Review

The insulin receptor: a prototype for dimeric, allosteric membrane receptors?

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The recent crystallographic structure of the insulin receptor (IR) extracellular domain has brought us closer to ending several decades of speculation regarding the stoichiometry and mechanism of insulin-receptor binding and negative cooperativity. It supports a bivalent crosslinking model whereby two sites on the insulin molecule alternately crosslink two partial-binding sites on each insulin-receptor half. Ligand-induced or -stabilized receptor dimerization or oligomerization is a general feature of receptor tyrosine kinases (RTKs), in addition to cytokine receptors, but the kinetic consequences of this mechanism have been less well studied in other RTKs than in the IR. Surprisingly, recent studies indicate that constitutive dimerization and negative cooperativity are also ubiquitous properties of G-protein-coupled receptors (GPCRs), which show allosteric mechanisms similar to those described for the IR.

Structure of the insulin receptor compared with other receptor tyrosine kinases and cytokine receptors

The insulin receptor (IR) and the closely related insulin-like growth factor-I (IGF-I) receptor belong to the superfamily of receptor tyrosine kinases (RTKs), which comprises 59 members in humans [1–3]. Unlike other RTKs, the IR and IGF-I receptors are disulfide-linked covalent dimers made of two extracellular α-subunits that contain the ligand-binding domains, and two transmembrane β-subunits that contain the intracellular RTK domain (reviewed in Refs [4–7]). All other RTKs, however, are noncovalent dimers or oligomers in the activated state [2,3].

It had long been predicted that the insulin and IGF-I receptors had a modular structure, as follows (Figure 1a). The extracellular portion is made successively (from the N-to-C terminus) of two leucine-rich repeat domains, L1 and L2, joined by a cysteine-rich domain (CR), followed by three fibronectin type-III repeats (FnIII-1, FnIII-2 and FnIII-3). FnIII-2 contains a 120-amino-acid insert (ID) of unknown structure, which contains the cleavage site that generates the α-subunits and β-subunits of the mature receptor. It also contains a 16-residue C-terminal sequence of the α-subunit (CT peptide at position 704–719) that is crucial for ligand binding in both the insulin and IGF-I receptors [8]. Contiguous to this CT peptide, a 12-amino-acid sequence (717–729) is alternatively spliced (coded for by exon 11),

Glossary

Allosteric: this refers to the regulation of the affinity or activity of a ligand binding to a protein (e.g. enzyme or receptor) by another ligand binding to a different site than the ligand of reference (referred to as the orthosteric-binding site). The name comes from the Greek *allos* (meaning 'other') and *stereos* (meaning 'space'). It was initially used in the restricted sense of the Monod–Wyman–Changeux model of concerted ligand-induced conformational and affinity changes in proteins like haemoglobin, which can only generate positive cooperativity in ligand binding, but has recently been more loosely used in a broader sense equivalent to homotropic or heterotropic cooperativity, both positive or negative, especially in the GPCR community.

Cooperativity: a mechanism whereby binding of a ligand to one site of a multimeric binding protein (like an enzyme or a receptor) affects the affinity of another ligand binding to another subunit of the same protein. Positive cooperativity means that the binding of one ligand increases the affinity of another ligand, whereas negative cooperativity means that it decreases the affinity of the other ligand. Homotropic cooperativity implies that both ligands are identical and bind to the same sites on protein subunits, whereas heterotropic cooperativity means that the two ligands are different (and bind to different binding sites on the same subunits).

Cytokine receptors: refers here to the receptors for a family of signalling protein hormones and other proteins involved in cellular communication having a four α-helix bundle structure, such as growth hormone, erythropoietin, interferons and some interleukins.

Dimer: an association of two protein molecules. A homodimer is made of two identical molecules, a heterodimer is made of two different molecules, and an oligomer is made of a few associated molecules.

Ectodomain: the extracellular portion of a cell-surface receptor.

Epidermal growth factor: a protein growth factor (part of a large family of related proteins) that has a role in the regulation of cell growth, proliferation and differentiation, in addition to a role in certain cancers.

G-protein: a guanine-nucleotide-binding protein that belongs to a family of protein enzymes called GTPases, which are involved in second-messenger-signalling cascades, stimulated by G-protein-coupled receptors (GPCRs), such as the β2-adrenergic receptor, that contain seven transmembrane domains (hence, they are also called '7TM receptors').

Insulin: a polypeptide hormone that is secreted by the β cells of the pancreas and is crucial in the regulation of glucose levels in the blood and overall metabolism. Its absence or deficient action or secretion results in diabetes mellitus.

Insulin-like growth factors (IGFs): IGF-I and IGF-II are peptide growth factors that are structurally related to insulin, which are involved in foetal growth and development, in addition to – in the case of IGF-I – being involved in postnatal growth under the control of pituitary growth hormone.

KIT: the receptor (RTK) for stem-cell factor; this was originally identified as a feline oncogene, hence the term 'KIT' (kitten?).

Nerve growth factor: a secreted protein (part of a family of neurotrophic factors) that induces the differentiation and survival of particular nerve cells.

Scatchard plots: plots of bound over free ligand as a function of receptor-bound ligand. This simple transformation of a binding equation yields a straight line in the case of a simple, mass-action-determined reaction with a slope that equals minus the affinity constant of the interaction and a horizontal intercept that equals the receptor concentration. Deviations from linearity denote cooperativity in binding: concave upwards indicates negative cooperativity, and concave downwards indicates positive cooperativity. A concave upward Scatchard plot can also arise from coexistence of high- and low-affinity sites.

TrkA: neurotrophin tyrosine kinase receptor type 1, also known as NTRK1; the receptor for nerve growth factor.

Tyrosine kinases: enzymes that phosphorylates protein substrates on tyrosine side-chains. Some are contained in the intracellular part of surface receptors (receptor tyrosine kinases or RTKs).

