Unforeseen conformational changes in insulin receptor on ligand binding: the emerging picture of insulin receptor activation

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Keywords: Insulin, insulin receptor, crystal structure, negative cooperativity, signalling mechanism, insulin-like growth factor, Type 1 insulin-like growth factor receptor

The authors declare that they have no conflict of interest.

Abstract

Unraveling the molecular detail of insulin receptor activation has proved challenging, but a major advance is the recent determination of crystallographic structures of insulin in complex with its primary binding site on the receptor. The current model for insulin receptor activation is that two distinct surfaces of insulin monomer engage sequentially with two distinct binding sites on the extracellular surface of the insulin receptor, which is itself a disulphide-linked $(\alpha\beta)_2$ homodimer. In the process, conformational changes occur both within the hormone and the receptor, the latter resulting in the disruption of the intracellular interactions that hold the kinase domains in their basal state and in the initiation of the phosphorylation events that drive insulin signaling. The purpose of this review is to summarize the extant structural data relating to hormone binding and how it effects receptor activation, as well as to discuss the issues that remain unresolved.

Introduction

Insulin binding to the insulin receptor homodimer (IR) is characterized by curvilinear Scatchard plots, negative cooperativity and a bell-shaped dose response curve for the dissociation-accelerating effect of unlabeled insulin [1]. These features can be explained by a model within which the receptor monomers are arranged in an anti-parallel fashion, with each receptor monomer having two distinct hormone binding sites (denoted Sites 1 and 2 on one receptor monomer and Sites 1′ and 2′ on the second receptor monomer) [2]. Hormone is proposed to bind first (with low affinity) to Site 1 of the first receptor monomer and then to form a cross-link to Site 2′ on the second receptor monomer, the resultant complex being of high affinity. Formation of the high-affinity cross-link of the Site 1 / Site 2′ pair reduces the capacity of ligand to

form a high-affinity cross-link of the alternate Site 1′ / Site 2 pair (= negative cooperativity), but without abrogating the ability of hormone to bind singly to the individual components of the alternate Site 1′ / Site 2 pair (= a bell-shaped dose response). This model has recently been refined to show that all of the kinetic data relating to hormone binding to IR can be accounted for by assuming that formation of the high-affinity cross-link is enabled by harmonic oscillation of the receptor (**Figure 1**) [3,4].

Understanding the molecular detail of the above events thus requires at least the following structural data: (a) the structure of insulin in its receptor-free form, (b) the structure of the receptor ectodomain in its hormone-free form, (c) the structure of insulin bound to receptor Site 1, and (d) the structure of the high-affinity complex wherein insulin cross-links receptor Sites 1 and 2′. Of these, (a), (b) and (c) now exist (see below), but not (d). In addition, to understand how insulin binding effects signal transduction across the membrane and subsequent receptor activation, detail is required regarding the changes in the trans-membrane and cytoplasmic regions of the receptor upon hormone binding. However, despite decades of endeavour and the recent elucidation of the structure of the hormone-bound IR Site 1 [5], the mechanism of insulin-initiated signal transduction remains largely elusive. Below we will review the existing structural data and discuss some of the complexities that are emerging regarding the nature of the hormone cross-linked receptor.

The structure of insulin in its receptor-free form

The first structure reported for insulin was that of the $2Zn^{2+}$-stabilised hexamer of porcine insulin [6]. This structure was understood to reflect the storage form of the hormone and revealed that the two chains (A and B) of the insulin monomer form a

two-layered sandwich (**Figure 2A**). The B-chain was seen to consist of an N-terminal segment (residues B1-B6), a type II β-turn (B7-B10), a central α-helix (B9-B19), a type I β-turn (B20-B23) and a C-terminal β-strand (B24-B28), followed by the less well-ordered residues B29-B30. The A-chain was seen to consist of an N-terminal α-helix (A1-A8), a non-canonical turn (A9-A11), a second α-helix (A12-A18) and a C-terminal segment (A19-A21). The structure displayed the expected disulphide bonds CysA6-CysA11, CysA7-CysB7 and CysA20-CysB19 [7]. Extensive biochemical and structural characterization of the hormone has since demonstrated that the Site 1 and Site 2 receptor-binding surfaces of insulin comprise residues predominantly drawn respectively from the hormone dimerization surface and from the hormone hexamerization surface (**Figures 2B, C**) [8-11]. These surfaces have both been shown to have closely-related counterparts in the insulin-like growth factors IGF-I and IGF-II [12,13].

The structure of the insulin receptor ectodomain homodimer in its insulin-free form

Sequence analyses reveal multiple domains

The determination of the cDNA sequence for human IR revealed that there are two isoforms of IR that differ by the absence (in the IR-A isoform) or presence (in the IR-B isoform) of a twelve-residue insert between residues 716 and 717 of the mature protein from alternate exon 11 splicing [14,15]. Sequence analysis simultaneously identified a single trans-membrane region and indicated that the intra-cellular C-terminal region contains a tyrosine kinase (TK) catalytic domain, flanked by a juxtamembrane region (~41 residues) and a C-terminal tail (~100 residues). Subsequent analysis identified the presence of two homologous leucine-rich repeat

domains (L1 and L2) within the N-terminal region of the receptor ectodomain [16], while the intervening, cysteine-rich region (CR) between L1 and L2 was shown to be comprised of eight disulphide-linked modules, similar to those within the tumour necrosis receptor and epidermal growth factor receptor; and to the epidermal growth factor itself [17]. The C-terminal half of the IR ectodomain was predicted to contain three fibronectin type III domains (FnIII-1, FnIII-2 and FnIII-3) [18-21]. The FnIII-2 domain contains a large (~120 residues) insert domain (ID) that includes the furin cleavage site that yields the α- and β-chains of the mature IR monomer [18]. The receptor is heavily glycosylated, with sixteen N-linked glycan moieties per monomer [22] and an O-linked segment near the N-terminus of the β-chain [23]. Thirteen potential tyrosine phosphorylation sites are distributed over the juxtamembrane, TK domain and C-terminal tail components of the intracellular region of the receptor β-chain and provide potential docking sites for SH2-containing [24,25] and PTB-containing signalling proteins [26,27]. Four of these sites are known to be functionally important, namely, Y972 in the juxtamembrane region and Y1158, Y1162, and Y1163 in the TK domain (IR-B numbering) [28].

