How ligand binds to the type 1 insulin-like growth factor receptor

Yibin Xu1,2, Geoffrey K.-W. Kong1,8, John G. Menting1, Mai B. Margetts1, Carlie A. Delaine3, Lauren M. Jenkin1, Vladislav V. Kiselyov4, Pierre De Meyts5,6,7, Briony E. Forbes3 & Michael C. Lawrence1,2

Human type 1 insulin-like growth factor receptor is a homodimeric receptor tyrosine kinase that signals into pathways directing normal cellular growth, differentiation and proliferation, with aberrant signalling implicated in cancer. Insulin-like growth factor binding is understood to relax conformational restraints within the homodimer, initiating transphosphorylation of the tyrosine kinase domains. However, no three-dimensional structures exist for the receptor ectodomain to inform atomic-level understanding of these events. Here, we present crystal structures of the ectodomain in apo form and in complex with insulin-like growth factor I, the latter obtained by crystal soaking. These structures not only provide a wealth of detail of the growth factor interaction with the receptor’s primary ligand-binding site but also indicate that ligand binding separates receptor domains by a mechanism of induced fit. Our findings are of importance to the design of agents targeting IGF-1R and its partner protein, the human insulin receptor.
The human type 1 insulin-like growth factor receptor (IGF-1R) is a homodimeric, disulphide-linked (αβ)₂ receptor tyrosine kinase implicated in normal human growth and development. Aberrant IGF-1R signalling is implicated in cancer proliferation and metastasis and IGF-1R has undergone extensive investigation as an anti-cancer target. IGF-1R is closely related to the human insulin receptor (IR): their ectodomains share 53% sequence identity, their respective monomers can form functional heterodimers and both receptors can bind all three ligands within the family (insulin and the two insulin-like growth factors, IGF-I and IGF-II), albeit with varying affinities. The bioavailability, activity and tissue distribution of the IGFs are controlled by a suite of six insulin-like growth factor binding proteins, as well as (in the case of IGF-II) by the type 2 insulin-microreceptor (μIR). Little is known about how either IGFs or insulin interact with the secondary binding site (site 2) of their cognate receptor, an event deemed essential for receptor activation.

Fig. 1 Structural biology of apo IRΔβ and insulin-bound μIR and the current model of ligand binding kinetics. a The Λ-shaped assembly of IRΔβ (PDB entry 4ZXB). Domain colours are L1 light blue, CR red, L2 orange, FnIII-1 green, FnIII-2 yellow, FnIII-3 dark blue, ID light magenta, αCT magenta. The foreground monomer is in ribbon representation, the background monomer in surface representation (apart from the ID element); dashed lines indicate disordered residues within the respective ID segments. b Human insulin (A chain grey, B chain black) bound to μIR (PDB entry 4OGA), coloured as in a. c Major pathway of ligand binding to IR and IGF-1R within the current kinetic model. S1, S2: site 1 and site 2 on one receptor monomer; S1’, S2’: site 1 and site 2 on the opposing receptor monomer. Red filled circle: ligand (i.e., IGF-1, IGF-II or insulin). d Steric overlap (asterisked) between insulin and the opposing fibronectin domain module of the structures depicted in a and b based on overlay of their common domain L1. αCT’ is shown in both its apo conformation (thin magenta ribbon) and its insulin-complexed conformation (magenta ribbon) in order to illustrate its altered disposition upon insulin binding.
Incoming ligand engages first with site 1, and then forms a cross-link to site 2, located on the alternate monomer to which it contributes domain L1 to site 1 (Fig. 1c), with the resultant cross-link being of high affinity18. An immediate conundrum is that overlay of the above two IR–based structures indicates that insulin is sterically prevented from forming a site 1 complex with apo IRAβ without displacement of the receptor L1-CR module away from domains FnIII-1’ and FnIII-2’ (Fig. 1d), i.e., the apo receptor structure is in a “closed” conformation. Resolution then requires either that (i) ligand binding itself separates domain L1 from the opposing FnIII’ domain module, (ii) ligand binds to only a dynamically transient population of receptor conformations that already have these domains displaced from each other (i.e., “open” receptors) or (iii) the apo-IRAβ structure does not reflect the ectodomain structure within apo holo-receptor. Biochemical and biophysical analyses of IGF-1R indicate that the separation of domain L1 from the opposing FnIII’ domain modules is in fact integral to IGF-1R activation19. These analyses suggest further that, in the ligand-free state, the receptor transmembrane domains are held apart by a Λ-shaped assembly of the ectodomain, but ligand-induced separation of domain L1 from the FnIII’ domain module then releases the conformational constraint on the latter, allowing the attached transmembrane (TM) helices to interact and autophosphorylation to occur19. Equivalent data do not exist for IR; however, there is indication that for IR, receptor activation may instead involve the separation (rather than coming together) of the transmembrane helices within the homodimer.20.

To address these issues and gain understanding of the mechanism of ligand binding, we have determined crystal structures of apo- and IGF-1-bound forms of IGF-1RAβ, the latter intriguingly obtained via crystal soaking. IGF-1RAβ, like IRAβ, is an ectodomain-only construct that lacks the likely disordered and non-functional segment near the N terminus of the receptor β chain.21. These structures, refined using data to resolution of 3.0 Å and 3.27 Å, respectively, were both obtained as co-crystals with an antibody variable-domain module (Fv). Not only do our structures provide a wealth of atomic detail regarding IGF-1R and its interaction with IGF-I, but they also lead to new insights into the receptor activation mechanism, relevant to those seeking to design novel agents targeting IGF-1R and/or IR.

**Results**

**Characterization of IGF-1RAβ.** Labelled-ligand competition binding assays show that IGF-I and IGF-II bind IGF-1RAβ with half-maximal inhibitory concentration (IC₅₀) of 0.14 nM (0.12–0.17 nM) and 0.33 nM (0.23–0.48 nM), respectively, with the values in parentheses being the 95% confidence intervals (Supplementary Figure 1). These values align closely with those reported for IGFBPs (0.41 ± 0.1 nM and 0.88 ± 0.6 nM, respectively) in similar assays of an isolated IGF-1R ectodomain devoid of the “Δβ” modification, demonstrating that the modification does not affect ligand affinity.

