight into the molecular mechanism of insulin signaling.



Insulin receptor (IR) is a $(\alpha\beta)_2$-type receptor tyrosine kinase that plays a central role in insulin-mediated regulation of cellular metabolism and growth. Each $\alpha\beta$ heterodimer is composed of an extracellular hormone-binding α-subunit and a transmembrane β-subunit that contains the intracellular tyrosine kinase (TK) domain. The α- and β-subunits are linked by a disulfide bond, and the two $\alpha\beta$ protomers are linked via two to four disulfides between the α-subunits (Figure 1). Binding of insulin to IR triggers transmembrane signaling and autophosphorylation of the β-chains at multiple sites. This initiates a cascade of protein−protein interaction and phosphorylation events, resulting in Akt2-mediated translocation of the glucose transporter (Glut4) into the plasma membrane and glucose intake, synthesis of glycogen, triglycerides, and proteins, and mitogen-activated protein kinase (MAPK)-mediated regulation of gene expression.[1−7]

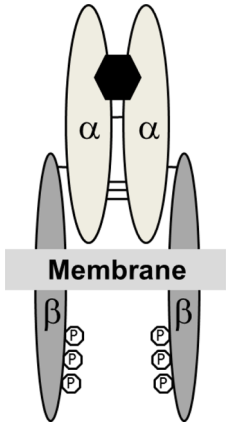


Figure 1. Cartoon of the insulin receptor. The two α-subunits are quadruply disulfide-linked, and each α-chain is disulfide-linked to a β-chain. A bound insulin molecule is shown as a hexagon, cross-linked to two α-chains. Upon activation, each β-chain phosphorylates its counterpart at tyrosines 1158, 1162, and 1163, depicted as small octagons with the letter P inside them.

Multiple genetic defects that result in mutations in insulin-signaling proteins, including a set of missense mutations in IR, have been associated with type 2 diabetes.[8−12] UniProt reports 56 naturally occurring mutations in human IR that cause various, often severe, insulin resistance syndromes (www.uniprot.org/uniprot/P06213). It has been suggested that up to 5% of type 2 diabetes cases, corresponding to more than 13 million people worldwide, may result from mutations and molecular dysfunction of IR.[9] The crystal structure of the TK domain of IR in the basal and phosphorylated states determined that the disease-related mutations either abolish functionally important H-bonds or cause steric clashes, thus destabilizing the TK domain structure.[11,13] Moreover, Cys-to-Ser mutations of cysteines involved in $\beta\alpha-\alpha\beta$ covalent dimerization of IR impair its kinase function.[14] These findings emphasize the importance of the structural integrity of IR in downstream cell signaling events. The success of rational drug design for diabetes heavily relies on the knowledge of the molecular structure of IR with and without bound insulin and,

Received: July 19, 2015
Revised: August 29, 2015
Published: August 31, 2015

DOI: 10.1021/acs.biochem.5b00805
Biochemistry 2015, 54, 5523−5532

most importantly, the conformational changes in IR underlying transmembrane signaling.

X-ray crystallographic studies have identified that the disulfide-bonded extracellular part of IR has a Λ-shaped structure, mostly composed of the two α-subunits engaged in an antiparallel arrangement.[15] Insulin was shown to directly interact with the C-terminal α-helix of the IR α-subunit (αCT), causing conformational changes in both insulin and the IR ectodomain upon hormone binding.[12,16,17] These data validate the "induced fit" concept of insulin−IR interaction and shed light on the negative cooperativity of ligand binding and other biochemical data.[5,12,17−19]

The TK domain of IR revealed two lobes, with the activation loop carrying the phosphorylation sites located in a cleft between them, preventing ATP binding and IR phosphorylation.[11] Tris phosphorylation caused major structural changes, i.e., reorientation of the two lobes and displacement of the activation loop, allowing unhindered access of ATP and the target insulin receptor substrate (IRS) proteins to the TK domain.[13] Further studies identified formation of a dimeric conformation of the phosphorylated kinase domain where the juxtamembrane region plays a central role.[20]

Despite these valuable structural data, the experts in the field acknowledge that the mechanism of insulin-initiated signal transduction remains largely elusive[5] and that the key mechanistic questions remain unanswered.[6,19,20] These questions are as follows. (i) What structural constraints prevent autophosphorylation and activation of ligand-free IR? (ii) What structural changes occur in the IR ectodomain upon insulin binding? (iii) How are these structural changes transmitted to the TK domain, temporally and spatially? (iv) What are the resulting conformational changes in the TK domain that entail receptor autophosphorylation and activation?

Circular dichroism (CD) and fluorescence studies identified changes in IR secondary structure upon insulin binding, possibly involving formation of more rigid helices,[21] and large-scale tertiary and quaternary structural changes consistent with a closer contact between the two protomers.[22,23] A complete understanding of the molecular details of IR function has not been reached, however.

Thus, the atomic resolution structures have been obtained for truncated fragments of IR, i.e., either the extracellular ectodomain or the intracellular TK domain. While these studies elucidate the conformational changes in individual domains, the structural connectivity between them remains unclear, leaving the molecular mechanism of transmembrane signaling unresolved. Spectroscopic studies, on the other hand, do not provide any clues regarding where the structural changes occur in terms of the α- and β-subunits of IR. The critical questions about the molecular mechanism of IR signaling can be answered by studies on the full-length $(\alpha\beta)_2$ IR molecule using structural techniques with appropriate time resolution.