Structures of the L1-CR-L2 domains of IGF-1R and IR

The first structural information regarding IR ectodomain arose from the crystal structure of the L1-CR-L2 module of the homologue IGF-1R [29]. The structure revealed that the L1 and L2 domains are β-solenoids comprising three β-sheet surfaces and also confirmed the predicted modular structure of the cysteine-rich region. Many of the sites of clinical mutation in IR that result in defective insulin binding, as well as sites within the L1 domain that are known to compromise insulin binding upon mutation, were seen to lie on the central β-sheet of the L1 domain (L1-

β_2), strongly suggesting its involvement in ligand binding. The subsequent structure of the L1-CR-L2 module of IR confirmed equivalent structural features to that of the IGF-1R module [30].

Structure of the IR ectodomain dimer

The structure of the IR ectodomain homodimer was reported in 2006 and revealed a folded-over "Λ" conformation [31]. Each "leg" of the "Λ" -shaped structure consists of the L1-CR-L2 module of one receptor monomer packed against the linearly-arranged FnIII-1, FnIII-2 and FnIII-3 domains of the alternate receptor monomer (**Figure 3**). The L2 domain of each receptor monomer is in contact with the FnIII-1 domain of the alternate receptor monomer at the apex of the "Λ", while the L1 domain of the each monomer is in contact with the FnIII-2 domain of the alternate monomer at the approximate mid-point of the leg [31]. The FnIII-1 and FnIII-2 domains both contain unusually large CC′ loops - that within the FnIII-1 domain contains the Cys524-Cys524 inter-α-chain disulphide bond, while that within the FnIII-2 domain contains the entire ID. Most of the ID was not resolved within the crystal structure [31]. At the base of the "Λ"-shaped structure, the C-termini of the respective FnIII-3 domains are poised to extend through the cell membrane to connect to the receptor´s cytoplasmic elements. The observed anti-parallel arrangement of the receptor monomers within the ectodomain agreed with an earlier negative stain electron microscopy study of insulin receptor ectodomain in complex with antibody fragments (Fabs) [32] and offered strong support for the model of receptor activation described in the Introduction.

The missing detail: nature of the L1 / αCT tandem element

Following release of the ectodomain structure [31], an orphan segment of difference electron density seen lying across the L1-β_2 sheet of each L1 domain in the original crystallographic maps of the IR ectodomain was shown to correspond to IR residues 693-710 from the C-terminal region of the IR α-chain, the so-called "αCT" segment [33] (**Figure 3**). This segment is essential for insulin binding [34-37] and is known from chemical cross-linking analyses (with insulin B24 and B25 derivatives) to lie in close proximity to the L1 domain [38]. The final nine residues of the IR α-chain (711-719, the crystallized construct being based on IR-A) were disordered in the crystal structure. Direct chemical cross-linking confirmed that the αCT segment associated with each L1 domain in the IR homodimer is contributed by the alternate receptor monomer to that which contributes the L1 domain [33], in line with an earlier prediction [39] and complementation analysis [40]. The observed association of the L1-β_2 sheet and the αCT segment as a tandem structural element thus provided the first view of IR Site 1 in its insulin-free form.

The structure of the insulin / Site 1 tandem element complex

Four structures, the same picture: αCT moves on insulin binding

In a major breakthrough, Menting and co-workers recently reported four structures that reveal the manner in which insulin engages Site 1 of IR. These structures include three of different insulins in complex with the IR L1-CR module in the presence of exogenous αCT peptide, and one structure of insulin in complex with an IR homodimeric domain-minimized construct termed IR593.αCT [5]. Together, they provide a consistent picture of the insulin / Site 1 interaction (**Figure 4A)**. The direct interaction of insulin with the L1 domain of IR is seen to be sparse, the hormone instead engaging predominantly with the αCT segment of the Site 1 tandem element.

Strikingly, the αCT segment is, with respect to its *apo*-IR counterpart, both displaced on the L1-β_2 surface and C-terminally extended to include residues 711-715 (**Figure 4A)**. The observed relocation was independent of whether the peptide employed was the "classical" 704-719 αCT peptide [34] or the N-terminally extended 697-719 αCT peptide [33]. Indeed, in the structure employing the latter peptide, residues 697-704, previously shown to be engaged with the ectodomain surface, were disordered. Relocation of αCT upon hormone binding is consistent with mutagenesis data that show that peptide substitutions in the αCT segment upstream of Phe705 that increase its affinity for the L1 domain concomitantly impair insulin binding [33]. Movement of the αCT peptide upon hormone binding also concurs with photo-cross-linking data [41] that show that IR residues Leu36, Leu37, Leu62 and Phe64 - which are substantially buried beneath the αCT segment in its *apo*-disposition on the L1 domain - are, upon mutation to *p*-azido-phenylalanine, capable of photo-cross-linking to insulin in the holo-receptor.

