The signalling conformation of the insulin receptor ectodomain

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Understanding the structural biology of the insulin receptor and how it signals is of key importance in the development of insulin analogs to treat diabetes. We report here a cryo-electron microscopy structure of a single insulin bound to a physiologically relevant, high-affinity version of the receptor ectodomain, the latter generated through attachment of C-terminal leucine zipper elements to overcome the conformational flexibility associated with ectodomain truncation. The resolution of the cryo-electron microscopy maps is 3.2 Å in the insulin-binding region and 4.2 Å in the membrane-proximal region. The structure reveals how the membrane proximal domains of the receptor come together to effect signalling and how insulin’s negative cooperativity of binding likely arises. Our structure further provides insight into the high affinity of certain super-mitogenic insulins. Together, these findings provide a new platform for insulin analog investigation and design.
The human insulin receptor is a homodimeric, disulphide-linked (αβ)₂ receptor tyrosine kinase. Despite the significance of the receptor's signaling in a number of major disease states, a complete, atomic-level understanding of the way in which insulin binds to the receptor and effects signal transduction has proved elusive. An obstacle is that the isolated, soluble receptor ectodomain (sIR), the entity most amenable to structural biology investigation, lacks the high affinity and the negative cooperativity of insulin binding that is characteristic of the holo-receptor (hIR). In particular, sIR binds two insulin molecules with equal nanomolar affinity with no negative cooperativity, whereas hIR binds one insulin molecule with picomolar affinity and displays negative cooperativity between its binding sites. A three-dimensional (3D) crystal structure of insulin-free (i.e., apo) sIR has been determined to 3.3 Å resolution, as well as single-particle cryoEM 3D structures of sIR in complex, respectively, with two insulin molecules (4.3 Å resolution; denoted here as “sIR + 2”) and with one insulin molecule (7.4 Å resolution; denoted here as “sIR + 1”). However, within both the latter cryoEM structures, the use of sIR precluded determination of how the high affinity and negative cooperativity of insulin binding are afforded to hIR. Furthermore, domains FnIII-2’, FnIII-3’, and FnIII-3’ are severely disordered (and hence unmodelled) within sIR + 1 and sIR + 2, precluding interpretation of the mechanism of transmembrane signal transduction. (receptor domain nomenclature is defined in Fig. 1a). By contrast with sIR, the only extant views of the apo- and insulin-bound hIR are at low resolution, being obtained by negative-stain transmission electron microscopy and two-dimensional (2D) class averaging of nanodisc-embedded hIR, without 3D reconstruction.

To overcome the above difficulties and to obtain a high-resolution 3D image of the receptor in its signaling-active conformation, we devised a construct termed “IRΔβ-zip” that consists of the receptor ectodomain followed by a 33-residue GCN4 leucine zipper segment at the C terminus of the β chain. The ectodomain component of IRΔβ-zip is based on the construct IRΔβ that was employed in the crystal structure determination of sIR, and lacks a short, highly glycosylated segment near the N terminus of the native β chain. Critically, attachment of the GCN4 zipper segment restores high-affinity insulin binding to IRΔβ (as has been shown for its attachment to sIR).

Subsequent single-particle cryoEM analysis of insulin-bound IRΔβ-zip reveals a structure that has insulin bound in a fashion similar to that within sIR + 1, but within which all six FnIII domains are resolved. The overall resolution of the structure (3.2 Å in the insulin-binding region and 4.2 Å in the membrane-proximal region) is significantly higher than both that of sIR + 2 and sIR + 1. Strikingly, the C-terminal FnIII-3’ domains are seen to be brought into association—an event that in the context of hIR will have the capacity to effect trans-phosphorylation of the intracellular tyrosine kinase domains.