**Structure of IGF-1RAβ + Fv 24–60.** The structure of the IGF-1R ectodomain was obtained by X-ray diffraction analysis of a crystal of IGF-1RAβ in complex with the Fv module of the monoclonal antibody (mAb) 24–60,22, the latter employed here as a crystallization chaperone.24. The crystal used displayed diffraction to a maximum resolution of ~3.0 Å (albeit anisotropically; see Methods and Table 1). Within the crystallographic unit cell, the IGF-1RAβ homodimer has twofold crystallographic symmetry and structure solution was by molecular replacement, employing as search objects the L1-CR and L2 fragments of IGF-1R (from the structure of the isolated L1-CR-L2 fragment of the receptor25) and homology models of Fv 24–60 and of IGF-1RAβ FnIII-1,2 and -3, the latter three based on their counterparts within IRAβ. The structure was refined using all data to a resolution of 3.0 Å; statistics are in Table 1 and representative difference electron density in Fig. 2a.

The quaternary structure of IGF-1RAβ (Fig. 3a) exhibits the same folded-over conformation as IRAβ, with the locations of secondary structural elements and domain boundaries being closely similar to those of IRAβ. No electron density is seen for Fv residues 642–690; these residues contain the inter-monomer disulphide bond motif at Cys669-Cys670-Ala-671-Cys672. Electron density is also poorly defined for residues 509–516 within domain FnIII-1, this loop contains the inter-monomer disulphide bond at residue Cys514. The equivalent disulphide bond regions are also poorly defined in the structure of IRAβ. In contrast, electron density for the α-chain to β-chain disulphide bond (linking Cys633 to Cys849) is well defined. N-linked glycan residues could be modelled convincingly at sites Asn21, Asn105, Asn304, Asn577, Asn610 and Asn883. Of the remaining potential N-linked sites apart from IGF-1RAβ, electron density features were seen extending from the respective side chains of Asn214, Asn284, Asn387, Asn408, Asn870 and (possibly) Asn592, but these were left unmodelled due to lack of adequate order. Some electron density was present in the vicinity of the side chain of Asn72—it is unknown whether this site is glycosylated in IGF-1R, though mass spectrometry has revealed that its counterpart

| **Table 1 X-ray diffraction data collection and refinement statistics**<sup>a</sup> |
|---------------------------------|---------------------------------|---------------------------------|
| apo IGF-1RAβ + Fv | IGF-1RAβ + Fv | apo IGF-1RAβ + Fv |
| 24-60 (PDB 5U8R) | 24–60 + IGF-I (PDB 5U8Q) | |
| **Data collection** | | |
| Space group | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> |
| Cell dimensions | | |
| a, b, c (Å) | 95.03, 201.73, 117.75 | 88.69, 197.66, 117.65 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 50.00–3.00 | 50–3.27 (3.46–3.27) |
| R<sub>rmerge</sub> | 0.25 (3.47) | 0.19 (2.07) |
| R<sub>fmean</sub> | 8.3 (9.6) | 9.1 (9.9) |
| Completeness (%) | 99.7 (99.5) | 98.6 (92.5) |
| Redundancy | 8.9 (7.2) | 7.2 (6.7) |
| R<sub>free</sub> = 0.256/0.285 | 22.18–3.27 |
| No. of reflections | 45,805 | 32,110 |
| No. of atoms | 123,112 | 148 |
| Protein | 8194 | 8537 |
| Carbohydrate | 112 | 148 |
| Solvent | 28 | 19 |
| B factors (Å<sup>2</sup>) | 134 | 177 |
| Protein | 146 | 146 |
| Carbohydrate | 125 | 178 |
| Solvent | 28 | 19 |
| R.m.s. deviations | 0.002 | 0.002 |
| Bond lengths (Å) | 0.48 | 0.52 |

<sup>a</sup> Each data set was collected from a single crystal
<sup>b</sup> Values in parentheses are for highest-resolution shell. The resolution limit was set at being the maximum at which the CC<sub>1/2</sub> statistic<sup>c</sup> remained significant at the P = 0.001 level of significance
<sup>c</sup> Maximum likelihood estimate of overall R<sub>free</sub> calculated using XTINiSAGE within the PHENIX suite
The designation of the αCT’ helix (residues 684–697) upon the surface of domain L1 is also closely similar to that within the structures of apo IRΔβ⁹ and apo µIR²⁷. The resolution of the current structure was sufficient to avoid ambiguities in the strand register within domains FnIII-1 and FnIII-2, an issue which bedevilled the original structure determination of IRΔβ⁸. We note further that, within the crystal lattice, substantial solvent volume exists in the vicinity of the first modelled residue (Glu744) of the β chain of IGF-1RΔβ, indicating that the observed structure is not in conflict with that which could be adopted by the intact IGF-1R ectodomain (i.e., one devoid of the “Δβ” mutation/deletion).

The crystallization chaperone, Fv 24–60, is seen attached to domain CR, consistent with the epitope reported for its parent mAb²³. Further detail of its epitope is provided below. Inspection of the unit cell reveals that Fv 24–60 mediates the majority of lattice contacts, consistent with its use to overcome the hindrance to crystallization posed by the N-linked glycans. The mAb 24–60 is reported to reduce by 90% the affinity of IGF-1 binding to a cell-bound receptor and to a lesser degree the affinity of binding to a soluble receptor²³. Here, the Fv module does not interact sterically with any receptor components beyond its epitope, suggesting that relative disposition of domains with the ectodomain has not been modulated by Fv attachment per se (see below for further discussion of the likely cause of ligand affinity reduction).