The detachment model for insulin is confirmed

A further key observation arising from these structures is that despite residues B22-B30 being unresolved at the reported resolution [5], the αCT helix occupies volume that would otherwise contain insulin residues B26-B30 if the latter retained their receptor-free (storage-form) conformation (**Figure 4B**). This observation confirms the so-called *detachment model* of insulin binding in which the C-terminal region of the B-chain displaces to expose the mostly hydrophobic core of the hormone [2,8,9,42-45]. The detachment model arose originally from a number of observations, including (a) the single-chain B29-A1 peptide-linked insulin molecule is completely devoid of biological activity despite retaining the same crystal structure as that of wild-type

insulin [46], (b) the B-chain C-terminal region (residues B20-B30) of the *active* PheB24Gly human insulin mutant is disordered in solution (**Figure 1A**) [42], and (c) the structure of the insulin ValA3Leu clinical mutant, which exhibits a 500-fold reduction in insulin affinity relative to wild-type, has a structure identical to that of the native hormone and within which the side chain of the variant residue remains buried [47]. Indirect evidence also suggests that the detachment may involve the formation of a β-turn at residues B25-B26 [45]. Within the four Site 1 complexed structures, the structure of the core of insulin remains closely similar to that in the receptor-free hormone - at least as far as can be discerned at the described resolution (3.9 Å) [5].

Critical interactions between αCT and insulin

Further analysis of the insulin / Site 1 interaction reveals the two most critical hormone-engaging residues in the αCT segment. The first is His710, which inserts into a pocket formed by invariant insulin residues ValA3, GlyB8, SerB9 and ValB12 [8]. The second is Phe714, which occupies a hydrophobic crevice formed by invariant insulin residues GlyA1, IleA2, TyrA19, LeuB11, ValB12 and LeuB15 (see **Box 1**). The critical role of His710 and Phe714 is consistent with alanine scanning mutagenesis of the IR ectodomain, wherein a His710Ala mutation results in no detectable hormone binding, while a Phe714Ala mutation confers an ~140-fold decrease in hormone affinity [36]. The restriction of the major αCT / insulin interaction to the 710-714 segment is also consistent with the observation that the residues structurally equivalent to IR 709-714 in the closely-related IGF-1R are significantly more protected from hydrogen / deuterium exchange upon growth factor binding than are residues upstream or downstream of this segment [48]. The

importance of Phe714 in ligand binding is further consistent with observations that IR / IGF-1R domain-minimized chimeras containing the αCT segment from the insulin-receptor-related receptor (IRR) in place of their cognate αCT segment are devoid of hormone-binding ability [49]. Replacement of IR Phe714 (≡ IGF-1R Phe701) by threonine in the alkali sensor IRR [50] is the salient difference between their respective αCT segments [49,51] and prevents IRR signaling in response to insulin and IGFs [50]. Residues GlyA1, IleA2, ValA3, TyrA19, GlyB8 and LeuB11, which are seen here to be in contact with αCT residues His710 and Phe714, have all been identified as resulting in a >10-fold reduction in insulin upon their mutation to alanine [52]. Further detail regarding the insulin / Site 1 interaction is provided in **Box 1.**

What about the C-terminal residues of the insulin B-chain?

Surprisingly, while individual alanine mutation of residues GlyB23, PheB24 and PheB25 also result in a > 10-fold reduction in insulin affinity, these residues were unresolved in the four Site 1 complexed structures (including the two utilizing [D-ProB26]-DTI-NH_2, whose free structure contains an ordered turn at B24-B26 [45]). Nevertheless, the disposition of the hormone in the Site 1 complexed structures suggest that, in holo-IR, the final location of the B-chain C-terminal segment is most likely between the αCT segment and the proximal CR domain. This conclusion is supported by the observations that different photo-probes attached to insulin LysB29 can label either CR [53] or L1 [54] and also by the fact that such a location for these residues must be the case for their counterparts in the single-chain molecules pro-insulin and the IGFs upon their binding to IR or IGF-1R.

Finally, in none of the four Site 1 complexed structures [5] does the insulin B-chain N-terminus display the so-called R-state conformation. In the hormone R state, the N-terminal portion of the B chain alters conformation to become part of an extended α-helix B1-B19 [55]. The R-state is considered less stable but more active than the so-called T-state conformation observed in the original crystal structure of insulin (**Figure 2A**) [56]. Failure to observe the R-state in the Site 1 bound complexes suggests either that the N-terminally extended B-chain helix is an artifact of the low-salt conditions of the original insulin R-state crystals [55], or that its formation requires formation of the high-affinity cross-link.

In summary, the Site 1 complexed IR fragment structures provide an explanation for a vast body of biochemical data relating to the insulin / IR interaction [5], strongly supporting the view that these structures adequately represent the structure of the Site 1 complex within the context of the holo-receptor.

The location of Site 2

No structure is currently available of the hormone cross-linking Sites 1 and 2′ or of the hormone bound to Site 2 (2′) alone. Nevertheless, the Site 1 complexed structures described above support the view that Site 2 most likely lies at the junction of the FnIII-1 and FnIII-2 domains of the IR monomer opposite to that providing the L1 domain to Site 1 [5,31]. This conclusion is based on the observations that (a) superposition (*via* their common L1 domains) of the structure of the hormone / Site 1 complex onto the structure of the *apo*-IR ectodomain places insulin in direct steric clash with the FnIII-1 / FnIII-2 domain junction, and (b) alanine scanning mutagenesis of this junction region reveals a reduction in insulin binding affinity upon individual mutation of residues Lys484, Leu552 and Asp591 (within the FnIII-1

domain) and residues Ile602, Lys616, Asp620 and Pro621 (within the FnIII-2 domain) [57]. Further supporting data for such a location for Site 2 include (a) bioinformatic analysis, which reveals a conserved patch of residues in the vicinity of the FnIII-1 / FnIII-2 junction [51] and (b) studies of whole IR / IGF-1R chimeras [58] and IR / IGF-1R hybrid receptors [59], which indicate that high-affinity insulin-binding requires insulin-specific components within the region 326-524 on the alternate receptor monomer to that providing the L1 domain. We note that while the observed steric clash in the overlay of the Site 1 complex onto that of the *apo*-ectodomain appears incompatible with the opened-up Site 1 / Site 2 conformation of the *apo*-receptor presented in Figure 1, the simplest explanation is that the observed ectodomain structure arises from the crystal lattice sampling only one state within the conformational ensemble accessible to the ectodomain homodimer and, indeed, that ensemble itself might be modulated by the four Fab domains employed to facilitate crystallization [31].