INSULIN SIGNALING

Upon dietary intake of sugar by mammals, the pancreatic β-islet cells secrete the peptide hormone insulin, which reaches all organs through the circulation system and binds to the ligand-binding domain of IR in cell plasma membranes. Insulin binding triggers a signaling cascade that involves multiple protein phosphorylation and activation events leading to translocation of Glut4 to the cell plasma membrane, glucose influx, regulation of gene expression, protein synthesis in myocytes, triglyceride synthesis in adipocytes, and glycogen synthesis in the liver.[1−4,24] These processes play a central role in preventing metabolic disorders such as hyperglycemia, diabetes, excessive fat deposition, and subsequent inflammatory responses.[25−28]

Insulin-mediated signal transduction to the intracellular TK domain of IR results in trans-autophosphorylation of the receptor at multiple tyrosine residues (the two juxtaposed β-chains phosphorylate each other). This is followed by phosphorylation of intracellular IRS proteins, resulting in an array of processes. In the phosphatidylinositol 3-kinase (PI-3K) pathway, phosphorylated IRS-1 binds to the regulatory domain of PI-3K, which then phosphorylates membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 recruits pleckstrin homology domain-containing enzymes, phosphoinositide-dependent kinase 1 (PDK1), and Akt2. PIP_3-bound Akt2 is phosphorylated by PDK1 and mediates plasma membrane translocation of Glut4, followed by glucose influx (Figure 2). Another function of Akt2 is triggering glycogen synthesis through activation of glycogen synthase kinase 3 (GSK3).[29,30]

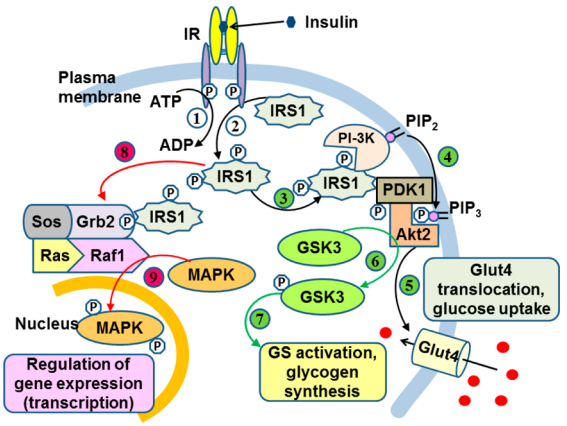


Figure 2. Activation of IR by insulin and downstream signaling events. IR activation is followed by phosphorylation of IRS proteins (steps 1 and 2). In the PI-3K pathway, phosphorylated IRS-1 binds to the regulatory subunit of PI-3K, which then phosphorylates membrane-residing PIP_2 to PIP_3. PIP_3 recruits the PH domain-containing kinases PDK1 and Akt2. PIP_3-bound Akt2 is phosphorylated by PDK1 and mediates plasma membrane translocation of glucose transporter Glut4, followed by glucose influx (steps 3−5). Akt2 also triggers glycogen synthesis through activation of GSK3 (steps 6 and 7). In the MAPK pathway, IRS-1 interacts with Grb2 protein, followed by a cascade of downstream protein−protein interaction and phosphorylation events resulting in activation of MAPK, which undergoes nuclear translocation and regulates distinct transcription factors, thus modulating gene expression, cell proliferation, and differentiation (steps 8 and 9). Protein-attached octagons with the letter P indicate phosphorylation.

In the MAPK pathway, phosphorylated IRS-1 interacts with Src homology 2 domain of the Grb2 protein, which then binds Sos, and the latter activates the Ras protein. A cascade of downstream events results in activation of MAPK, which undergoes nuclear translocation and regulates distinct transcription factors, thus modulating gene expression, cell proliferation, and differentiation.[30,31]

Thus, the insulin signaling machinery is central to cell metabolism and growth, and IR plays a pivotal role in transduction of the insulin signal across the plasma membrane. Analysis of the structural basis of IR signaling is crucial to understanding the molecular mechanisms underlying the

DOI: 10.1021/acs.biochem.5b00805
Biochemistry 2015, 54, 5523−5532

function of IR and homologous receptors such as insulin-like growth factor 1 receptor (IGF1R) and insulin receptor-related receptor (IRR).

MECHANISTIC INSIGHT FROM X-RAY CRYSTAL STRUCTURES

In contrast to the monomeric, single-chain receptor tyrosine kinases such as the epidermal growth factor receptor, the IR family receptors, i.e., IR, IGF1R, and IRR, belong to a family of proteins that upon maturation become $(\alpha\beta)_2$-type tetramers. The hormone-binding α-subunit and the tyrosine kinase β-subunit of IR result from the cleavage of the receptor precursor and are covalently linked by a single disulfide bridge between α- and β-chains (α-chain Cys^{647} and β-chain Cys^{860} for human IR isoform A, UniProt entry P06213-2, disregarding the N-terminal 27-residue signal sequence). The $(\alpha\beta)_2$ tetrameric mature protein is formed by two to four disulfides between the α-chains (Figure 1).[6,32−34] IR is a glycosylated protein with 19 *N*-glycans and six *O*-glycans spread over the α-subunit and the extracellular part of the β-subunit in a way that does not interfere with hormone binding.[35,36] Glycosylation is believed to facilitate protein folding, processing, and membrane translocation.[36] IR is expressed in A and B isoforms resulting from alternative splicing of exon 11, the latter containing 12 additional amino acid residues at the carboxyl terminus of the α-chain.[33,37] The IR-A isoform binds insulin as well as insulin-like growth factor (IGF) with high affinity and performs major insulin signaling and mitogenic functions.[37] All discussion in this article refers to the A isoform of IR.