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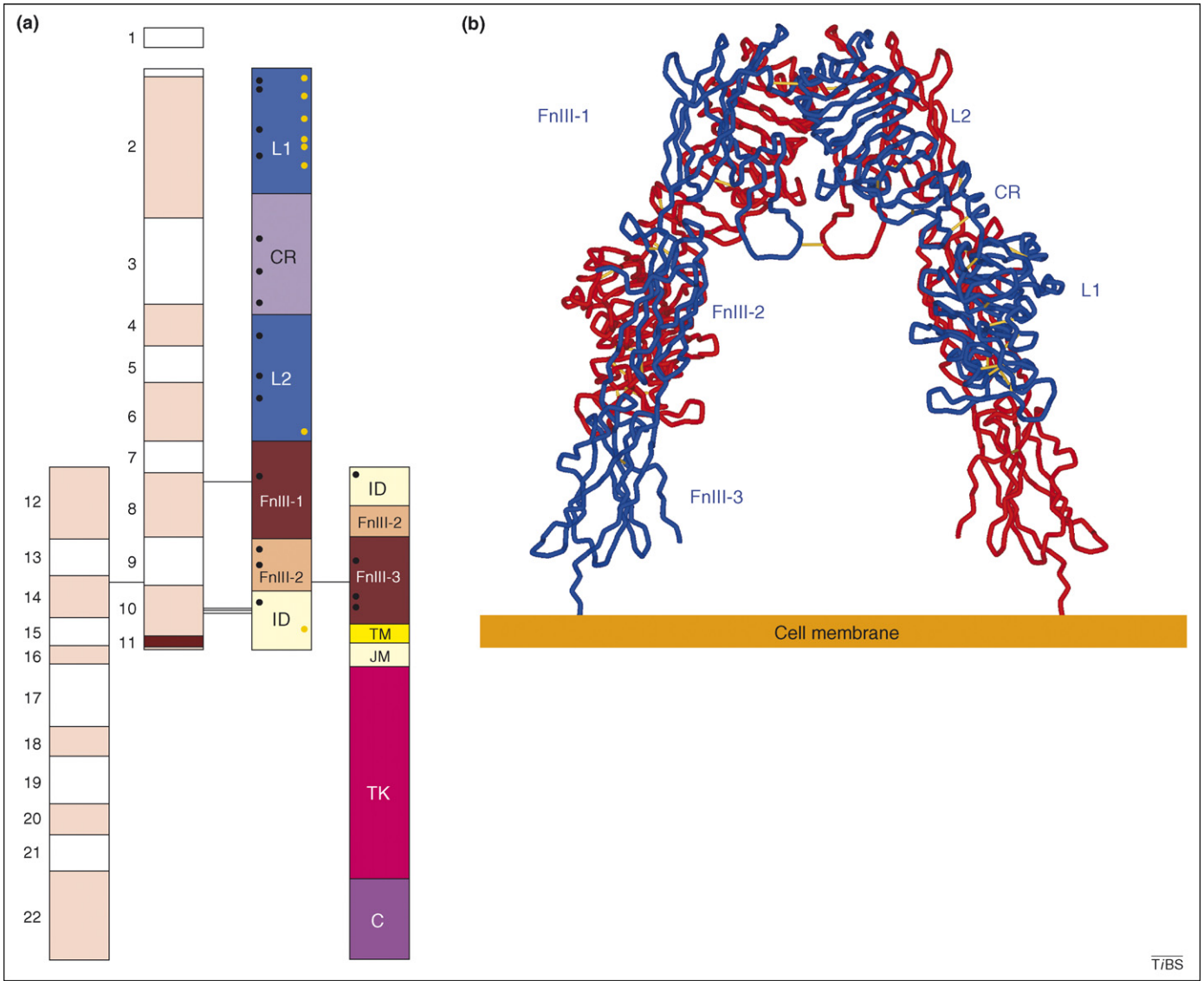


Figure 1. Comparison of the modular structure of the entire insulin receptor as predicted from the primary sequence (a), with the actual crystallographic structure of the receptor ectodomain (b) (PDB code: 2DTG) [29]. **(a)** Shows a schematic extended view of the $\alpha 2\beta 2$ structure of the receptor as predicted from primary cDNA and gene-sequence information. On the left half of the receptor, spans of the sequences encoded by the 22 exons of the IR gene are shown in alternating white and pink boxes. The alternatively spliced exon 11 is highlighted. On the right half, spans modules predicted from secondary-structure predictions. Module boundaries mostly correspond to exon boundaries. C, C-terminal tail; CR, cysteine-rich domain; FnIII-1, FnIII-2, FnIII-3: fibronectin type III domains; ID, insert domain in FnIII-2; JM, juxtamembrane domain; L1 and L2, large domains 1 and 2, containing leucine-rich repeats; TK, tyrosine kinase domain; TM, transmembrane domain. All modules are drawn to scale. Black dots: N-glycosylation sites. Yellow dots: ligand-binding 'hotspots' identified by single amino acid site-directed mutagenesis. The disulfide bridges are shown. See Ref. [4] and text of this review for more explanations. **(b)** Shows a side view of the structure of the IR ectodomain (tube model): one α subunit (blue) runs antiparallel to the second one (red) and shows an inverted-V folded-over conformation. The various modules are denoted for the blue subunit only.

resulting in isoform A (sequence absent) or B (sequence present). The presence of this sequence has minor consequences for insulin-binding affinity but results in enhanced affinity for IGFs for isoform A. The transmembrane helical domain of the β-subunit is followed by a juxtamembrane domain, the tyrosine kinase domain for which crystal structures exist in both the inactive and active state (reviewed in Ref. [9]), and a C-terminal region. The α-subunits are linked by disulfide bonds [10] at Cys524 in FnIII-1 and Cys682, Cys683 and Cys685 in the FnIII-2 insert domain. A single disulfide bond, Cys647–Cys860 (note, the numbering is based upon the IR–A nomenclature), links the α- and β-subunits.