Despite the above similarities, two salient differences emerge between the structures of IGF-1RΔβ and IRΔβ. First, the sites of membrane entry (i.e., the respective C termini of domains FnIII-3 and FnIII-3’) are substantially closer together in IGF-1RΔβ (~67 Å) than in IRΔβ (~115 Å), i.e., the overall shape of IGF-1RΔβ is more “closed” (Fig. 3a) than that of IRΔβ (Fig. 1a). The altered spacing reflects cumulative differences in the relative orientations of consecutive domains within the receptor monomers, with the largest being a 26° difference between the two receptors in the relative orientation of the L1-CR module with respect to its downstream domain L2 (Fig. 3b). These intra-monomer differences in domain orientation accumulate to provide a 17° difference between the two receptors in the alignment of domain L1 of one monomer with respect to domain FnIII-2’ of the adjacent monomer (Fig. 3c).

The second difference lies in the IGF-1RΔβ αCT’ residues 698–704, which are located C terminal to the αCT’ helix (residues 684–696). These residues are well resolved (Fig. 2b) and pack

Fig. 2 Stereo view of representative αC-weighted (2F – F) difference electron density. a The αC-weighted (2F – F) difference electron density in the vicinity of IGF-1RΔβ L1 domain residues 28–34 within the crystal of apo IGF-1RΔβ + Fv 24–60. The density is sharpened (B_{sharp} = –60 Å²) and displayed at a contour level of 1.7 σ (σ = root-mean-square deviation of the sharpened map). Density is shown only for volume within 2.0 Å of the atoms displayed.

b The αC-weighted (2F – F) difference electron density in the vicinity of IGF-1RΔβ αCT residues 700–704 within the crystal of apo IGF-1RΔβ + Fv 24–60. The density is sharpened (B_{sharp} = –60 Å²) and displayed at a contour level of 0.33 σ (σ = root-mean-square deviation of the sharpened map). Density is shown only for volume within 2.5 Å of the atoms displayed.

c The αC-weighted (2F – F) difference electron density in the vicinity of IGF-1RΔβ αCT residues 700–704 within the crystal of apo IGF-1RΔβ + Fv 24–60. The density is sharpened (B_{sharp} = –60 Å²) and displayed at a contour level of 1.7 σ (where σ is the root-mean-square deviation of the sharpened map). Density is shown only for volume within 2.0 Å of the atoms displayed.
within domain FnIII-2 L1 and FnIII-2 only limited loss of diffraction resolution occurred (compared to where such a large moiety as IGF-I (molecular weight module of IGF-1R 10-12) the basis of corresponding residues within the L2 domain, showing the 26° correlation ( see Methods). Difference maps revealed IGF-I bound to the single site 1 within the asymmetric unit, in a fashion effectively identical to that seen in liganded-μIR structures 10,27, allowing its ready incorporation into the atomic model. Refinement statistics are in Table 1 and representative difference electron density in Fig. 2c.

Analysis of the structure reveals that IGF-I binding is accompanied by a separation of the IGF-1-bound L1-CR module away from domain FnIII-2' (Fig. 5a, b), this displacement being effected largely by a “hinge” motion close to the junction between domains CR and L2 (Fig. 5c). Conformational variation at this junction has been seen across extant structures of the IR ectodomain and its fragments 7,10,30. The site-1-bound IGF-I also interacts with domain FnIII-2', the interface involving residues Ile583, Ser788, Asn789 and Phe790 of the receptor and residues Asp53, Leu54 and Arg55 of IGF-I. This interface is remarkably sparse (Fig. 5d) and hence, in our judgment, does not likely reflect the site 2 interaction—indeed, of the IGF-I residues involved in its formation, only Leu54 is deemed on the basis of alanine scanning mutagenesis 15,16 to engage site 2. Whereas at physiological concentrations of ligand, only one IGF molecule is anticipated to be bound to the cell-surface expressed receptor 18, the presence here of two IGF-I molecules within the homodimer is likely a consequence of the supra-physiological concentration of IGF-I soaking and its subsequent binding within the crystal.

N-linked glycan could be convincingly modelled at sites Asn21, Asn105, Asn504, Asn577, Asn870 and Asn883, with weaker density suggestive of carbohydrate (and left unmodelled) seen extending from the side chains of sites Asn214, Asn284, Asn408, Asn592 and Asn610. No indication of glycan was apparent at site Asn72. None of the N-linked glycan (modelled or otherwise) appeared to be in the immediate vicinity of the bound IGF-I. Again, substantial solvent volume is apparent in the vicinity of the first modelled residue Tyr745 of the β chain of IGF-1R, indicating that the observed structure is not in conflict with that which could be adopted by an intact IGF-1R ectodomain devoid of the Δβ modification.

The mode of engagement of IGF-I with site 1. Conformational changes are seen in both the αCT' helix and IGF-I upon IGF-I binding to site 1 of IGF-1RΔ. These changes largely mimic those seen in the structure of IGF-I bound to the IR L1-CR+IGF-1R αCT hybrid-microreceptor complex 27 and in the structure of insulin bound to the μIR 10,11. In particular, they include remodelling of the αCT’ helix on the L1-β2 surface and a folding out of the C-terminal region of the B domain of IGF-I away from the hormone core in order to allow its engagement by key residues within the αCT’ helix.