The disulfide-bonded IR ectodomain (i.e., the α-subunit and the extracellular part of the β-subunit) showed a Λ-shaped structure engaged in an antiparallel arrangement with extensive intermolecular contacts between the two protomers (Figure 3).[15] Each ectodomain monomer is composed of two leucine-rich repeat domains (L1 and L2) between which a cysteine-rich region (CR) intervenes, and three fibronectin type III domains (FnIII-1, FnIII-2, and FnIII-3). It should be noted that the full-length ectodomain has been crystallized in the presence of antigen-binding fragment (Fab) molecules that might have altered the structure, as noted by Menting et al.[18] and Whitten et al.[38] This potential issue has been settled by the crystal structure of an IR microreceptor (L1-CR + αCT$^{697−719}$ domains) that was crystallized without Fab and showed a disposition of the αCT segment similar to that in the full-length ectodomain.[18] However, the fact that in the structure of the full-length IR ectodomain the C-terminal region of the α-chain was poorly resolved[15] calls for reassessment of the topic. On the basis of this structure and data reported previously,[21,22,39−42] a model of binding of insulin to IR and the resulting conformational changes was proposed.[15] Insulin was suggested to bind to the L1 and CR domains of one IR protomer, followed by cross-linking to the junction of FnIII-1 and FIII-2 domains of the other protomer, resulting in high-affinity divalent binding. The cross-linking thus causes intermolecular movements between the two IR subunits that facilitates signaling, eventually resulting in trans-autophosphorylation of IR β-chains.[15,19,33]

The structure of a fragment of the human IR TK domain that contained the activation loop (residues 1149−1170) carrying the important phosphorylation sites (Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}) in unphosphorylated form revealed an N-terminal lobe that comprised a twisted, five-stranded β-sheet structure and an α-helix (named αC) and a C-terminal lobe containing eight α-helices and four short β-strands.[11] The activation loop was between the two lobes, preventing ATP binding and IR phosphorylation by occupying most of the ATP-binding pocket. It was proposed that binding of insulin to IR causes rearrangements in the quaternary structure of the receptor, positioning the phosphorylation sites of one subunit against the kinase domain of the other and thus facilitating trans-autophosphorylation.

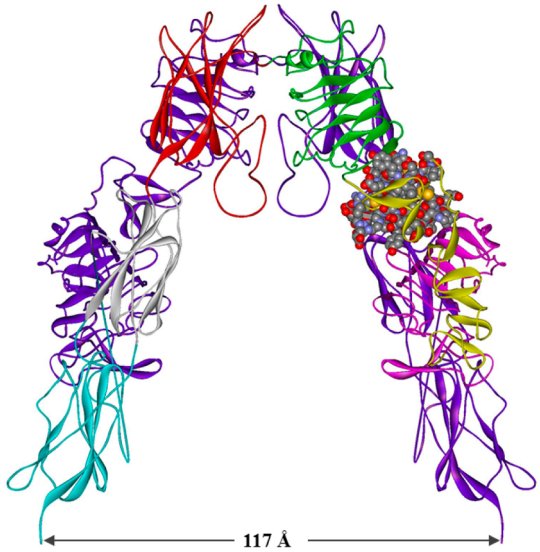


Figure 3. Human IR ectodomain in the ligand-free state (PDB entry 2DTG), shown in ribbon format. One protomer is colored purple, and the other is colored by domain: L1, magenta; CR, yellow; L2, green; FnIII-1, -2, and -3 domains, red, gray, and cyan, respectively. The two protomers run antiparallel and are symmetry-related through a vertical 2-fold rotational axis. One insulin molecule is shown in ball and stick format (PDB entry 1ZNJ) between the L1 and CR domains of one protomer and the FnIII-1 domain of the other. Note that the ectodomain structure is that of an apo receptor, and the insulin molecule is shown to merely indicate the hormone-binding site rather than to present a structure of the receptor−hormone complex. The ectodomain structure with a bound hormone is likely different (see Figure 4b and ref 44) but has not been determined. The membrane would be below the structure, perpendicular to the plane of the picture.

Major structural changes in the same IR fragment upon activation were identified by the X-ray crystal structure of the tris-phosphorylated TK domain (TK3P), complexed with an ATP analogue and a peptide substrate.[13] The structural changes involved large-scale (up to 30 Å) displacement of the activation loop in TK3P and reorientation of the two lobes, allowing an unhindered access of ATP and the target proteins to the active site of the IR β-chain. Structural consequences of Tyr phosphorylation, e.g., breakage of the Tyr^{1162}−Asp^{1132} H-bond (accompanied by protonation of the catalytic Asp^{1132}) and formation of P-Tyr^{1163}···Arg^{1155} and P-Tyr^{1162}···Arg^{1164} H-bonds, have been identified. A rearrangement of the β-strand pairing in the TK3P left the β10 strand (part of the activation loop) unpaired and available for interaction with IRS proteins.