The epidermal growth factor (EGF) receptor (EGFR) family members comprise four receptors in vertebrates – EGFR, ErbB2, ErbB3 and ErbB4 (reviewed in Refs [11,12]) – that, unlike the insulin and IGF-I receptors, are monomeric but form both homo- and heterodimers that are stabilized by a subset of a dozen potential ligands. The N-terminal portions of the members of the EGFR family have an arrangement of L1–CR–L2 domains (called domain I, II and III) similar to the IR and IGF–IR, but they are linked to the cell membrane by a second CR domain (domain IV) rather than three FnIII repeats.

Most RTKs, like the cytokine (haematopoietic) receptors for which liganded crystal structures have been determined (examples are shown in Figure 2), have been found to form 'face-to-face' symmetrical structures united by a monomeric or dimeric ligand sitting between the two receptor moieties, as exemplified by the growth-hormone-

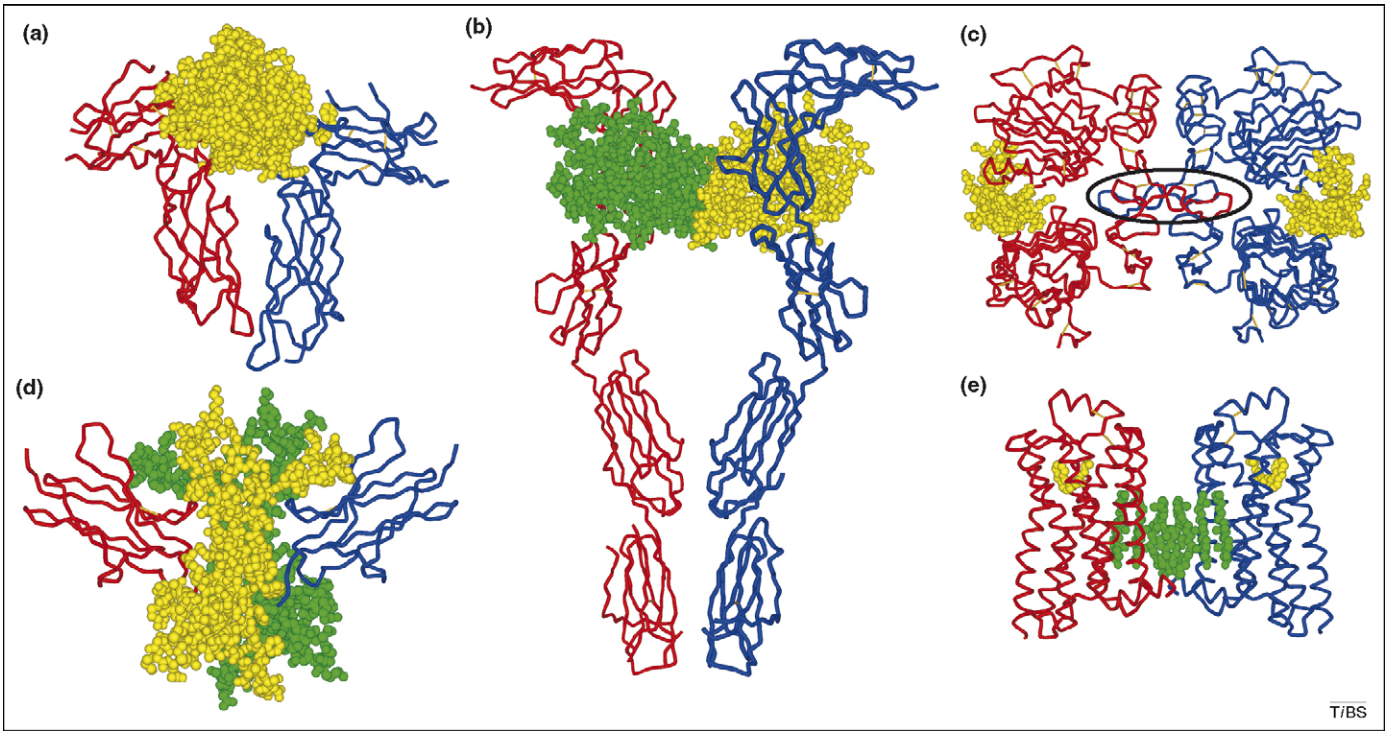


Figure 2. Examples of dimeric membrane-receptor complexes. A sample of the various architectural modes of dimerization found in the crystallographic structures of liganded extracellular domains of three classes of membrane receptors: cytokine receptors (a), RTKs (b–d) and GPCRs (e). Receptor structures are shown as tubes (one monomer red, one blue), and the ligands are shown as CPK spheres (which are yellow when monomeric and yellow and green when dimeric) **(a)**. Structure of human-growth hormone bound to two growth-hormone receptor ectodomains (PDB code: 3HHR) [81]. **(b)** Structure of a dimeric SCF bound to two KIT ectodomains (PDB code: 2E9W) [14]. **(c)** Structure of two monomeric EGFs bound to two EGF-receptor ectodomain molecules (PDB code: 1 IVO). The black ellipse denotes the contact zone between the two autoinhibitory hairpin loops of the EGFR [82]. **(d)** Structure of a dimeric NGF bound to two TrkA-receptor ligand-binding domains (PDB code: 1WWW) [83]. **(e)**. Structure of the β2-adrenergic receptor embedded in a lipid membrane (not shown) and bound to a diffusible ligand (yellow), with cholesterol and palmitic acid (green) between the two receptor molecules (PDB code: 2RH1) [71].

receptor complex [13] (Figure 2a) and the recent structure of the complex between the KIT RTK and stem-cell factor [14] (Figure 2b). The original paradigm, which was proposed first for the RTKs [2,3,9] and further validated for the cytokine-receptor family by the crystal structure of the growth-hormone-receptor complex [13], was that the ligand induced the dimerization of monomeric unliganded receptors. This concept was hugely influential in understanding the mechanism of activation of several classes of membrane receptors and for designing ligand agonists and antagonists [15]. More recently, however, this concept has been challenged, and in both growth-hormone and erythropoietin receptors [16], in addition to some RTKs such as the EGFR [17–19], it has been proposed that the ligand might instead stabilize a pre-existing equilibrium between monomers and dimers and induce conformational changes within the dimer.