In contrast to the dramatic effect on insulin binding of mutation of key residues within Site 1 (*e.g.*, individual mutation of His710 and Phe714 to alanine), the reduction in insulin affinity upon individual mutation to alanine of the putative Site 2 residues does not exceed 5-fold [57]. While such limited reduction in affinity is consistent with the hormone / Site 2 interaction being weaker than that of Site 1 (μM compared to nM [3]), a question thus remains as to whether the residues detected in the alanine scan actually define Site 2. One way to resolve this issue would be to undertake a similar study to those described in refs [5,41], wherein residues within IR are replaced by photo-probes (using amber-suppression technology) and then probed for their ability to photo-cross-link to insulin.

The degree of receptor conformational change - large or small?

The IR ectodomain is flexible

The existing structural data have highlighted sites of inter-domain flexibility within IR. Four distinct arrangements of the L2 domain with respect to the CR domain have now been observed (two in the IR485 crystallographic asymmetric unit [30], one in the *apo*-IR ectodomain [31] and one in the IR593.αCT insulin complex [5]). Furthermore, the relative arrangement of the constituent domains of the $(L2/FnIII\text{-}1)_2$ IR module (located at the head of the "Λ", **Figure 3**) differs in the IR593.αCT insulin complexed structure compared to its counterpart in the *apo*-IR ectodomain structure [5]. Such movement is consistent with the observation that the interfaces within this module are not well packed [31]. Finally, removal of the glycan at IR Asn624, which lies at the junction of the FnIII-1 and FnIII-2 domains [31], leads to an increase in basal signalling [60]. Removal of glycan at this location has the potential to increase the scope for relative movement of these domains. We speculate that such increased flexibility allows the receptor greater capacity to access conformations similar to those brought about by formation of the high-affinity cross-link at this site.

The case for limited conformational change

Given that Site 1 (defined by the L1 domain and its associated αCT segment) and Site 2′ (defined by the FnIII-1 / FnIII-2 junction of the opposing monomer) are already in close proximity in the *apo*-ectodomain structure [31, 33], conformational change in the receptor upon hormone binding may be small. Limited conformational change in IR upon hormone binding is compatible with a small-angle X-ray scattering study of IGF-I binding to the soluble IGF-1R ectodomain, wherein very little change in the radius of gyration was observed in the ectodomain upon binding of IGF-I [61]. Soluble IGF-1R ectodomain shows similar ligand-binding kinetics to those of holo-

IGF-1R [62] and thus the conformational change in the IGF-1R ectodomain upon IGF-I binding may conceivably mimic that in the holo-receptor. Limited conformational change is also suggested by the bioinformatics analysis referred to in the previous section, which indicates that only a slight "twist" of the last two FnIII domains is required to align the proposed Site 2 with the second binding surface of insulin [51]. The conformational change associated with opening up the L1 domain / FnIII-2 domain interface to allow hormone cross-linking would certainly be less than the major rotation and movement of the L1-CR1 domains with respect to the L2-CR2 domains seen in the related epidermal growth factor receptor (EGFR) family upon ligand binding [63]. Limited, indeed facile, conformational change may reflect the fundamentally different mechanism of receptor activation in the IR family compared to the EGFR family: - in the IR family, receptor activation occurs *via trans*-phosphorylation within the pre-formed disulphide-linked receptor homodimer [64], while in the EGFR family, receptor activation occurs upon ligand-induced non-covalent dimerization of receptor monomers.

The case for more extensive domain movements

Nonetheless, limited conformational change (corresponding to facile opening of the interface between Site 1 and the FnIII-1 / FnIII-2 domain junction to accommodate hormone) appears to be at odds with the following observations. (a) Two severely insulin-resistant patients with Rabson-Mendenhall syndrome exhibit the IR L2 domain mutation Ser323Leu [65]. Given that the L2 domain is not involved in the Site 1 interaction, Ser323 must therefore be involved in the generation of the high-affinity state. Facile opening of the interface between the L1 domain and FnIII-2 to allow hormone to cross-link Sites 1 and 2′ does not, *prima facie*, bring hormone or receptor elements into the vicinity of Ser323. (b) The clinical IR mutations

Phe382Val, Lys460Glu and Asn462Ser are located remote from Site 1 and from the proposed Site 2′, but result in either impaired insulin binding or reduced signalling [31,66-69]. Furthermore, a hydrogen / deuterium exchange study of IGF-1R found that the region 428-448 in IGF-1R (equivalent to IR 438-458; immediately upstream of residue Lys460 referred to above), showed increase protection from exchange on ligand binding [48]. (c) In addition, the hydrogen / deuterium exchange study referred to above found that residues 628-648 (equivalent to IR 634-654; lying at the N-terminal region of the ID) are protected from exchange on ligand binding. In IR, this segment lies close to the first cysteine-rich module (residues 158-189), which is intimately associated with the L1 domain [30]. Displacement of the L1 domain away from the fibronectin leg would lead to this segment having increased exposure to exchange rather than increased protection. (d) IR residue Asp707 and insulin N-terminal A-chain helix are implicated in signalling [41], yet these residues are remote from the proposed Site 2′ interaction. (e) Studies using detergent-solubilized holo-IRs and truncated membrane-anchored IR ectodomains indicate that insulin binding results in significant re-orientation of the IR subdomains [70,71], evidenced by a reduction in Stokes radius and an increase in sedimentation coefficient, the precise values depending on the experimental conditions employed. Interestingly, in these studies, no equivalent change was observed in solubilized IR ectodomain upon hormone binding. This observation is consistent with the fact that within the solubilized IR ectodomain system, insulin is capable of binding only with low affinity and negative cooperativity is absent [72], *i.e.* high-affinity cross-linking and the associated conformational changes are abolished by removal of the trans-membrane anchors.