A recent X-ray crystallographic study of a larger (328-residue) fragment of the IR β-chain that comprised the tris-phosphorylated TK domain and the cytoplasmic juxtamembrane region identified a dimeric structure formed by engagement of the juxtamembrane segment of one molecule with the N-lobe of the TK domain of the other by means of hydrophobic and ionic contacts.[20] It was inferred that the

DOI: 10.1021/acs.biochem.5b00805
Biochemistry 2015, 54, 5523−5532

dimeric conformation of TK3P played an important role in receptor activation by the following mechanism. In the basal state, the juxtamembrane region interacts with the αC helix of the N-lobe within the same protomer and stabilizes the kinase domain in a nonproductive conformation, as proposed previously.[43] Receptor phosphorylation repositions the juxtamembrane region into the N-lobe of the counterpart protomer, with a concomitant tertiary structural transition of the αC helix to a conformation that facilitates ATP binding and substrate phosphorylation. Thus, the juxtamembrane region is believed to play a *cis*-inhibitory and *trans*-activating function.[20]

The structures of the TK domain in unphosphorylated and phosphorylated states have thus provided important clues to the structural basis for IR tyrosine kinase activation. However, as underscored by Cabail et al.,[20] it has not been determined unequivocally if the diminutive activity of the basal state receptor is a result of spatial separation of the kinase domains or formation of an inhibitory dimer. Moreover, the major question of how insulin binding triggers the conformational changes leading to receptor activation remains unanswered.[19,20,44]

SPECTROSCOPIC STUDIES

Spectroscopic and other methods have been employed to assess the structural basis of IR signaling.[16,21−23,40−42,45] Lee et al.[22] observed a significant increase in tryptophan fluorescence intensity and anisotropy of lipid-reconstituted human IR upon insulin binding, which was interpreted in terms of large-scale conformational changes. Binding of ATP to IR induced a rapid increase in tryptophan fluorescence anisotropy, as well, and this effect was larger in the presence of IR-bound insulin, indirectly implying that the insulin-induced conformational changes in the α-subunit reach the ATP-binding site of the IR β-subunit. Insulin binding was proposed to cause tryptophan burial and a closer or tighter contact between the two αβ protomers. This conjecture echoes with findings that insulin binding causes a decrease in the Stokes radius and an increase in the sedimentation coefficient of the $(\alpha\beta)_2$ form of IR but not the αβ protomer or the IR ectodomain.[40−42]

CD studies of IR truncated right after the transmembrane domain and reconstituted in Triton X-114 micelles indicated changes in the protein secondary structure upon insulin binding, possibly involving formation of more rigid helices, and fluorescence data indicated a slight blue shift of tryptophan emission.[21] Insulin dose-dependent changes in near-UV CD spectra of the IR ectodomain provided further evidence for insulin-induced tertiary structural changes in IR.[23] It was suggested that insulin binding resulted in reorientation of the α-subunits and formation of a less elongated and more globular IR structure where the transmembrane (TM) domains are moved farther apart.

Rearrangements in the relative positions of TM domains of IR and IGF1R during receptor activation remain unresolved. Two recent publications reached mechanistically divergent conclusions regarding this issue. A synthetic peptide corresponding to the IR TM domain was shown to activate IR in the absence but not in the presence of insulin.[46] The peptide did not remove IR-bound insulin, and peptides mimicking the TM domains of homologous or heterologous receptors failed to activate IR, suggesting specific interaction of the peptide with a region distinct from the insulin-binding site of IR. The authors concluded that IR TM domains are dimerized in the basal state, keeping the TK domains apart, and receptor activation by hormone binding or by exogenous TM peptides involves dissociation of the TM domains and merger of the TK domains (Figure 4a).

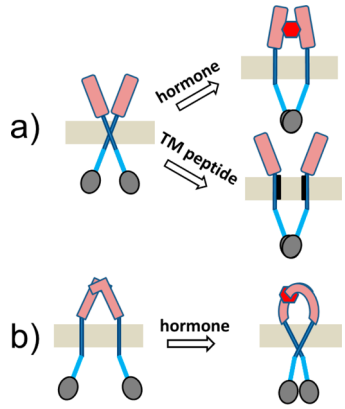


Figure 4. Schematic presentation of two proposed mechanisms of rearrangements of the TM domains of IR or IGF1R during receptor activation. Model a suggests TM domains in close contact in the basal state; receptor activation by hormone or by TM peptide causes TM domain dissociation and TK domain rendezvous (adapted from ref 46). Model b suggests dissociated TM domains in the basal state; high-affinity hormone binding results in conformational changes in the ectodomain involving movements of the two FnIII-2/FnIII-3 modules toward each other, and a closer contact between the TM domains and the TK domains of the two protomers (adapted from ref 44). Color code: receptor ectodomain, pink; TM domain, blue; juxtamembrane region, cyan; TK domain, gray oval; membrane, gray slab; hormone, red hexagon; TM peptide, black stick.