The concept of a single ligand molecule crosslinking two receptor moieties with a 1:2 stoichiometry was the basis of models proposed in the 1990s to explain the complex kinetics of insulin and IGF-I-receptor binding (see later), in which a single ligand molecule crosslinked two different binding sites within the covalent receptor dimer [20,21]. I proposed that to explain the negative cooperativity observed in IR binding, the two receptor halves should display an antiparallel symmetry [20], as shown in Figure 3a. This was first supported by single-molecule images of Fab-decorated IR ectodomain molecules [22].

The determination of the crystal structure of the insulin and IGF-I receptors proved to be a formidable challenge. A partial structure of the IGF-I receptor L1–CR–L2 domain was determined ten years ago [23] and later modelled in combination with IGF-I [24]. More recently, the structure of the same fragment of the IR was solved [25]. These IR and IGF-receptor fragments did not bind the ligand in the absence of the aforementioned CT peptide [8].

A major step towards understanding the structural biology of this class of receptors was the elucidation in 2002 of the crystal structure of the EGFR in combination with EGF or transforming growth factor (TGF)-α by two different groups (reviewed in Refs [11,26,27]). A major surprise was that the EGFR molecules are arranged "back-to-back' in a 2:2 stoichiometric complex with the ligand molecules (Figure 2c), which are clamped between the L1 and L2 domains from the same EGFR molecule, and make contact with only one receptor moiety in the dimer on the outside face of the complex. The dimer interface is made of EGFR–EGFR contacts only. The contacts occur mostly through a hairpin loop (residues 242–259) in the CR1 domain that is tethered in the unliganded monomer but 'pops out' of the receptor structure upon ligand binding (plus additional contacts between the CR2 C-terminal modules).

If such a structure applied to the structurally related IR and IGF–IR, it would invalidate the proposed model for negative cooperativity [20] and suggest that a more clas-