IGF-1R is different

No data yet exist for the location of Site 2′ on IGF-1R, and that despite the close sequence relationships both between insulin and the IGFs and between IR and IGF-1R, it remains possible that the location of Site 2′ is different in the two receptors [62,73]. Indeed, the retention of the ability of ligand to bind the solubilized IGF-1R ectodomain with high affinity and negative cooperativity [62], in contrast to the loss of these characteristics in the solubilized IR ectodomain system [72], may align with differences in their respective Site 2′ elements. These latter differences may also suggest that the inter-domain flexibility of the solubilized IR ectodomain is greater than that of the solubilized IGF-1R ectodomain, with trans-membrane anchors being required to restore high-affinity binding in the case of IR.

A number of soluble IR and IGF-1R constructs with both high-affinity binding and negative cooperativity are described in the literature [62,74-76] - their structural study may ultimately provide insights into the Site 1 / Site 2′ cross-linked state. However, as has been shown in the case of the high-affinity construct IR593.αCT [5,76], correspondence of the ligand-bound conformation of these structures to that of the native receptor is not guaranteed.

Changes in the trans-membrane region and intracellular components

Very little if anything is known about how the IR trans-membrane domains transfer signals across the membrane. The trans-membrane segments have been shown to be tolerant of significant change in sequence [77,78] and also tolerant of significant insertion at the ectodomain junction [79]. As far as we are aware, no data exist in the literature that indicate that the trans-membrane segments of IR interact with each other, contrasting with the recent report that such association occurs between the trans-membrane segments of EGFR upon growth factor binding [80,81].

With regard to the cytoplasmic domains, we have argued previously [39,82-84] that in the basal state, the kinase domains of IR are unlikely to be simply "suspended" from an extended, disordered juxtamembrane segment, as it would then be difficult to see how any structural change in the extracellular domains on ligand-binding could significantly increase the propensity of the kinase domains to interact with each other. The kinase domains must therefore be constrained in some way in the basal state, with these constraints being removed upon ligand binding, allowing the catalytic domains to be released in "yo-yo"-like fashion [70,71] (**Figure 5A**), resulting in *trans*-phosphorylation *via* both TK domains [64,85].

Understanding these processes remains a major goal. The critical role of the IR juxtamembrane region in regulating kinase activity is seen in the structure of an extended IR kinase construct that contained part of the juxtamembrane region [86]. This structure (**Figure 5B)** implied an inverted orientation of the kinase domain lobes relative to the cell membrane. In IR and IGF-1R, the juxtamembrane regions proximal to the tyrosine kinase domain inhibit its activity through the highly-conserved residue Tyr984 (IR-B numbering, equivalent to Tyr972 in IR-A and Tyr957 in IGF-1R). This tyrosine residue interacts with several conserved residues in the N-terminal lobe of the IR kinase domain, stabilizing a non-productive position of the αC helix [86]. Mutation of this residue in the full-length IR or IGF-1R increases basal auto-phosphorylation substantially [86,87]. Juxtamembrane inhibition in IR is more significant than that contributed by the activation loop, since, in the full-length IR found on cells, mutation of Tyr984 to Ala increases the basal phosphorylation state thirty fold, ten times greater than the three-fold increase seen following mutation of the activation loop residue Tyr1162 to Asp [86]. An additional role may be played by the C-tail, as it has been shown that insulin binding initially induces a short-lived

conformational change in the C-tail. This facilitates ATP binding and subsequent auto-phosphorylation, effecting additional conformational change in the juxtamembrane region, activation loop, other parts of the catalytic domain and the C-tail itself [88]. In the case of EGFR, data exist that an LRRLL helical element within the N-terminal juxtamembrane region is membrane-associated in the ligand-free form of the receptor and that part of the activation process involves release of this element from the membrane and its dimerization with the equivalent element in the second EGFR monomer [80,81]. In addition, such activation is shown to require an N-terminal association of the trans-membrane helices *via* a classical GXXXG motif within these segments [80,81]. Neither of these motifs is present in the equivalent regions of IR or IGF-1R, underscoring again that IR and IGF-1R most likely have a fundamentally different mode of activation to EGFR [84].

Conclusions

Significant progress has been made in understanding the structure of the insulin receptor and the mode of insulin engagement with the receptor, and there is considerable cause for optimism that the remaining pieces can now be determined. Importantly, there have been a number of major technological advances in structural biology over the past decade, in particular, improvements in single-particle cryo-electron microscopy [89], allowing significantly higher-resolution reconstructions to be obtained from particles that lack internal symmetry. We are thus cautiously optimistic that the remaining pieces of the puzzle can be assembled on a time scale much shorter than that required to date.

Acknowledgments

This work was supported by Australian National Health and Medical Research Council Project Grant 1005896 and Independent Research Institutes Infrastructure Support Scheme Grant 361646, the Hazel and Pip Appel Fund and a Victorian State Government Operational Infrastructure Support Grant.

FIGURE CAPTIONS

Figure 1 The insulin / IR interaction at physiological concentration as represented by the harmonic oscillator model. S1, S2: primary and secondary binding sites on one receptor monomer; S1′, S2′: primary and secondary binding sites on the second receptor monomer. a_1, d_1: association and disassociation constants for Sites 1 and 1′; a_2, d_2: association and disassociation constants for Sites 2 and 2′. k_{cr}: cross-linking constant describing the rate of transition of the receptor from the inactive to the active conformation. Based on ref. [3], Figure 6.