Kavran et al.[44] arrived at a diametrically opposite conclusion about the role of TM domain movement in activation of IR and IGF1R. On the basis of a detailed analysis of IR ectodomain structures in liganded and ligand-free states, they postulated that the Λ-shaped architecture of the hormone-free ectodomain of IR or IGF1R that keeps the TM and TK domains spatially isolated was stabilized by extensive interactions between the L1 domain of one protomer and the FnIII-2 and FnIII-3 domains of the other. Hormone binding disrupts this interaction by recruiting the L1 domain, resulting in a dramatic rotation of the L1 and CR domains and allowing for conformational transition of the FnIII-2 and FnIII-3 domains toward the central axis of the receptor, TM domain association, a closer contact between the two TK domains, and receptor autophosphorylation (Figure 4b). This mechanism was substantiated by a range of biochemical and biophysical data, such as a constitutively phosphorylated IGF1R construct lacking the L1 domain and a ligand-induced increase in fluorescence resonance energy transfer between probes that replaced the intracellular domains of IGF1R expressed in CHO cells.[44] It should be noted that similar receptor activation scenarios have been proposed previously.[22,36] On the basis of spectroscopic studies, Lee et al.[22] inferred that insulin binding resulted in a closer contact between the αβ-subunits of IR, thereby facilitating trans-autophosphorylation. Proteomics analysis suggested an inward rotation of the FnIII-2/FnIII-3 module upon hormone binding,[36] reminiscent of the model of Kavran et al.[44] If ligand-induced receptor asymmetry loosens the intersubunit interaction at the opposite side of the ectodomain (see below), this might result in disengagement of the respective FnIII-2/FnIII-3 module and a subsequent inward rotation, thus bringing

DOI: 10.1021/acs.biochem.5b00805
Biochemistry 2015, 54, 5523−5532

the two TK domains into contact, as shown in the graphical abstract. A definitive answer to this question might have stemmed from the structures of IR or IGF1R ectodomains in the hormone-free and high-affinity hormone-bound states. However, while the hormone-free structure has been determined for the whole ectodomain,[15,16] the hormone-bound structure lacked the FnIII-2 and FnIII-3 domains,[12] preventing direct assessment of movements of these domains and hence the downstream TM and TK domains upon receptor activation.

INSULIN BINDING TO INSULIN RECEPTOR

Insulin is a peptide hormone synthesized in the pancreas as a zinc-stabilized hexamer of disulfide-linked A- and B-chains.[47] Secretion of insulin into the circulation system is followed by hexamer dissociation to the physiologically active form of the A-B structure (Figure 5).[47−50]

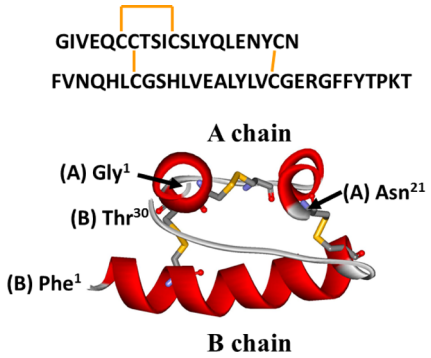


Figure 5. Amino acid sequence and disulfide bond formation of mature human insulin (above) and a ribbon representation of its X-ray crystal structure (PDB entry 1ZNJ) (below). The disulfide bridges between A-Cys6 and A-Cys11, A-Cys7 and B-Cys7, and A-Cys20 and B-Cys19 are shown as yellow lines (top) and in stick format (bottom).

The available data indicate that each IR $(\alpha\beta)_2$ unit can bind one insulin molecule with high affinity and an additional one or two molecules with lower affinities.[19,33,51] More specifically, each α-subunit of IR presents two insulin-binding sites, 1, 2 on one subunit and 1′, 2′ on the other. IR site 1 (or 1′) is composed of the L1 domain of one protomer and the αCT′ segment of the other, and site 2′ (or 2) involves the junction between FnIII-1′ and FnIII-2′ (or FnIII-1 and FnIII-2) domains (the prime symbol indicates the opposite protomer). The dimeric, antiparallel arrangement of the α-subunits (Figure 3) positions 1 against 2′ and 2 against 1′.[19,33,39,52] Experiments with intact IR, using IR-expressing cell cultures, have suggested that insulin's dimerization surface binds to IR site 1 with an affinity of ∼6 nM, while the hormone's hexamerization surface interacts with IR site 2′ with an affinity of ∼400 nM, resulting in a productive-mode, divalent binding with an overall affinity of 0.2 nM.[12,19,39,51,52] This breaks the 2-fold rotational symmetry of the IR ectodomain, moving sites 1 and 2′ closer to each other and sites 1′ and 2 farther apart and available for low-affinity (i.e., $K_D \approx 6$ and 400 nM) binding of two more ligand molecules (Figure 6). These low-affinity binding events can take place at higher insulin concentrations, resulting in a maximum of three insulins bound to IR. This can be followed by dissociation of the first and either second or third ligands, subsequent high-affinity cross-linking of sites 1′ and 2 by one insulin, and an inverse asymmetry of the IR ectodomain (Figure 6), generating negative hormone binding cooperativity.[39,53,54] At still higher ligand concentrations (∼1 μM), two ligand molecules can bind to sites 1 and 2′ with lower affinities, thus producing the bell-shaped ligand concentration dependence of the ligand dissociation rate.[53]