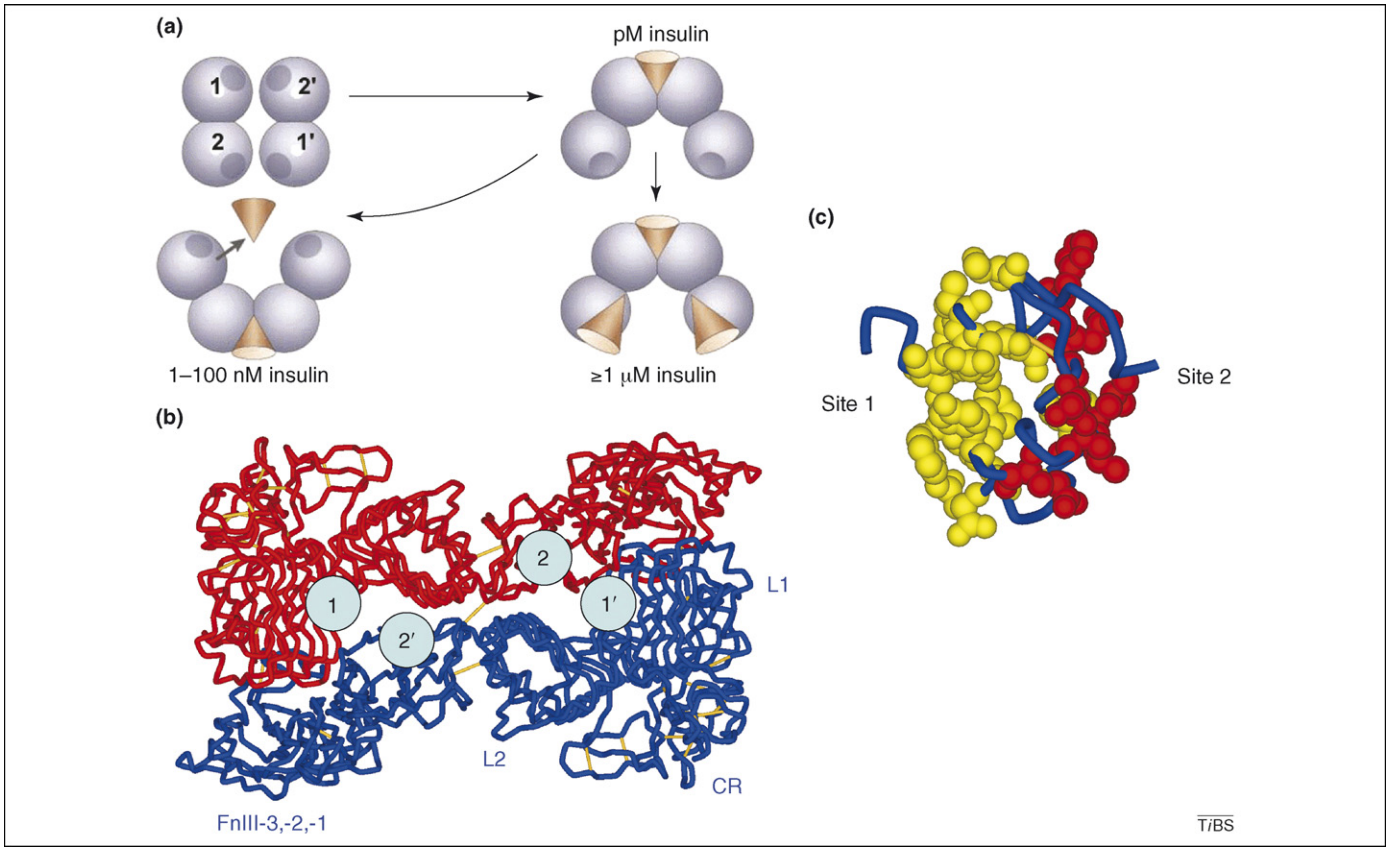


Figure 3. The alternative bivalent binding model of the IR interaction. I compare here my theoretical model of 1994 (a) with a more realistic model based on the actual receptor structure (b), and the mapping of the two insulin-binding sites by alanine scanning mutagenesis is shown in (c). **(a)** The De Meyts model for negative cooperativity [14], or 'alternative bivalent crosslinking model', which was proposed before the crystal structure shown in (b) was solved: the model depicts schematically the alternative crosslinking of two different receptor subsites (shown by the spheres) on each α subunit by the bivalent insulin monomer (shown by the cones). The receptor is viewed from the top (looking down towards the plasma membrane) and shows how the accelerated dissociation of the first bound insulin (to sites 1 and 2′) is generated when the second insulin crosslinks the alternative pair of receptor subsites (sites 2 and 1′). The key aspect of the model besides ligand bivalency is the antiparallel disposition of the pairs of receptor subsites (i.e. site 1 is opposite site 2′ and site 2 is opposite site 1′). Reproduced from Ref. [4]. **(b)** Top view of the IR structure shown in Figure 1b; the approximate location of the two ligand-binding sites is shown by blue circles. This is a structurally realistic depiction of the model in (b) and confirms the antiparallel disposition of the pairs of binding sites. **(c)** The structure of the receptor ligand, insulin, showing the two binding surfaces mapped by alanine-scanning mutagenesis [5] as CPK spheres. The 'classical binding surface' (known as site 1) is in yellow: Gly A1, Ile A2, Val A3, Glu A5, Thr A8, Tyr A19, Asn A21, Val B12, Tyr B16, Gly B23, Phe B24, Phe B25, Tyr B26. The 'novel' binding surface (known as site 2) is in red: Ser A12, Leu A13, Glu A17, His B10, Glu B13, Leu B17.

sical "half-of-the-sites reactivity' model might apply, as it does in the bacterial aspartate receptor [28].

In fact, the structural arrangement of the IR ectodomain, solved in 2006 at 3.8-Å resolution [29], proved to be different from that of the EGFR ectodomain (reviewed in Refs [26,30,31]) and is depicted in Figure 1b. The receptor ectodomain was not crystallized in the presence of insulin but, instead, was crystallized as a complex with four Fabs from monoclonal antibodies [32] (not shown in Figure 1b) and a fragment of an insulin mimetic peptide [33], which was not resolved in the structure. Each monomer of the IR adopts a folded-over conformation, making an 'inverted V' arrangement relative to the cell membrane (Figure 1b); one leg, made of the three FnIII domains, stems from the membrane (the α-subunit C-terminal portion being superimposed linearly on top of the extracellular part of the β-subunit), and the other leg is formed by the L1–CR–L2 domains. The two monomers are disposed in an antiparallel symmetry that is consistent with the model proposed previously [20] (Figure 3b). A crucial element of the binding mechanism is unfortunately not resolved because of a disordered structure – the insert domain (ID) in FnIII-2 that contains the α–β cleavage site in addition to the CT peptide that is part of binding-site 1 and the triplet of α–α disulfide bonds Cys682, Cys683 and Cys685. This structure is not consistent with that proposed from a 3D reconstruction from scanning electron microscopy, which showed a more 'collapsed', globular structure [34].

In the next section, I show that despite the above IR structure being unliganded, combining this structural information with a variety of different approaches (including ligand-binding kinetics, biochemical approaches, site-directed mutagenesis and photoaffinity crosslinking) over the past three decades to study the IR interactions has provided a credible and quite detailed model of the molecular mechanisms governing insulin binding and receptor activation.

Mechanistic aspects of IR-binding kinetics

The mechanism of insulin binding to the IR is complex, as was revealed in the early 1970s by curvilinear Scatchard plots (denoting the coexistence of high- and low-affinity binding sites) and ligand-induced acceleration of dissociation in an 'infinite' dilution, the hallmark of negative cooperativity (reviewed in Refs [4,5]). Similar properties have been shown for IGF-I binding to the IGF-I receptor

[35,36]. I proposed in 1994 [20] that this results from alternative bivalent crosslinking of two distinct binding sites on each α-subunit (Figure 3a). Insulin was proposed to contain two distinct binding surfaces, site 1 (also termed the 'classical binding surface') and site 2 (Figure 3c). Each monomer of the IR dimer was proposed to contain two distinct binding regions (site 1 and site 2 on one monomer and site 1′ and site 2′ on the other) [4,5]. The proposed antiparallel symmetry of the two pairs of receptor sites brings site 1 and 2′ and site 1′ and 2 in sufficient proximity for the low-affinity binding of one insulin molecule (e.g. to site 1), and then for the bound ligand to crosslink to site 2′, resulting in a high-affinity crosslinked complex (K_d ~0.2 nM) with slow dissociation. The receptor structure is postulated to enable only one such crosslink, at either pair of sites. Binding of a second insulin molecule to site 1′ (which results in curvilinearity of the Scatchard plot) will enable alternative crosslinking to site 2 upon partial dissociation of the first crosslink, resulting in destabilization of the first crosslink and accelerated dissociation of the first bound insulin molecule. At supraphysiological concentrations of insulin >100 nM, monovalent binding of two insulin molecules to sites 1′ and 2 will block the second crosslink and stabilize the first one, which explains the bell-shaped dose–response curve for negative cooperativity (Figure 3a, see also the enlightening structure-based model in Figure 3 of Ref. [30]). Bell-shaped curves are a hallmark of mechanisms that implicate bivalent crosslinking [37]. IGF-I binding to the IGF-I receptor has similar kinetic properties to insulin binding but does not show a bell-shaped curve for negative cooperativity [35], probably because the larger size of the ligand owing to the permanent C-domain does not allow two ligand molecules to coexist within the site 1 – site 2′ (or site 1′– site 2) interstice (or because of some steric hindrance due to differences in the local receptor structures).