Details are as follows. In the apo IGF-1RΔβ structure, the αCT’ helix spans residues 684–696 and engages (via the side chains of residues Tyr688, Phe692 and Phe695) a hydrophobic trough formed by the side chains of residues Leu32, Leu56, Phe58, Phe82, Tyr83, Val88 and Phe90 on the surface of L1-β2 (Fig. 6a). A potential salt bridge occurs between the side chains of αCT’ residue Glu685 and L1 residue Arg112. Residues 681–683 are in an extended conformation N terminal to the αCT’ helix, while C terminal to the helix, residues 697–704 order on the surface of the adjacent FnIII-2’ domain (see above). Upon IGF-I binding, αCT’ remodels, its helix now spanning residues 688–701, i.e., αCT’ unwinds by one turn at its N-terminal end and extends by one turn at its C-terminal end (Fig. 6b). Concomitantly, the helix re-orientates to lie approximately perpendicular to the direction of the

Structure of IGF-1RΔβ + Fv 24–60 + IGF-I. Crystals of the complex of IGF-I with the Fv-bound IGF-1RΔβ were obtained by soaking IGF-I directly into crystals of the receptor ectodomain/Fv complex. Incorporation by soaking of IGF-I into the apo crystals is remarkable, and is presumably facilitated by the crystal’s high solvent content (~75%). Soaking resulted in altered unit cell dimensions (Δa = 6.3 Å, Δb = 2.3 Å, Δc = 0.3 Å) without change in space group. We are not aware of any other instance where such a large moiety as IGF-I (molecular weight = 7.7 kDa) has been incorporated into crystals by soaking. Despite cracking, only limited loss of diffraction resolution occurred (compared to the resolution typical in our hands of the parent crystals), but anisotropy persisted. Diffraction data were processed to 3.27 Å resolution (Table 1). The structure was solved by molecular replacement, using the domains of the apo IGF-1RΔ + Fv 24–60 structure as search objects (see Methods). Difference maps revealed IGF-I bound to the single site 1 within the asymmetric unit, in a fashion effectively identical to that seen in liganded-μIR structures 10,27, allowing its ready incorporation into the atomic model. Refinement statistics are in Table 1 and representative difference electron density in Fig. 2c.
L1-B2 strands and to engage the hydrophobic L1-B2 surface via the side chains of residues Phe692, Phe695, Leu696, Ile700 and Phe701 (Fig. 6b).

IGF-I, upon engagement of IGF-1Rαβ site 1, is also seen to undergo a conformational change similar to that seen in the hybrid microreceptor complex of IGF-I with IR L1-CR + IGF-1R αCT (and analogously in the microreceptor complex of insulin with IR L1-CR + IGF-1R αCT). IGF-I residues Tyr24 and Phe25 are displaced from the core of the growth factor, with the side chain of IGF-I αCT residue Phe701 now locating into volume originally occupied by the side chain of IGF-I Phe25 (Fig. 6c). The side chain of IGF-I Phe23 undergoes rotameric re-arrangement to bury in a largely hydrophobic pocket formed by the side chains of domain L1 residues Asn11, Leu33, αCT’ residue Phe701 and IGF-I residues Leu14, Gln15 and Cys18 (Fig. 6c), as well as by the main chain atoms of IGF-I residue Tyr60. The side chain of IGF-I residue Tyr24 interacts with the side chains of αCT’ residues Val702 and Arg704 and with that of IGF-I residue Asn26. The side chain of IGF-I residue Phe25 stacks against those of domain L1 residues Asp8 and Arg10 and αCT’ residue Val702. (Fig. 6c).

No interpretable electron density is apparent for IGF-I B-domain residues 27–30 nor C-domain residues 31–38, the only C-domain residues in interpretable density thus being residues Pro39, Gln40 and Thr41. Of these latter residues, only Gln40 interacts here with the receptor, via αCT’ residues Phe695 and Ser699 (Fig. 6d). The absence of density for IGF-I residues 27–38 is important, as Tyr31, Arg36 and Arg37 have been shown by site-directed mutagenesis to be critical contacts for high-affinity IGF-I binding (reviewed in Denley et al.31). In particular, grafting the IGF-I C domain into the insulin molecule raises the affinity of insulin for IGF-1R to 19–28% of that of IGF-I32. The absence here of a visible interaction between elements of the C domain and IGF-1R may be caused by the attachment of Fv 24–60 (see below). While the absence of density for the IGF-I C domain does not formally resolve the issue as to whether or not the αCT peptide “threads” through the loop formed by the C domain and the helical core of IGF-I, residual electron density between IGF-I residues 26 and 39 in the vicinity of IGF-1R domain CR suggests that such threading occurs. Contacts between IGF-I and the site 1 elements of the receptor are summarized in Supplementary Table 1. Also included in Supplementary Table 1 are literature-derived data regarding the effect of mutations on IGF-I binding to IGF-1R—these data indicate that many of the site-1-engaging residues of IGF-I are critical to the interaction. In particular, IGF-I residue Val44 (Fig. 6d) is a critical site 1 contact; mutation of this residue to (the larger) methionine causes dwarfism31. Mutation to (the larger) leucine at the equivalent ValA3 position in insulin Wakayama causes diabetes33.

Inhibitory nature of mAb 24–60. The mAb 24–60 has been shown to inhibit IGF-I (but not IGF-II) binding to IGF-1R by up to 90%22,23. The Fv 24–60 epitope is seen here to comprise primarily the residue 254–265 loop of domain CR (Fig. 7a). This loop contains a number of acidic residues implicated in IGF affinity and selectivity, potentially through interaction with basic residues within the C domain of IGFs30,34,35. The residue 254–265 loop has an effectively identical conformation in the two
Mutational analysis of residues within FnIII-2'. Our structures suggest that a number of residues within domain FnIII-2' may play a role in receptor functioning. These residues include (a) His774, which interacts with αC' and FnIII-2' and FnIII-3', showing separation (asterisked) of the L1-CR module away from FnIII-2' and IDN'. (b) Overlay of one ‘leg’ of the IGF-1RΔβ homodimer in its IGF-1-bound form (coloured ribbon) onto the corresponding domains of the apo IGF-1RΔβ homodimer (white ribbon). Alignment is based on domains FnIII-2' and FnIII-3'. (c) Overlay (via L2) of the L1-CR-L2 module of IGF-1–bound IGF-1RΔβ (coloured ribbon) onto that of apo IGF-1RΔβ (white ribbon). Pro297, the hinge point, is in black and asterisked. (d) Interaction between the site-1-bound IGF-1 (A domain black, B domain white) and FnIII-2'.