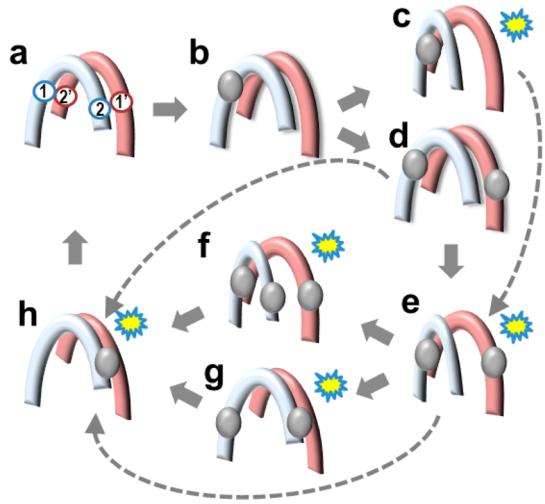


Figure 6. Schematic models for hormone binding to IR or IGF1R ectodomains. Part a shows a symmetric dimer of two protomers, colored pink and cyan, each with hormone-binding sites 1 and 2 or 1′ and 2′. The hormone (gray oval) can bind with low affinity to any of these four sites, or with high affinity to sites 1 and 2′ or 2 and 1′ (cross-linking, or divalent binding), causing asymmetry, i.e., closer contact between one pair of binding sites (1 and 2′ or 2 and 1′) and a larger gap between the other pair. Part b shows the hormone bound to site 1, which can undergo a transition to either 1, 2′ cross-linking (c) or binding of a second hormone molecule to site 1′ (d) (or site 2, not shown). The 1, 2′ cross-linked molecule can bind another hormone at site 1′ (e) (or site 2, not shown), and then a third hormone can bind to site 2 (f). The complex (e) can undergo a transition to step g by dissociation of the 1, 2′ cross-link and formation of a 2, 1′ cross-link, followed by dissociation of the hormone from site 1 (h). Transitions can also occur between different complexes skipping the intermediates, some of which are shown by dashed arrows. All transitions are reversible. Receptor activation is reached by high-affinity hormone binding, indicated by yellow polygrams. For more details, see refs 5, 15, 19, 51−54, and 57.

Insulin binding affinities have also been measured for IR that was expressed in mammalian cells, solubilized in detergent (Triton X-100), and affinity-purified. These studies reported somewhat higher affinities, e.g., 6 pM for the overall dissociation constant[21] as opposed to 200 pM derived from cell-based assays (see above). Analysis of binding of insulin to solubilized/purified IR by Whittaker and co-workers yielded K_D values of 0.03−0.04 and 0.4−0.5 nM,[55,56] which could be compared with De Meyts values of 0.2 nM (overall binding affinity) and 6 nM (IR site 1 binding affinity).

Insulin's interaction with IR has been analyzed using a systems biology approach, where the extracellular domains of IR have been treated as rigid bodies connected by springs, allowing ligand-assisted, but largely stochastic, transitions between the basal (symmetric) and active (asymmetric) configurations of IR.[51] This treatment, which was termed a "harmonic oscillator model", was able to reproduce all prior biochemical and kinetic data on ligand binding to IR as well as IGF1R. The stochastic nature of this approach, i.e., the active role of random events such as collisions with the solvent molecules, may be reconsidered.

DOI: 10.1021/acs.biochem.5b00805
Biochemistry 2015, 54, 5523−5532

Recent crystallographic studies revealed significant conformational changes in insulin upon binding to the truncated IR ectodomain. Insulin was found to open up via detachment of the C-terminal segment of the B-chain, resulting in exposure of the nonpolar residues to make intimate physical contact with the IR αCT segment, which underwent displacement toward the β_2 surface of the L1 domain of IR and was C-terminally extended by five residues.[12,17] A similar mode of binding of IGF-1 to a hybrid receptor fragment (L1-CR of IR + αCT of IGF1R) has been identified.[18] These findings supported the "detachment" and "induced fit" mechanisms of insulin−IR interactions proposing a conformational switch in insulin to fit the hormone-binding site of IR.[5,12,17−19,49,57]

■ DYNAMICS OF INSULIN SIGNALING

The rate of conformational change in reconstituted human IR following insulin binding was characterized by a time constant of ∼6 min by fluorescence spectroscopy, corresponding to slow backbone motions of proteins.[22] IR autophosphorylation approached completion within 6−10 min.[22] Similar time courses of insulin-induced fluorescence changes and IR autophosphorylation indicated a possible correlation between these two events. Studies of 3T3-L1 adipocytes showed that phosphorylation of IR reached a maximal level in ∼1 min following insulin binding and stayed at a high level for ∼10 min.[58] Mobilization of the IRS molecules that directly interact with the IR β-subunit occurred with a $t_{1/2}$ of 3.5 min, and binding of insulin to IR of muscle cells resulted in protein phosphorylation events occurring within 2−5 min.[59] Differences in experimental conditions, including temperature, should be taken into account in interpreting the dynamics of IR activation. These findings indicate that the signal transduction events in the insulin cascade are relatively slow, providing an opportunity to study the kinetics of insulin signaling by conventional spectroscopic methods.

A steady state and stopped-flow fluorescence analysis of binding of fluorescent dye-labeled insulin to a truncated IR ectodomain (Midi-IR) indicated a biphasic binding process that could be characterized with two time constants (τ), a fast interaction (τ_1 < 1 ms) with a low-affinity site, and a slower interaction ($\tau_2 \approx$ 2−5 s) with a high-affinity site that caused downstream conformational changes and IR activation.[23] It was concluded that these two kinetically distinct processes were related to sequential interaction of insulin's two receptor-binding sites with two hormone-binding motifs of IR, constituting the basis of the induced fit and the negative cooperativity discussed above.