Sites 1 and 2 have been precisely mapped on the surface of the insulin molecule (for details, see Figure 3c). Site 1 was inferred from consideration of conservation in the evolution of vertebrate insulin sequences, in addition to chemical or semi-enzymatic synthetic approaches in the early 1970s and, for that reason, is usually referred to as the 'classical binding surface' of insulin [38,39] (reviewed in Refs [5,40,41]). It largely overlaps with the insulin surface involved in dimerization. The involvement of these residues was confirmed by alanine-scanning mutagenesis of insulin [5,42] (Figure 3c). Alanine-scanning mutagenesis is a simple and widely used technique in determining the functional role of protein residues. Alanine substitution eliminates the side chain beyond the β carbon but does not usually alter the main-chain conformation [43]. The alanine-scanning mutagenesis of insulin using the high-affinity cell-bound receptor revealed a second binding surface that overlaps with the hexamerization surface of the molecule [5,44] (Figure 3c). A second binding surface was also mapped recently on the IGF-I molecule [44]. A variety of evidence indicates that insulin undergoes a change in conformation on binding to the IR [45].

Photoaffinity crosslinking studies, in addition to alanine-scanning mutagenesis of specific receptor-surface residues and binding studies using minimized receptor constructs, helped to identify the approximate receptor locations where the 'classic' site 1 and novel site 2 of insulin bind (reviewed in Refs [5,30,40,41]). In brief, site 1 of the IR has two components: the central β sheet of L1 [46] (Figure 3b) and the CT peptide from the ID [8,47–49]. Recent complementation experiments indicate that the CT peptide of one α subunit complements the site 1 of the other α subunit in the dimer, as previously predicted [26], rather than being a *cis*-interaction [50]. Although the FnIII-2 insert is essentially invisible in the IR structure, it probably corresponds to the tube of electron density seen

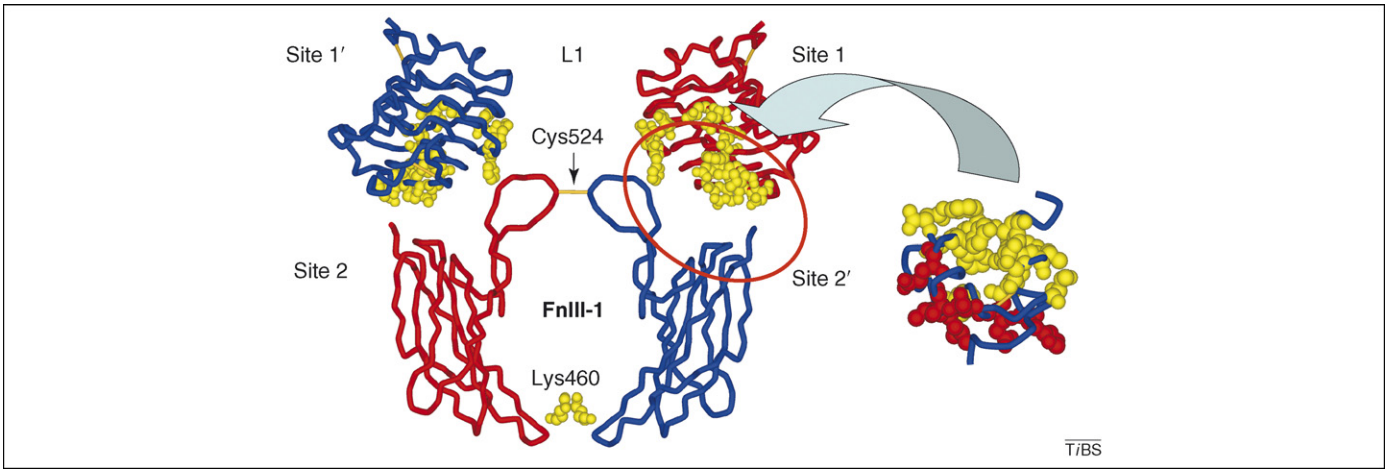


Figure 4. The essential architectural components of the allosteric mechanism of the IR. Here, the structural features that compose the core of the allosteric mechanism in the IR interaction are highlighted. The L1 modules (containing sites 1 or 1′) and the FnIII-1 modules (containing sites 2 or 2′) are shown in red for one α subunit and in blue for the second one (Figure 3b). The remainder of the α subunits are not shown, except for Cys524, which appears as a 'hinge' for the see-saw mechanism for the alternative crosslinking. An essential component of site 1, the CT peptide from the FnIII-2 ID, is missing because this domain was poorly resolved in the crystal structure. The residues from site 1 involved in insulin binding, as mapped by alanine-scanning mutagenesis, are shown as yellow CPK spheres. They are Asp12, Arg14, Asn15, Gln34, Leu36, Leu37, Phe64, Leu87, Phe89, Asn90, Tyr91, Glu97, Glu120 and Lys121 [46] (reviewed in Ref. [4]). The putative insulin-binding area between the two subsites is shown by the red ellipse (the insulin-molecule-binding surface, as shown in Figure 3c, is depicted in the orientation it is expected to bind at this site as a blue arrow). The residues from site 2 in FnIII-1 are unpublished at the time of writing. The two Lys460 from the L2 domains (which are otherwise not shown) located near the base of the FnIII-1 domains are shown as yellow CPK spheres. They seem to have an important role in modulating the cooperativity: mutation to arginine completely suppresses the negative cooperativity, whereas mutation to glutamic acid (as in the Arkansas leprechaun patient or in the *Drosophila melanogaster* IR) enhances it [84].

lying across the face of each L1 domain in the crystal structure [30] (see Figure 8 of Ref. [41]). In the IGF–IR, the L1 component of site 1 is extended to a part of the CR domain that binds the C-domain of IGF-I [30] (Figure 1 of Ref. [4]). Docked models in which the classical binding surface of insulin (site 1) and the corresponding surface of IGF-I bound to their receptor sites 1 have been proposed [24,25].