Single-particle cryoEM analysis of IRΔβ-zip. IRΔβ-zipInsFv was analysed by single-particle cryo-EM and the resulting density map was used to build an atomic model of the entire complex (Supplementary Figs 2 and 3 and Supplementary Table 2). The resolution of the initial map was sufficient to resolve the receptor domains (Fig. 1c, d). Only a single insulin was seen attached to the complex, consistent with IRΔβ-zip mimicking the negative cooperativity of hIR, i.e. binding of one insulin molecule to IRΔβ-zip markedly decreases its affinity for a second insulin. Improved maps were then obtained by focused refinement of “upper” and “lower” volumes of the complex, the upper volume encompassing domains L1, CR, L2, FnIII-1, L2’, FnIII-1’, αCT, and insulin and the lower volume domains FnIII-2, FnIII-3, L1’, CR’, FnIII-2’, and ‘3’, with the polypeptide without the ‘ symbol being that which contributes domain L1 to the insulin-binding site. Resolution of the resultant upper and lower maps was assessed as 3.2 Å and 4.2 Å, respectively, using the gold-standard Fourier shell correlation coefficient (FSC; Supplementary Fig. 3 and Supplementary Table 2). Representative volumes of the maps are given in Fig. 1e–h. The angular particle distributions are very similar for both maps (Supplementary Fig. 3), exhibiting some preferential orientations but, most importantly, exhibiting no “missing view” areas on the Euler sphere. In our judgment, the resolution discrepancy between the “upper” map and the “lower” map likely results from flexibility of the FnIII-2 and FnIII-3 domains within the “lower” part of the structure. Density corresponding to the Fv modules was apparent adjoining the known epitope on domains CR (Supplementary Fig. 4), but their associated volumes were excluded from the focused refinement as they showed signs of disorder. No density was apparent at any stage that could be attributed to the leucine zipper.

Conformational change with respect to apo-receptor. Significant conformational changes are seen in IRΔβ-zipInsFv with respect to apo sIR. Strikingly, the fibronectin domain modules, i.e., (FnIII-1-2-3) and (FnIII-1’-2’-3’) are no longer in a Λ-shaped arrangement, instead, they are folded inwards in a pincer-like fashion that brings domains FnIII-3 and FnIII-3’ into contact (Fig. 1c, d and Fig. 2a–f). The association is effectively symmetric and occurs between the canonical C and ‘C’ strands and EF loop of FnIII-3’ and their counterparts in FnIII-3’, involving residues Tyr849, Arg851, Asp854, Glu855, Glu856, Leu857, Leu859, Arg875, and Ser878 of each domain (Fig. 2f). Critically, we note that the distance between the respective most C-terminally resolved residues of domains FnIII-3 and FnIII-3’ (i.e., Asp907 and Asp907’) is ~15 Å (Fig. 2e), well short of the maximum (~70 Å) that would be permitted for the total of eighteen residues that connect these termini to the N termini of the leucine zipper segments of IRΔβ-zip. The FnIII-3:FnIII-3’ association is thus not a hard constraint of zipper attachment. Indeed, a transition upon insulin binding in hIR from the open Λ-shaped structure of apo sIR to one in which the FnIII-domain modules have been brought together has been recently imaged at low (negative-stain electron-microscopy) resolution. The bringing together of the FnIII-3 domains by insulin binding seen here is thus proposed to be the key event that concomitantly brings together the intracellular tyrosine kinase domains, to permit trans-phosphorylation and initiation of the signaling cascade. The insulin-complexed L1-CR-L2 module itself is folded out of the L2-[FnIII-1:]-L2’-[FnIII-1’] module of the receptor (Fig. 1c, d and Fig. 2c, d), re-positioning the insulin-bound L1 + αCT’ tandem element in such away that insulin makes contact with residues that lie within the canonical (and membrane-distal) BC and C’E loops of domain FnIII-1’ (Fig. 3a). While such re-positioning is similar to that seen in the cryoEM structures
sIR + 2 and sIR + I7 (Fig. 4a), the higher resolution of our structure clarifies detail of the hormone’s interaction with FnIII-1’ (Fig. 3a). In particular, an interaction is discerned between Arg539’ and insulin residue HisB10, with the side chain of Arg539’ stabilized by interactions with that of Trp493 and Phe497’. Mutation of insulin HisB10 to acidic residues is known to result in insulins with slower off-rates and mitogenic signaling properties16,17. A reduced off-rate would be consistent with the formation of a salt bridge between the mutant Asp/GluB10 and Arg539’. The insulin-bound aCT’ helix is extended (with respect to its structure in apo sIR) in an N-terminal direction back to residue Asp689’, with the polypeptide being traceable back to the triple-Cys motif at residues 682/683/685’ (Fig. 3b). In the cryoEM structure sIR + 2’, the helix is also N-terminally extended, but only reportedly back to residue Ile692, suggesting that here it is in a yet more stable conformation.