Fig. 5 Mode of IGF-I binding to IGF-1RΔβ. a Bridge formed by IGF-I (black) between the site 1 components L1 and αC‘- and FnIII-2', showing separation (asterisked) of the L1-CR module away from FnIII-2' and IDN'. b Overlay of one ‘leg’ of the IGF-1RΔβ homodimer in its IGF-1-bound form (coloured ribbon) onto the corresponding domains of the apo IGF-1RΔβ homodimer (white ribbon). Alignment is based on domains FnIII-2' and FnIII-3'. c Overlay (via L2) of the L1-CR-L2 module of IGF-1–bound IGF-1RΔβ (coloured ribbon) onto that of apo IGF-1RΔβ (white ribbon). Pro297, the hinge point, is in black and asterisked. d Interaction between the site-1-bound IGF-1 (A domain black, B domain white) and FnIII-2'.
engagement of the two-chain insulin molecule. Nevertheless, the IGF-1 complexed structure demonstrates that the mode of IGF-I engagement with the site 1 components (i.e., domain L1 and αCT’) of the receptor is closely similar to that of insulin’s engagement with the corresponding components of IR10,11, again aligning with the ability of the receptors to bind each other’s ligand(s)37.

Together, the structures demonstrate that IGF-I binding obligates a separation of IGF-1R domain L1 away from the FnIII domain module of the adjacent monomer19. The manner in which this has occurred within the apo crystals raises the interesting issue as to what extent it reflects binding in vivo. The fact that binding can occur within the crystal suggests that the ligand binding process is one of induced fit that results not only in conformational change in the ligand and the receptor site 1 elements, but also in the concomitant separation of domain L1 from the adjacent FnIII’ domains. Such induced fit must by its nature involve initial (likely metastable) engagement of the IGF-1 with structural elements of the receptor ligand binding cavity, the latter being in the form visualized in the apo receptor structure.

The existence of such a ligand/receptor pre-complex that, upon relaxation, directs the receptor into an “open” conformation has not been considered prior in the literature. It is thus opportune to ask whether such binding is compatible with the extant kinetic data for the receptor. IGF-1R and IR exhibit complex kinetics, characterized by a curvilinear Scatchard plot and negative cooperativity. The latter is best exemplified by the accelerated dissociation of a pre-bound tracer (e.g., I125-labelled ligand) in the presence of unlabelled (“cold”) ligand under conditions of “infinite” dilution that preclude tracer rebinding38. These observations can be explained by the so-called harmonic oscillator (HO) model18, which assumes that the apo receptor exists in a continuum of energetic states that can be modelled as arising from harmonic oscillation of the receptor domains. The majority (~95%) of these conformations, under physiological conditions, are postulated to be “open”, i.e., all four sites (1, 1’, 2 and 2’) are exposed to incoming ligand (Fig. 1c). Within the HO model, conformational oscillation of the open receptor results either (i) in the presence of ligand, a ligand cross-link to site 2’ (Fig. 1c), or (ii) in the absence of ligand, a low level of constitutive activity.

**Fig. 6** Dissection of the interaction of IGF-I with binding site 1 of IGF-1R. **a** Conformation of L1-β2 (light blue) and αCT’ (magenta) in the apo IGF-1RΔβ structure, compared with **b**, its conformation in the IGF-I liganded IGF-1RΔβ structure. **c** Interaction between B domain of IGF-I (black) and the IGF-1R site 1 elements of L1-β2 (light blue) and αCT’ (magenta). The A domain of IGF-I (located in the foreground) is omitted for clarity. **d** Interaction between A domain of IGF-I (black) and the IGF-1R site 1 element αCT’ (magenta); no interaction is observed between the A domain and L1-β2 (light blue). The IGF-I B domain is in white.

**ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-03219-7**
However, while the HO model provides an accurate fit to the kinetic data, it cannot readily be mapped to the structures of apo IGF-1RΔβ or apo IRAβ, as in these structures, sites 1 and 1′ (and possibly also sites 2 and 2′) are partly occluded from the incoming ligand (Fig. 1d). Two resolutions are proposed. The first is to assume that in vivo the receptor oscillations are such that 95% of receptor conformations are “closed” (i.e., inactive), with the incoming ligand being able to access site 1 only within the 5% of receptor conformations that are “open” (i.e., the inverse conformation percentages to those in the HO model). The second resolution is that physiological ligand binding occurs by a process similar to that observed in the crystal, i.e., that ligand binds to the “closed” receptor by a process of induced fit that concomitantly results in separation of domains L1 and FnIII-2. These models are not mutually exclusive and both may occur under physiological conditions. We note that regardless of the mode of binding, no more than one mole equivalent of ligand is expected to bind to the receptor (αβ); homodimer at physiological ligand concentrations, given that both IGF-1R and IR display negative cooperativity.

However, the induced fit mechanism requires reformulation of the kinetic model. We have thus tested whether such reformulation agrees with the receptor binding and negative cooperativity data (see Methods). Inclusion of a doubly liganded, symmetrical receptor conformation under appropriate experimental conditions allows substantial simplification of the ligand binding scheme compared to that of the HO model (Fig. 8a, with detailed description provided in the Methods section). If rate constants are chosen to reflect a high-affinity site for ligand of $K_4 \approx 0.2$ nM and a low-affinity site of $K_4 \approx 6$ nM (i.e., approximately those values derived from the HO model analysis), with insulin having an additional binding site with a $K_4 \approx 1000$ nM, then simulation with these values is seen to yield good agreement with the experimental negative cooperativity data for both receptors (Fig. 8b).

We note that if physiological IGF-I binding to IGF-1R is indeed by a process of induced fit, then a corollary is that certain IGF-I residues may interact only transiently with receptor, i.e., during formation of the pre-complex, and it thus cannot be ruled out that some (even all) of the IGF-I residues currently understood to interact with site 2′ (i.e., IGF-I residues Glu9, Asp12, Phe16, Leu54 and Glu58)$^{15}$ may fall into this category. Such interactions would enhance the ligand on-rate to site 1 and contribute to high affinity.