Site 2 of the IR (Figure 3b) and IGF–IR is currently less well defined, but there are strong indications from the IR structure and other photoaffinity crosslinking and biochemical evidence [51,52] (reviewed in Ref. [30]) that it involves the loops at the junction of FnIII-1 and FnIII-2.

Thus, despite the lack of a still much-needed structure of the complex between insulin and IGF-I with their receptors, the current structural and biochemical information has converged into a near-complete picture of the interactions of the ligand- and receptor-binding sites. Moreover, it provides a realistic, structure-based model for the architecture of the allosteric mechanism that explains negative cooperativity in the IR (Figure 4). It should be mentioned that, in addition to homotropic negative cooperativity, insulin binding to the IR exhibits a variety of heterotropic, positive, cooperative interactions with other ligands such as monoclonal antibodies, lectins and insulin-mimetic peptides ([33]).

It remains a matter of debate whether the negative cooperativity in insulin and IGF-I binding is a correlative mechanistic phenomenon that reflects the nature of the binding mechanism or whether it has a physiological meaning *per se* in regulating insulin actions. Clearly, the second phase of the bell-shaped curve for the dose–response curve lies well outside the physiological range of insulin concentrations, much as the self-antagonistic effects seen at high concentrations in the bell-shaped curves of growth-hormone effects *in vitro* (which also reflect a crosslinking binding mechanism) [53,54] are supraphysiological. However, the acceleration of dissociation, which limits the residence time of insulin on the receptor at higher ambiant concentrations, might have a role in limiting the mitogenic potency or other long-term actions, such as gene regulation, of insulin [54,55]. Metabolic responses such as glucose transport show a high degree of 'receptor spareness', meaning that the response is already maximal at fractional receptor occupancy. Therefore, the relevance of negative cooperativity to such responses is likely to be minimal.

Bivalent receptor crosslinking: implications for other RTKs and cytokine receptors

A comprehensive review of the RTK and cytokine receptor families is outside the scope of this brief article; instead, here, I wish to make the point that structural information does not automatically translate into a full understanding of receptor kinetics. Shortly after negative cooperativity was demonstrated in 1973 for the IR [56] by showing accelerated ^{125}I-ligand dissociation in the presence of unlabelled ligand in an 'infinite dilution', it was also shown for nerve growth factor (NGF) binding to the NGF receptor [57], long before structural information became available on the ligands and receptors. It is now known that the neurotrophins bind to two completely different cell-surface receptors – the Trk tyrosine kinase receptors (TrkA, TrkB and TrkC) and the shared p75 receptor [58]. The NGF dimer binds to a dimeric form of the TrkA receptor in a "face-to-face' bivalent mode (Figure 2d) but binds to p75 in a monomeric complex. It is still unclear how these binding mechanisms relate to the curvilinear Scatchard plots and complex dissociation kinetics in this system, and more work is clearly necessary here to reconcile structural and kinetic information, including mathematical modelling.

Likewise, EGFR binding displays curvilinear Scatchard plots that implicate the coexistence of high- and low-affinity sites, as well as ligand-accelerated dissociation [59,60]. How that relates to the different monomeric and dimeric complexes and how ligand acceleration is induced given the back-to-back crystal structure of the dimeric complexes remain unclear [61]. The analysis of EGFR binding to whole cells is complicated by extensive endocytosis and trafficking. Further work is needed here also to reconcile the structural and kinetic information. In this respect, two recent breakthroughs have occurred this year. Macdonald and Pike [60] showed that ^{125}I-EGF-binding isotherms (yielding curvilinear Scatchard plots) from cells expressing increasing levels of EGFR could be simultaneously fitted to a simple EGFR-aggregation model (monomer–dimer equilibrium). They also showed negative cooperativity in dissociation kinetics by using the 'infinite dilution' procedure (discussed earlier) but did not derive differential equations from their model to attempt to fit the kinetic data. In another important study, Webb *et al.* [62] used single-molecule imaging and fluorescence-lifetime imaging microscopy to investigate the *in situ* structures of high- and low-affinity EGFR in A431 cells. Their data strongly indicate that high-affinity EGFR ectodomains have the exclusive ability to interact head-to-head via asymmetric surfaces in tetramers with an antiparallel symmetry. It is tempting to speculate that this mechanism could be compatible with concepts developed for the IR to explain the negative cooperativity [20], which is not easily explicable in the context of the back-to-back crystal structure. A ligand-induced EGFR dimer–tetramer transition has also been inferred recently by a multidimensional microscopy analysis [63].

The signalling pathways of the EGF receptor have been extensively explored and modelled in systems biology approaches (reviewed in Ref. [64]); it would be nice to be able to integrate models of the initial receptor-binding kinetics in such a perspective.

Bivalent binding of growth hormone to the growth-hormone receptor, the prototype of the cytokine receptor family and an inspiration for our 1994 insulin-binding model [20], was shown in 1973 to exhibit linear Scatchard plots and no accelerated dissociation [56]. In contrast to the IR, it is essentially the same receptor site that binds both sites of the ligand (Figure 2a) and there is no alternative ligand binding to a second pair of sites, which is the mechanism that causes the accelerated dissociation in the IR system. However, it has been shown recently that the high- and low-affinity sites of the growth-hormone receptor are allosterically coupled [65].