Figure 2 The structural biology of insulin. **A)** The tertiary structure of insulin. The A chain (21 residues) is shown in gold and the B chain is shown in grey (residues B1-B19) and yellow (B20-B30). Cysteine side chains forming disulfide bonds are shown in green [6]. Residues B20-B30 have been shown to be capable of dissociating from the helical core of the hormone upon the mutation PheB24Gly (see text) [42]. **B)** The "classical" receptor binding site of insulin, comprising residues GlyA1, IleA2, ValA3, GlnA5, ThrA8, TyrA19, AsnA21, ValB12, TyrB16, GlyB23, PheB24, PheB25 and TyrB26. **C)** The secondary receptor binding site of insulin, comprising residues SerA12, LeuA13, GluA17, HisB10, GluB13 and LeuB17. Schematics are based on Protein Data Bank (PDB) entry 4INS.

Figure 3 The spatial "Λ"-shaped arrangement of the constituent domains of the insulin receptor ectodomain $(\alpha\beta)_2$ homodimer [31]. The zoom-in shows the Site 1 tandem element formed by the (most hydrophobic) association of αCT segment of one receptor monomer with the L1 domain of the alternate receptor monomer [33]. Schematic is based on PDB entry 3LOH.

Figure 4 Structural transitions accompanying the formation of the insulin / Site 1 complex [5]. **A)** Quaternary association of the insulin helical core structure with the L1-CR domain and αCT segment receptor. InsA (gold): insulin A chain residues A1-A21; InsB (black): insulin B-chain residues B7-B21; L1 (cyan): first leucine-rich repeat domain of IR; CR (orange): cysteine-rich domain of IR; αCT (mauve): residues 705-715 of the IR insert domain αCT segment. The transparent mauve segment shows the location of αCT residues 693-710 in the structure of the *apo*-IR ectodomain. The respective N-terminal residues of the insulin A and B chains are indicated by small blue spheres and the respective C-terminal residues by small red spheres. **B)** Implied steric clash between insulin B-chain residues B25-B30 (shown in yellow) and the αCT segment if insulin were to remain in its receptor-free conformation within the structure of the Site 1 complex shown in (**A**). Panels are based on Figure 1 of ref. [5] (used by permission).

Figure 5 Receptor activation. **A**) The left-hand Panel shows the intra-cellular tyrosine kinase (TK) domains of the receptor homodimer held in an inactive conformation by the respective juxtamembrane segments of the receptor β-chain monomers. Upon insulin binding, an as-yet unknown conformational transition in the receptor ectodomain results in the release of the juxtamembrane segments from the respective TK domains, allowing these domains to *trans*-phosphorylate. **B)** Structure of the IR TK domain showing the association between its N-terminal domain (brown) and the juxtamembrane segment residues 978-988 (indicated by a solid red tube); the dashed red line indicates the speculative direction of the N-terminal connection back to the *trans*-membrane segment. Panels are reprinted from Current Opinion in Structural Biology, 22(3), CW Ward and MC Lawrence, "Similar but different: ligand-induced

activation of the insulin and epidermal growth factor receptor families", pp360-366, Copyright (2013), with permission from Elsevier..

BOX 1 The hormone / Site 1 interaction [5]

Panel A. *The interface between the αCT segment (mauve) and the insulin A-chain (orange) and the insulin B-chain (black).* Two key residues of the αCT segment are seen to interact with insulin. The first is His710, the side chain of which inserts into a pocket formed by insulin residues ValA3, GlyB8, SerB9 and ValB12, and the second is Phe714, the side chain of which is positioned in a pocket formed by insulin residues GlyA1, IleA2, TyrA19, LeuB11, ValB12 and LeuB15. The side chain of Asn711 is directed towards with insulin residues GlyA1, ValA3 and GluA4. Val712 and Val715 are the only residues within αCT that have little or no interaction with either insulin or the L1 domain, aligning with the fact that these are the only residues within the 704-715 segment that are not highly conserved across species and across IR and IGF-1R [50]. The disorder of αCT beyond Val715 is consistent with observations that individual substitution by Ala at sites 716-719 in the ectodomain causes minimal reduction in insulin affinity [36] and with the fact that this segment is tolerant of the insertion of twelve residues at the 716 / 717 junction in IR-B. In the Panel, the white transparent surface is that of insulin, while the cyan surface is that of the L1-β_2 surface, the view direction being parallel to the L1-β_2 strands.

Panel B. *The interface between the αCT segment (mauve) and the receptor L1 domain (white surface with cyan side chains).* The side chains of residues Phe701 and Phe705 are packed adjacent to each other in a hydrophobic pocket formed by the side chains of the L1 domain residues Leu62, Phe64, Phe88, Phe89, Tyr91, Val94, Phe96 and Arg118. The side chain of Tyr708 lies approximately parallel to the strands of L1-β_2, in proximity to the side chains of L1 domain residues Arg14, Gln34, Leu36 and Phe88. The side chains of Glu698 and Arg702 lie close to each other and interact

with the side chains of the L1 domain residues Arg118 and Glu120, respectively, with the four side chains appearing to form a charge-compensating cluster. The side chain of Leu709 is in hydrophobic contact with the side chains of Leu37 and Phe64. The αCT helix appears to be stabilized further by "clamps" formed by Arg118 and Arg14, with the conformation of Arg118 stabilized by interactions with Tyr91, Glu120, His144 and Phe705 and the conformation of Arg14 stabilized by interactions with Asp12 and the insulin A-chain C-terminus.

Panel C *The interface between insulin B-chain (black with green side chains) and the receptor L1 domain (white surface with cyan side chains).* The side chain of insulin residue ValB12 is positioned between L1 domain residues Phe39, Phe64 and Arg65, while that of insulin residue TyrB16 adjoins that of the L1 domain residue Phe39.

The Panel schematics are from ref. [5], used with permission.