In summary, our structure provides the first view of IGF-I in complex with site 1 of its receptor and the serendipitous way in which it was obtained by in situ crystal soaking has led us to propose a previously unconsidered mechanism of receptor activation. While the HO model remains a valid and general conceptual model (and was indeed the first concerted allosteric model able to incorporate negative cooperativity arising from ligand-stabilized asymmetry and bivalent cross-linking), its mathematical formalism has here been adapted and mapped onto the structural detail that has emerged for IGF-1R and for IR. The next challenge in the structural biology of this receptor family will be to understand the pathway by which the final ligand complex is formed and how it enables the intricate conformational change that directs the receptor to its final, activated state.

**Methods**

**Expression and purification of IGF-1RΔβ.** A CHO Lec8 cell line stably expressing IGF-1RΔβ (a construct of the human IGF-1R ectodomain comprising residues 1–903 but with the highly glycosylated segment (residues 718–741) near the N terminus of the β chain replaced by the quadraplet AGNN) was originally obtained from CSIRO (Parkville, Australia) by the corresponding author’s laboratory$^{25}$. Cells were thawed into Dulbecco’s modified Eagle’s medium F12 + GlutaMAX medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies) and expanded by passing several times in T150 tissue culture flasks at 37°C/5% CO₂. Cells from the T150 flasks were then used to seed 850 cm² roller bottles (Corning, Sigma-Aldrich); these were grown at 37°C for 21 days but in the absence of CO₂.

IGF-1RΔβ was recovered from harvested media by IGF-I affinity chromatography on a column of immobilized LON-13-IGF-I (GroPep Bioreagents; Australia) and then further purified by size-exclusion chromatography (SEC) as follows: the LON-R3-IGF-I affinity column was prepared by covalently binding 40 mg of media grade LON-R3-IGF-I (GroPep Bioreagents; Australia) to 50 ml Mini-Leak Medium Agarose (Kem-En-Tec; Denmark) as per the manufacturer’s instructions. Typically, 10 L conditioned medium containing IGF-1RΔβ was pumped through a 50 ml column of Sepharose CL-4B (Sigma-Aldrich) to remove non-specifically binding material and then a 50 ml affinity column at 4°C, followed by extensive column washing to remove unbound protein. Both columns were equilibrated with Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 8.0) containing 0.02% sodium azide (TBSA). The affinity column was eluted with 0.4 M NaCl, 0.2 M tri-sodium citrate adjusted to pH 5.0 with HCl and also containing 0.02% sodium azide. Eluate was collected into 3 M

![Fig. 7 Interaction of Fv 24–60 with IGF-1RΔβ. a Interaction of residues of domain CR of IGF-1RΔβ (red) with Fv 24–60 (variable heavy chain domain pink; variable light chain domain light grey). b Displacement by Fv 24–60 of the peptide loop formed by IGF-1RΔβ CR residues 254–265 towards the IGF-I binding site. The yellow ribbon is that of IGF-1R domain CR within the crystal structure of the isolated L1-CR-L2 fragment of IGF-1R obtained in the absence of attached Fv (PDB entry 1IGR25), overlaid onto that of the IGF-I-bound IGF-1RΔβ structure (red) on the basis of common domain L1. Red sphere: IGF-I Asn26; blue sphere: IGF-I Pro39. The connecting IGF-I domain C residues 27–38 (indicated putatively by a blue dashed line) are disordered.](image-url)
Fig. 8 Induced fit binding of ligand to IGF-1R and IR. a) Proposed kinetic scheme. S1, S2: site 1 and site 2 on one receptor monomer; S1', S2': site 1 and site 2 on the opposing receptor monomer. Black filled circle: hot ligand (i.e., IGF-I, IGF-II or insulin). Grey circle: cold ligand (i.e., IGF-I, IGF-II or insulin). $a_1$ and $d_1$: ligand association and dissociation rate constants for the high-affinity site. $a_2$ and $d_2$: ligand association and dissociation rate constants for doubly liganded, symmetrical receptor conformation. $a_3$ and $d_3$: association and dissociation rate constant for binding of a third insulin molecule (IR only, not applicable to IGF-1R). b) Plot for accelerated dissociation of a pre-bound tracer-labelled ligand by cold (unlabelled) ligand. The dissociation time was 20 min. The experimental data were as described previously (reproduced in Supplementary Table 4) and are shown as blue triangles for IGF-1 and red diamonds for insulin, with the fit of the induced-fit model to these data shown as lines (IGF-1 blue; insulin red).
were integrated and scaled using the XDS package42; statistics are presented in Table 1. The resolution limit was set at being the maximum at which the CC1/2 statistic is marginally significant at the F = 1 level. The structure of the ligand-receptor replacement, the diffraction data sets were further subjected to ellipsoidal truncation and anisotropic scaling using the Diffraction Anisotropy Server44,45. Structure determination and refinement. Molecular replacement (MR) structure solution of the apo form of IGR-1Δαβ in complex with Fv 24–60 was undertaken with Phaser46. Search objects for the L1-CR module and the L2 domain were obtained directly from the structure of the IGF1R L1-CR-L2 fragment (PDB entry 1GCR2); search objects for IGF-1R Fni1-L1 domain and the (Fnn3–II)–(Fnn3-II) module were generated from their counterparts in the IRα δ ectodomain structure (PDB entry 3LOH); whereas a search object for Fv 24–60 was generated from the structure of an anti-BcA scFv (PDB entry 3UMT; unpublished), modified to replacing all non-identical residues with serine using the FEAS03 server47,48. The MR search employed the anisotropy-corrected diffraction data set, as attempts using the complete data set failed. The initial model was refined against all data to 3.0 Å resolution using PHENIX49,50 (terated with manual rebuilding using COOT50). N-linked glycan residues were included where evident at Asn-X-Thr/Ser/ser sequences. Refinement included translation / libration / screw (TLS) parameters, using TLS groups assigned by PHENIX. Within the refinement, the relative weighting of the X-ray and stereochemistry terms and of the X-ray and atomic displacement parameter terms were determined using the “automatic” protocol with PHENIX, rather than the program default option. Ramachandran plot percentages are favourable 92.3, allowed 6.6, outliers 1.1, rotamer outlier percentage MolProbity51 all-atom clash score is 6.6. Final refinement statistics for both structures are in Table 1. Figures here and elsewhere were generated using Chimera52.