Constitutive oligomerization and negative cooperativity are widespread properties of GPCRs

Soon after the demonstration in 1973 of negative cooperativity in IRs by using the 'infinite dilution' procedure [56], the same procedure showed accelerated dissociation in β-adrenergic receptors [66] and thyroid-stimulating hormone (TSH) receptors (for review, see Ref. [67]), both of which showed curvilinear Scatchard plots. The issue, however, became contentious, especially after these receptors were shown to be GPCRs. GPCRs were for a long time thought to be monomeric, which would not offer a mechanism for site–site interactions. Nevertheless, the infinite-dilution procedure subsequently also showed accelerated dissociation in muscarinic M2 receptors [68] and bradykinin B2 receptors [69]. The clues to a possible allosteric mechanism were provided by the pioneering work of Bouvier and colleagues, who demonstrated unequivocally with bioluminescence resonance energy transfer that the β2-adrenergic receptor is constitutively dimeric (reviewed in Ref. [70]). The recent high-resolution structure of the β-2 adrenergic receptor shows a crystal lattice of parallel dimers stabilized by lipids [71] (Figure 2e). It is unclear whether these relate to the physiological dimer. Constitutive dimerization or oligomerization of GPCRs recently became the dominant paradigm, and the allosteric properties of GPCRs now seem to be generally accepted [72,73]. A clear link between homo- and heterodimerization and negative cooperativity was shown recently for the glycoprotein-hormone receptors, including the TSH receptor [74] and chemokine receptors [75]. The same phenomenon has been shown this year for receptors of the relaxin-like family of peptides [76,77]. It has also been suggested that allosteric interactions might occur across GPCR dimers in receptor arrays [73]. Agnati and colleagues have championed the concept of higher-order oligomers (which they have termed 'receptor mosaics') [78] and have published numerous papers on this concept, but the concept of cooperative receptor clusters was, in fact, introduced much earlier by Alex Levitzki, a pioneer of negative cooperativity [79].

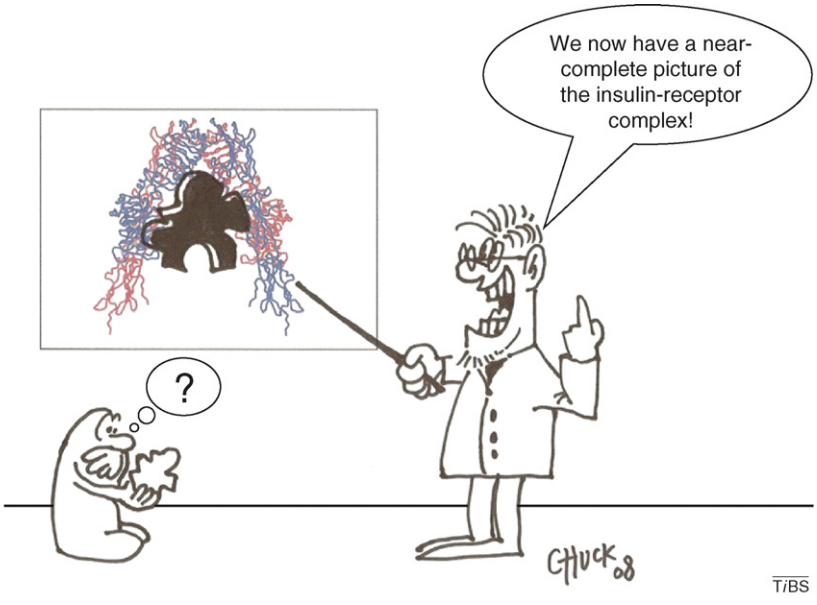
Concluding remarks and future perspectives

Constitutive or ligand-induced oligomerization associated with kinetic cooperativity or allosterism, for many years the apanage of the IR, might well be the rule rather than the exception among membrane receptors, as I predicted >30 years ago [67]. It is in that sense (and not as an 'architectural blueprint') that I present the IR in the title as a 'prototype' for other receptors with similar behaviour because it was the first shown to exhibit negative cooperativity [56] and the first suspected to be oligomeric [67]. It is clear, however, that the architectural modes of receptor oligomerization and activation are diverse and, in many cases, unique, as exemplified by the small sample shown in Figure 2.

The functional consequence of ligand activation of receptor dimers or oligomers is the activation of the initial steps in signal transduction. In the RTKs, the tyrosine kinase is usually activated by transphosphorylation of a kinase-activation loop, the EGFR being a notable exception (reviewed in Ref. [9]), whereas cytokine receptors recruit intracellular tyrosine kinases.

The physiological relevance of negative cooperativity is usually thought of as resulting from spreading the binding curve over a larger range of ligand concentrations – in effect, buffering the system against a rise in ligand concentration. However, the modulation of the dissociation rate might be crucial in a different way. It has been suggested that the residence time on the receptor might affect signalling pathways that differ between transient versus sustained occupancy [55]. Thus, insulin analogues with prolonged residence times have been shown to exhibit enhanced mitogenicity.

It is clear that, so long as the structure of a liganded IR has not been solved, our proposed binding mechanism remains, in part, speculative. Moreover, it is possible that the current unliganded structure does not represent the active conformation of the receptor. Obtaining such a structure, including the receptors of species other than human, should be pursued as a high priority.



However, targeting the receptor sites 1 and 2 described here with peptides obtained from phage libraries has resulted in high-affinity dimeric peptide ligands that exhibit either agonist or antagonist properties, which helps to validate the bivalent-binding concept [20] and reflects its usefulness.

A structure-based mathematical model that is able to mimic the complex binding kinetics of the IR and other RTKs and to provide robust estimates of kinetic parameters would also be very helpful. For many RTKs, the wealth of structural information has not really translated into a clear understanding of the kinetic-binding properties, and more detailed kinetic studies, in addition to novel biochemical approaches, are clearly warranted.

The pharmacological implications of allosterism in receptor binding for the design of allosteric modulators targeting RTKs, GPCRs and other receptors have been the focus of much recent research [72,73,80] and should yield new approaches to drug design and discovery.

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