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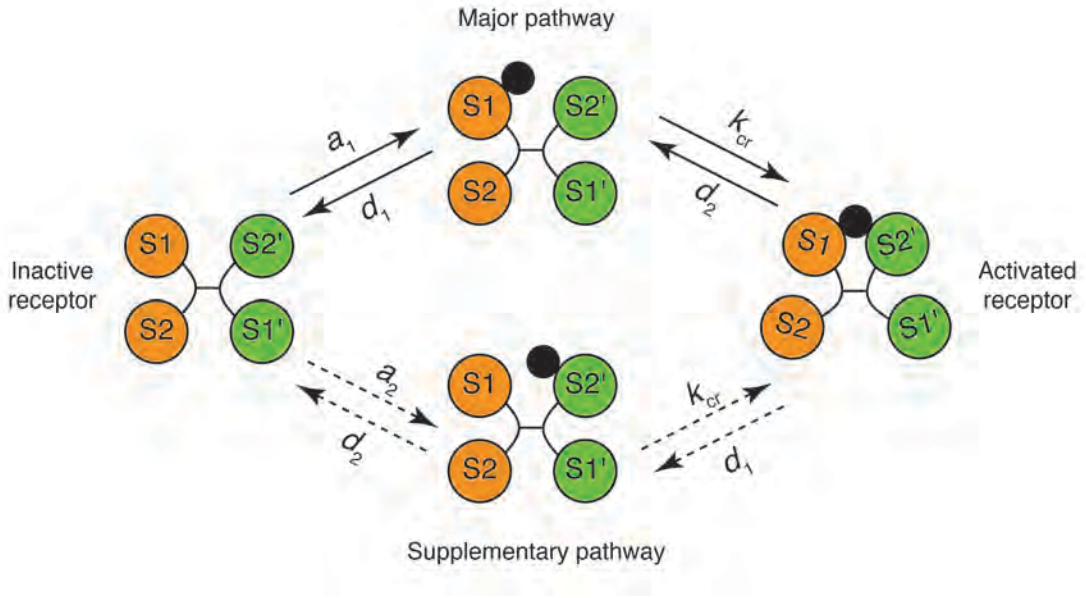

Major pathway
S1
S2'
S2
S1'
a_1
d_1
k_{cr}
d_2
Inactive receptor
Activated receptor
a_2
d_2
k_{cr}
d_1
Supplementary pathway

FIGURE 1

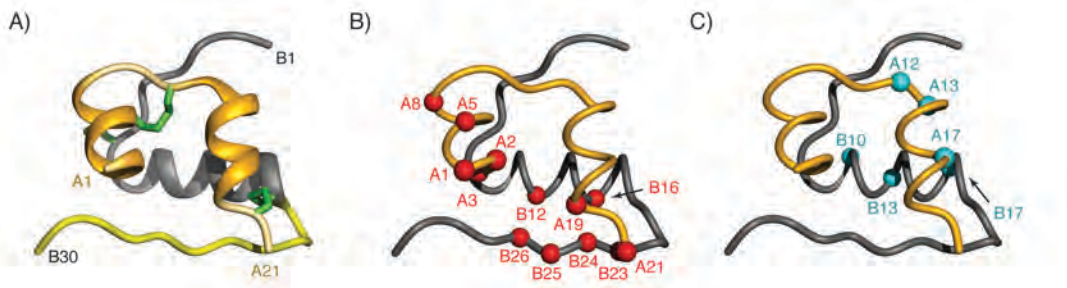
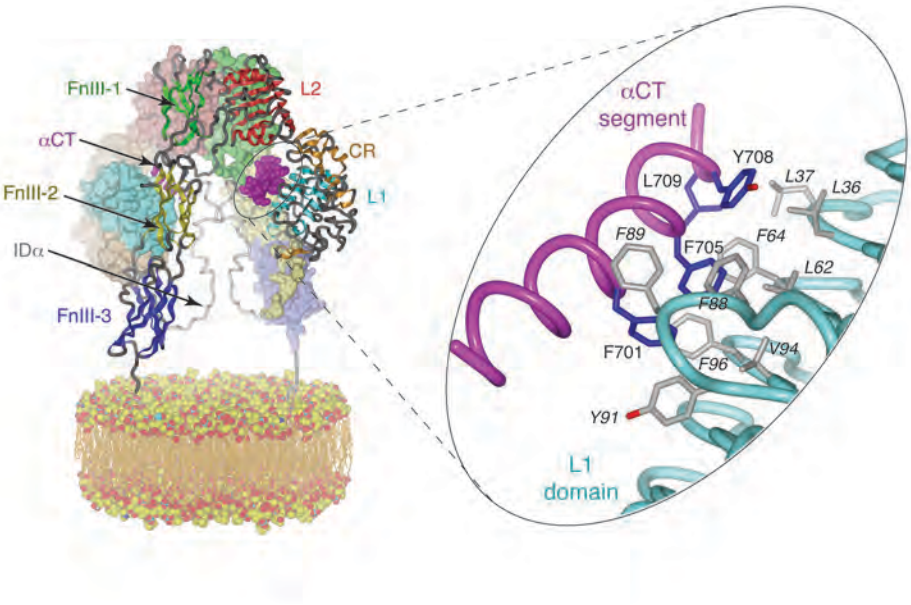

A)
B1
A1
B30
A21
B)
A8
A5
A2
A1
A3
B16
B12
A19
B26
B25
B24
B23
A21
C)
A12
A13
B10
A17
B13
B17

FIGURE 2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L2
αCT
CR
FnIII-2
L1
IDα
FnIII-3
αCT
segment
Y708
L709
L37
L36
F89
F705
F64
L62
F88
F701
V94
F96
Y91
L1
domain