Structure solution for the IGF-1-bound crystals of IGF-1Δαβ – Fv 24–60 employed Phaser (again employing an anisotropy-corrected data set), searching with individual domains from the already refined Fv-complexed apo IGF-1Δαβ structure. Electron density for the IGF-I ligand was readily visible, bound to the L1 domain and αC helix in a fashion effectively identical to that seen in its complex with the human insulin receptor domain 1 plus IGF-1R αCt (PDB entry 4SSS53), allowing ready model building and structure refinement within PHENIX and COOT as above, using all data to 3.26 Å resolution. N-linked glycan residues were included where evident at Asn-X-Thr/ser sequences. Ramachandran plot percentages are favourable 92.2, allowed 7.3, outliers 0.5, rotamer outlier percentage MolProbity51 all-atom clash score is 6.3. Final refinement statistics for both structures are in Table 1. Comparison of IGR-1R and IR quaternary structure. The relative dispositions of domains within IGF-1R-Δαβ compared to those of their counterparts in IRαδ were computed using ProSMART53, using a fragment length of 15 residues. Mutant IGF-1R activation assays. Synthetic double-stranded complementary DNA (cDNA) fragments (Supplementary Table 3) incorporating either His774Ala, Ser878Ala, Asp879Ala, Phe879Ala or Phe879Ala/Pho879Ala (Integrated DNA Technologies) were cloned into an existing pcDNA3.1 plasmid incorporating the expression signal of IRαδ (DNase I-EndoH-sensitive plasmid) and subsequently transfected into Rβ cells (fibroblasts derived from IGF-1R knockout mice54, a gift from Dr Renato Baserga) and after 48 h cells expressing the IGF-1R mutants were stimulated with 100 nM IGF-I for 10 min. Cells were lysed in 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM MgCl2, 10% glycerol, 1% Triton X-100, 1 mM EGTA, pH 7.5 (lysis buffer) containing Complete protease inhibitor cocktail and PhosSTOP (Sigma-Aldrich), 2 mM Na3VO4, 100 mM NaF for 1 h at 4 °C; lysates were stored at −80 °C. Activation of the IGF-1R mutants was assessed by immunoblotting lysates with an antibody (APC–448–4069), Invitrogen; 1:2000, raised in rabbit) specific for phosphorylated Tyr1158/Tyr1162/Tyr1163 (in the IGF-1R kinase activation loop). Total receptor expression was measured by probing with an IGF-1R antibody (3027S, Cell Signaling; 1:10000 dilution, raised in rabbit) and an anti-tubulin antibody (Invitrogen; 1:10000 dilution, raised in mouse) was used as a loading control. Anti-rabbit IR Iy dye 680RD and anti-mouse IR Iy dye 800CW (Llicer; 1:50000 dilution) were used as secondary antibodies. Quantiﬁcation of the blots was achieved using the Image Studio Lite quantitation software (LI-COR Biosciences) as follows. First, the IGF-1R expression levels were normalized to the tubulin expression in each lane, as were the levels of Tyr1158/Tyr1162/Tyr1163 phosphorylation. The fold activation above basal was then calculated by comparing levels of Tyr1158/Tyr1162/Tyr1163 phosphorylation in non-stimulated and stimulated samples. Data represent n = 3 technical replicates and were analysed using one-way analysis of variance within Prism 7.0 (GraphPad).

Mutant IGF-1R affinity assays. After 72 h of transfection of WT and mutant IGF-1R into Rβ cells, cells were lysed with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM EGTA (pH 7.5)) for 1 h at 4 °C. Lysates were centrifuged for 10 min at 3500 rpm and then 100 μl was added per well to all a 96-well plate, and triplicates were fixed with anti-IGF-1R antibody 24–3153 and blocked with 0.5% bovine serum albumin in TBST (20 mM Tris, 150 mM NaCl, 0.05% v/v Tween-20). P6 cells (a gift from Dr Renato Baserga) were used as a positive control for over-expression of the wild-type IGF-1R55. Plates were incubated at 4 °C for 16 h. Horseradish-peroxidase labelled secondary antibody (goat anti-mouse IGF-1R (Groeppe Bioreagents; Australia) was prepared as instructed by the manufacturer (DELFIA Eu-labelling kit, Perkin Elmer). Approximately 3 x 10^6 fluorescent counts of europium-labelled IGF-1R were to each well along with IGF-1 competitor (0–300 nM) in triplicate and incubated for 16 h at 4 °C. Wells were washed three times with TBST, and the plate was treated by addition of DELFIA Eu-labelling solution. After 10 min, time-resolved fluorescence was measured using 340 nm excitation and 612 nm emission filters with a Victor X4 2030 Multilabel Reader (Perkin Elmer). IC50 values were calculated using Prism 7.0 (GraphPad) for curve-fitting of a one-site competition model.