■ THE OTHER FAMILY MEMBERS: IGF1R AND IRR

The IR family includes two other highly homologous receptor tyrosine kinases, IGF1R and IRR, that share significant structural and mechanistic features with IR. IR and IGF1R are present in all vertebrates, but IRR has not been identified in teleost fish.[60] These three receptors form unique disulfide-linked $(\alpha\beta)_2$ tetramers of similar size and structure. The major functional distinctions are that IGF1R performs cellular proliferation and antiapoptotic (i.e., survival) functions and IRR is an extracellular pH sensor in contrast to the major metabolic function of IR. The aberrant overexpression of IGF1R can lead to cancer.[61−63] IR and IGF1R are able to swap ligands; i.e., they both can bind insulin, IGF-1, and IGF-2 with varying affinities and physiological consequences,[18,38,63] whereas IRR binds none of these ligands and is considered an orphan receptor.[64] Hybrid receptors composed of one $\alpha\beta$ heterodimer from IR and another from IGF1R occur in cells expressing both receptors, bind all three hormones, and perform signaling functions that have not been fully characterized.[65] Attempts to inhibit IGF1R in cancer therapy can be curtailed by activation of the mitogenic function of IR by IGF-2.[63,66,67] Insulin-like growth factor-2 receptor is a nonsignaling receptor of divergent structure and does not belong to the IR family. It binds IGF-2 and mannose 6-phosphate-tagged proteins and facilitates lysosomal degradation of IGF-2, thus attenuating IGF-2 signaling.[68]

According to ExPASy (http://www.expasy.org), mature IR (isoform A), IGF1R, and IRR comprise 1339, 1333, and 1267 amino acid residues per $\alpha\beta$ unit, respectively. IR shares 58% sequence identity and 80.4% similarity with IGF1R and 54.4% sequence identity and 77.2% similarity with IRR (all human isoforms). Sequence identity between IR and IGF1R reaches 84% when the TK domains of the receptors are compared.[69] The domain structure and the overall three-dimensional architecture of IGF1R and IRR are believed to resemble those of IR.[18,33,36,38,44,64,70,71] Their glycosylation patterns are different, with IR being more heavily, albeit redundantly, glycosylated with no apparent biological benefit.[36]

There are similarities and differences between modes of binding of the hormone to IR and IGF1R. While both receptors demonstrate negative cooperativity of hormone binding,[39] IGF1R displays a sigmoidal, or monophasic, hormone concentration dependence of hormone dissociation rate, in contrast to IR that shows a bell-shaped, biphasic dependence.[52,72] As described above, the bell-shaped feature of IR has been interpreted in terms of an initial displacement of labeled ligand from a high-affinity site (e.g., 1, 2′) at a relatively low concentration of unlabeled ligand, when the latter cross-links sites 1′ and 2, and further reassociation of two ligand molecules to sites 1 and 2′ with lower affinity at higher ligand concentrations (0.1−1.0 μM).[39,53] IGF1R is believed to lack this feature because of its inability to bind three ligands at a time (see Figure 6f) because of the larger size of IGF-1 compared to that of insulin and/or structural differences between hormone-binding sites of IR and IGF1R.[18,51,71] Indeed, the crystal structure of the N-terminal half of the IGF1R ectodomain (residues 1−462) suggested that CR and parts of L1 and L2 domains constituted the primary hormone-binding site,[73] which is not identical to the mode of binding of insulin to IR.[70,71] However, small-angle X-ray scattering studies of IR and IGF1R ectodomains in solution countered the notion, showing that (a) both IR and IGF1R ectodomains are more flexible than the respective crystal structures, allowing for rotation of the FnIII-2/FnIII-3 module away from the symmetry axes by 10−25°, and (b) IGF1R can actually bind three molecules of IGF-1.[38] A wider opening of the Λ-shaped configuration of IR and IGF1R ectodomains in solution by outward motion of the FnIII-2/FnIII-3 modules described by Whitten et al.[38] echoes the hormone-induced transition of FnIII-2 and FnIII-3 domains toward the central axis of IGF1R during activation proposed by Kavran et al.,[44] adding credibility to a mechanism in which a hingelike motion of the C-terminal part of the ectodomain is an important part of conformational changes underlying hormone-induced receptor trans-autophosphorylation.

Despite the structural similarities between IR and IGF1R,[12,18] the precise modes of binding of ligand to IR and

DOI: 10.1021/acs.biochem.5b00805
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IGF1R are likely to be different. One line of evidence supporting this surmise is that while the solubilized IGF1R ectodomain maintains the high ligand affinity and negative cooperativity,[74] solubilized IR loses both its high-affinity insulin binding capability and negative cooperativity.[39,75] Conceivably, this difference may imply greater interdomain motional freedom of IR compared to that of IGF1R, or a more critical role of the TM domains of IR in hormone-triggered activation.[5,75]

The X-ray crystal structure of the IGF1R kinase domain revealed a mostly β-sheet N-lobe and a mostly α-helical C-lobe with the ATP-binding site and the catalytic center between the two lobes.[76,77] Although IGF1R revealed a narrower substrate-binding cleft between the N- and C-lobes and altered structures of the activation and nucleotide-binding loops as compared to IR, X-ray studies of the tris-phosphorylated TK domain of IGF1R in complex with an ATP analogue and an IRS-1-derived peptide substrate identified ATP and substrate binding modes similar to those of IR.[78] There is structural and biochemical evidence that the TK domains of both IR and IGF1R in the basal state are inhibited by extensive interactions of the juxtamembrane region with the N-lobe, keeping its αC helix in a nonproductive conformation.[5,43] Activation of both receptors is believed to involve relocation of the two juxtamembrane segments onto the N-lobes of opposite, rather than the same, protomers.[20] All in all, IR and IGF1R appear to have more structural similarities than differences, and this makes targeted, rational drug design a more challenging task.[67]