FIGURE 3

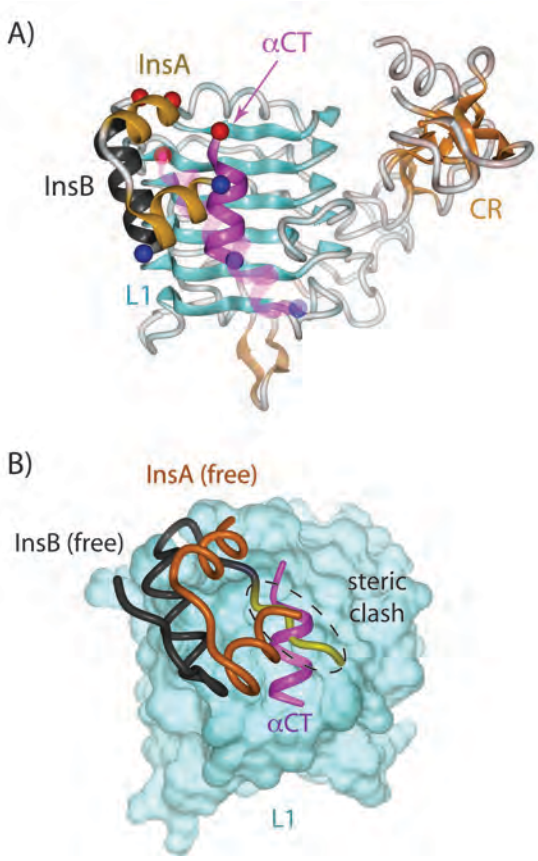


FIGURE 4

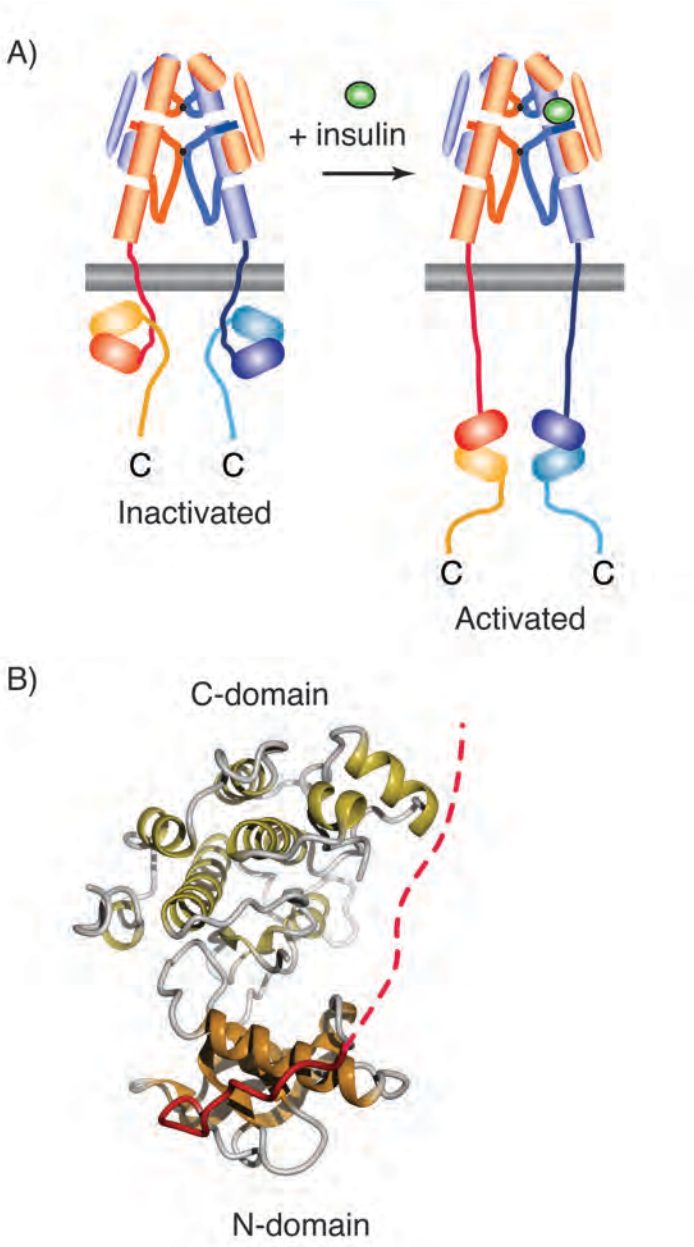
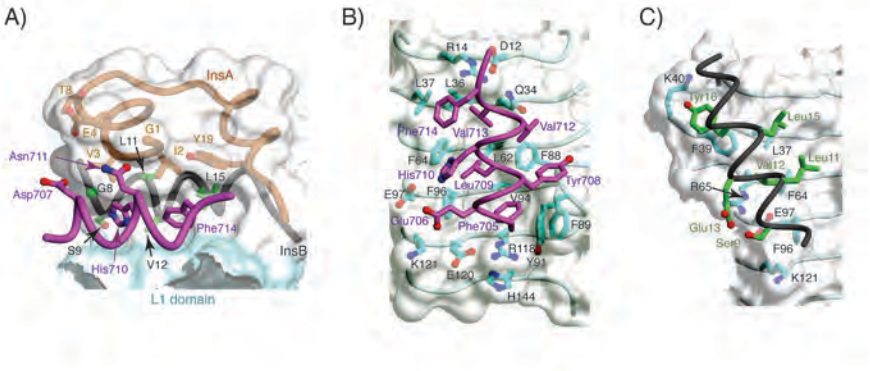


FIGURE 5


A)
InsA
T8
E4
G1
L11
Y19
I2
V3
Asn711
L15
G8
Asp707
Phe714
S9
His710
V12
InsB
L1 domain
B)
R14
D12
L37
L36
Q34
Phe714
Val713
Val712
F64
L62
F88
His710
Tyr708
E97
F96
Leu709
V93
Glu706
Phe705
F89
R116
K121
Y91
E120
H144
C)
K40
Tyr18
Leu15
F39
L37
Val12
Leu11
R65
F64
E97
Glu13
Ser9
F96
K121

BOX 1