Modelling negative cooperativity of IGF-1R and IR. The experimental data for negative cooperativity of IGF-1R and IR were those described previously38 and are provided for completeness here in Supplementary Table 4 by permission of Professor Pierre De Meyts. As indicated in the Discussion, the initial binding of ligand to the “closed” form of receptor can be explained by an induced fit model or by transient receptor opening, the latter effectively being described by the HO model38,59 upon reversal of the percentage of times that the receptor spends in its respective open and closed conformations. The exact nature of this binding event is not important for the modelling presented here, as its sequential components can be grouped into a single reaction (Supplementary Figure 5a) that represents high-affinity receptor binding (and receptor activation). Binding of a second ligand would then lead to either an asymmetric or symmetric receptor conformation. Both cases need to be considered. (i) In case of an asymmetric conformation, the second ligand hypothetically binds to a partially open site 1 of the alternate pair of binding sites, without it engaging site 2 (Supplementary Figure 5b). This interaction is expected to have a lower affinity to that of an interaction with two high-affinity sites, contradicting binding data that demonstrate that there is only one high-affinity site per holo-receptor38,59, unlike the soluble IR ectodomain that has two equal lower-affinity sites67. Indeed, it is plausible that symmetrical opening of the receptor domains to accommodate two ligands requires distortion of the receptor structure in energetically costly fashion that reduces ligand affinity. The binding of a third insulin molecule is proposed to account for the ascending phase of accelerated dissociation for IR59. IGF-I lacks this part of the curve and thus, for simplicity, binding of the third ligand will be considered only in the case of insulin binding to IR. Additional separation (“opening”) of the receptor domains may be required to accommodate the third ligand (Supplementary Figure 5c), presumably via an energetically unfavourable process that results in very low affinity for that ligand. It is proposed that binding of the third ligand “locks” the tracer in the bound state in the experiment for accelerated dissociation, and tracer dissociation can only occur after the cold ligand dissociates67. Taking into account the acceleration of the bound binding revealed in the model, we propose a two-state model of doubly liganded, symmetrical receptor conformation leads to a compact binding scheme of the ligand–receptor interaction (Supplementary Figure 5e). It should be noted that this binding scheme is applicable only to the experimental conditions described above. For example, receptor intermediaries with two or three hot ligand molecules bound were excluded from the reaction scheme, since such intermediates would not be formed in any significant quantities at 10 pM ligand concentration. Similarly, intermediates with only cold ligand molecules bound were eliminated due to tracer pre-binding. Endocytosis is, however, included, as even though the binding data were derived from experiments performed at 16 °C, endocytosis at this temperature is known to be minimally excluded67, as within the HO framework, that upon activation of inactive receptor intermediary, Rfree, the active intermediaries such as Rfree, Rfree-Rfree or Rfree-Rfree (see Supplementary Figure 5e) are internalized with an internalization rate constant kfree-on. Upon internalization, it is assumed that ligand dissociates instantly which leads to accumulation of hot ligand, Ligand, and internalized receptor, Rfree, inside the cells. The internalized receptor, Rfree, is recycled back to the plasma membrane with an exocytosis rate constant, krec. The internalized ligand, Lig, is recycled out of cells (either intact or degraded) with an exocytosis rate constant, krec. The binding of two species of ligand (hot and cold) in the presence of endo- and exocytosis and under conditions of no ligand depletion can be described by a system of ordinary differential equations shown in Supplementary Figure 6. The rate constants for endocytosis and exocytosis in IM9 cells were taken from the HO model38. The initial values for α and d (high affinity site), αΔ and dΔ (low affinity symmetrical conformation) and αΔ and dΔ (describing binding of the third insulin molecule) were also taken from the HO model38 and were modified to achieve a new experimental data for accelerated dissociation at 20 min while keeping the high-affinity site constrained to Kass = 0.12 mM for IGF-1 and Kass = 0.2 mM for insulin and the low-affinity site to Kass = 4.3 mM in case of...
of IGF-I and $K_d = 6$ nM in case of insulin. Simulations were performed using Mathematica v11.0 (Wolfram). The optimized parameter values are shown in Supplementary Table S5. No attempt was made to obtain a best fit of parameters or to establish if the identified parameter set is unique; nevertheless, the identified set of parameters leads to good agreement with experimental data (Fig. 8b).

Data availability. The coordinates of the structures determined here and their associated structure factors have been deposited in the Protein Data Bank (accession codes 5URJ and 5URQ). Other data are available from the corresponding author upon reasonable request.

Received: 11 October 2017 Accepted: 29 January 2018
Published online: 26 February 2018

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Acknowledgements

This article is dedicated to Dr Colin Ward, our colleague who initiated the study but sadly passed away before its conclusion. The work was supported by grants APP1128456 and APP1128553 to M.C.L. and APP1069328 to B.E.F. from the Australian National Health and Medical Research Council (NHMRC) and made possible at WEHI through Victorian State Government Operational Infrastructure Support and the Australian NHMRC Independent Research Institutes Infrastructure Support Scheme. Diffraction data were collected at the Australian Synchrotron beamline MX2 and crystallization screening was conducted at the CSIRO Collaborative Crystallization Centre (Parkville, Australia). We thank Professor Kenneth Siddle (University of Cambridge) for providing the hybridoma cell lines from which the sequences of mAb 24–60 were determined. Figure 1a is adapted from Fig. 1e of ref. 8 (copyright 2016) with permission from Elsevier.

Author contributions

Y.X. and G.K.-W.K. performed crystallographic experiments; Y.X., J.G.M., M.B.M. and L. M.I. produced and purified protein; C.A.D. performed experiments; B.E.F. contributed to experimental design; Y.X. and M.C.L. analysed crystallographic data; V.V.K. undertook the kinetic analysis; B.E.F., V.V.K., P.D.M. and M.C.L. wrote the manuscript. All authors commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-03219-7.

Competing interests: Part of the activity of M.C.L.’s laboratory is supported by Sanofi (Germany); V.V.K. is an employee of Eli Lilly and Company and P.D.M. is the Director of De Meyts R&D Consulting SPRLU and an external consultant to Novo Nordisk A/S. The remaining authors declare no competing financial interests.

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Author/s:
Xu, Y; Kong, GK-W; Menting, JG; Margetts, MB; Delaine, CA; Jenkin, LM; Kiselyov, VV; De Meyts, P; Forbes, BE; Lawrence, MC

Title:
How ligand binds to the type 1 insulin-like growth factor receptor

Date:
2018-02-26

Citation:
Xu, Y., Kong, G. K. -W., Menting, J. G., Margetts, M. B., Delaine, C. A., Jenkin, L. M., Kiselyov, V. V., De Meyts, P., Forbes, B. E. & Lawrence, M. C. (2018). How ligand binds to the type 1 insulin-like growth factor receptor. NATURE COMMUNICATIONS, 9 (1), https://doi.org/10.1038/s41467-018-03219-7.

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