IRR is considered an orphan receptor with no identified ligand, and its function has remained undefined until recently. Deyev et al.[79] discovered the agonist of IRR; it turned out to be the hydroxyl ion. They identified tyrosine kinase activation of IRR by extracellular alkaline media (pH ≈8.5−9.5), which was reversible and dose-dependent and involved conformational changes in the receptor and downstream IRS-1/Akt2 mobilization events. When alkaline media used to activate IRR in CHO cells were collected, neutralized, and added to a fresh cell culture, no response was elicited, indicating that the agonist was the alkaline pH itself rather than an alkali-induced IRR ligand. Thus, IRR functions as an alkali sensor and plays a role in handling metabolic alkalosis in organs exposed to elevated pH, such as bicarbonate excretion in kidneys.

Alkali-induced activation of IRR displayed positive cooperativity with a Hill coefficient of ∼3.5, in contrast to negative cooperativity of IR and IGF1R.[79,80] The search for an alkali-sensor motif, which involved domain-swapped IRR/IR and IRR/IGF1R chimeras and a set of point mutations, identified the L1, CR, and L2 domains of IRR as carriers of the pH sensors.[81−83] It was inferred that IRR activation by basic pH resulted from structural changes involving multiple domains of the receptor's ectodomain rather than deprotonation of defined amino acid residues. These studies indeed provided a major breakthrough in understanding the biological function and the mechanism of IRR. However, the absence of atomic resolution structure of IRR or its domains prevents a deeper glance into the detailed molecular mechanism of alkali-triggered activation of IRR.

OUTLOOK AND CONCLUSIONS

The frequency of type 2 diabetes is increasing at an alarming rate, and a considerable fraction of cases is related to mutations in IR and resulting receptor dysfunction.[6,8,9] Novel and more potent agents need to be developed to modulate the function of IR and IGF1R.[67] Unfortunately, the flexible, multidomain nature of these proteins, their large size, the hydrophobic TM domain, extensive ectodomain glycosylation, and disulfide-bonded architecture have prevented crystallization of the full-length receptors for structural analysis.[12] Multimethod structural approaches are especially promising. For example, Menting et al.[17] employed solution nuclear magnetic resonance, X-ray crystallography, and molecular modeling computational methods to identify the structural details of the "induced fit" insulin−IR interactions. Utilization of other methods, including time-resolved spectroscopy, may yield more valuable data. Structural analysis of selectively isotope-labeled proteins by vibrational spectroscopy is a powerful technique that has not been fully utilized in structural biology. Fourier transform infrared (FTIR) spectroscopy is capable of detecting changes in the structure and dynamics of proteins in real time.[84] Moreover, isotopic labeling of a certain stretch, or a domain, or a subunit of a protein with ^{13}C allows determination of the local structure and dynamics of the labeled region.[85−88]

In the case of IR family receptors, the full-length protein can be produced in which the α- and β-subunits are isotopically distinct; i.e., one subunit is selectively labeled with the ^{13}C stable isotope, while the other is unlabeled. For both unlabeled and ^{13}C-labeled receptors, the disulfide bridges can be reduced, followed by H_2O_2-assisted reassociation of the ^{13}C-labeled β-subunit with the unlabeled α-subunit or the unlabeled β-subunit with the ^{13}C-labeled α-subunit. Similar protein engineering procedures have been reported for other proteins.[89,90] The subunit-specific ^{13}C-labeled receptor can be reconstituted in lipid membranes and real-time conformational changes in each subunit following hormone binding detected by FTIR. Insulin mimetic peptides can be used to stimulate IR that incorporates diabetes-related mutations, in an attempt to recover the structural and functional features of the wild-type protein. Thus, insulin surrogates for the treatment of type 2 diabetes related to IR dysfunction can be identified.

In closing, efficient rational drug design requires a detailed knowledge of the molecular basis of insulin signaling, including the function of IR, the IRS proteins, and the downstream molecular machinery.[67,91] Such knowledge may be generated through extensive basic research that is both transformative and translational in nature, i.e., provides a higher-level, comprehensive understanding of the detailed molecular mechanisms underlying insulin signaling and leads to effective clinical applications and new therapies. Multiscale studies, combining atomic resolution techniques with time-resolved spectroscopy, computational methods, and structure−function analysis seem to be a promising avenue that may lead to advancements in antidiabetic therapeutics.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

αCT, C-terminal 16-residue α-helical segment of the α-subunit of insulin receptor; CD, circular dichroism; CR, cysteine-rich region; Fab, antigen-binding fragment; FTIR, Fourier transform infrared; FnIII, fibronectin type III domain; Glut4, glucose transporter; GS, glycogen synthase; GSK3, glycogen synthase

DOI: 10.1021/acs.biochem.5b00805
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kinase 3; IGF, insulin-like growth factor; IGF1R, insulin-like growth factor 1 receptor; IR, insulin receptor; IRR, insulin receptor-related receptor; IRS, insulin receptor substrate; L1 and L2, leucine-rich repeat domains 1 and 2, respectively; MAPK, mitogen-activated protein kinase; PDB, Protein Data Bank; PDK1, phosphoinositide-dependent kinase 1; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI-3K, phosphatidylinositol 3-kinase; TK, tyrosine kinase; TK3P, tris-phosphorylated TK domain; TM, transmembrane.

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