The (insulin-free) domain L1’ is observed in our structure to retain an apo-like association with domain FnIII-2 (Fig. 2c, d). By contrast, domain L1’ in the 7.4 Å structure of sIR + 1 is located in the vicinity of, though not interacting with, domain FnIII-1’ (Fig. 4b). Furthermore, domain L1’ has here, on the surface of its central β-sheet, a large density feature (Fig. 3c) which we interpret as likely arising from the aCT segment seen in that location in the crystal structure of apo IRAβ. The density is, however, poorly resolved and its longer axis is offset somewhat with respect to that of the aCT helix in apo IRAβ. Also apparent in the vicinity is a central β-sheet, a large density feature (Fig. 3c) which we interpret as likely arising from the aCT segment seen in that location in the crystal structure of apo IRAβ. The density is, however, poorly resolved and its longer axis is offset somewhat with respect to that of the aCT helix in apo IRAβ.

Several density features are apparent in the vicinity of the pseudo-two-fold axis that relates the pair of FnIII-2:FnIII-3 modules (Fig. 3d). These features likely arise inter alia from the components of α- and α’-chain insert domains that connect the

**Fig. 1** Insulin receptor structural biology. a Receptor domain layout5. L1 and L2: first and second leucine-rich repeat domains, CR: cysteine-rich domain, FnIII-1, -2 and -3: first-, second and third fibronectin Type-III domains, ID: insert domain, asterisk: α-chain C-terminal region (αCT), TM: trans- and juxta-membrane domains, TK: tyrosine kinase domain, C-tail: β-chain C-terminal segment. Black lines: inter-chain disulphide bonds; Δβ: location of the glycosylated segment mutated/deleted in IRΔβ5. Domains within the second αβ-polypeptide are distinguished by a prime (‘) symbol. b Competition-binding curve for insulin binding to IR, IC50 values are identical at the 95% degree of confidence based on an F test (P = 0.171; no. of degrees of freedom = 80). c, d Orthogonal views of cryoEM structure of IRΔβ–zipInsFv. Circle: location of insulin. Fv modules are not shown but are attached to the respective CR domains as in prior X-ray crystal structures5,38 (see Supplementary Figure 4). e–h Sharpened density derived from the focused maps, covering (respectively) segments of the insulin B chain, domain L1 (insulin-bound), domain L1’ (insulin-free), and the dimeric FnIII-3:FnIII-3’ assembly.

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**Image 1** CryoEM structure of sIR + 1 reveals an apo-like association of domain L1’ with domain FnIII-2. This structure is compared to that for hIR-A immuno-captured from solubilized cell membranes (IC50 covering (respectively) segments of the insulin B chain, domain L1 (insulin-bound), domain L1’ (insulin-free), and the dimeric FnIII-3:FnIII-3’ assembly.

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**Image 2** Orthogonal views of cryoEM structure of IRΔβ–zipInsFv. Circle: location of insulin. Fv modules are not shown but are attached to the respective CR domains as in prior X-ray crystal structures5,38 (see Supplementary Figure 4).
Fig. 2 Comparison of the cryo-EM structure of IRΔβ-zipInsFv with the crystal structure of apo IRΔβ. a Domain arrangement in apo IRΔβ (left) with domains colored as in Fig. 1a. b Apo IRΔβ but with only domains L1, CR, L2, L1′, CR′, and αCT′ shown in color (for comparison with panel (d)). c Domain arrangement in IRΔβ-zipInsFv with domains colored as in Fig. 1a. d IRΔβ-zipInsFv but with only domains L1, CR, L2, L1′, CR′, and αCT′ shown in color in order to highlight the rearrangement of the insulin-bound (L1-CR-L2) + αCT′ module with respect to apo IRΔβ (panel (b)). e Comparison of the relative disposition of domains FnIII and FnII′ in apo IRΔβ (grey) and in IRΔβ-zipInsFv (colored). f Pseudo-two-fold-symmetric interaction of domains FnII′-3 and FnII′-3 within IRΔβ-zipInsFv. For clarity, only one member of each pseudo-symmetry-related residue pair is labeled. Green arrows in all panels indicate the direction of membrane entry. Panel (a) is adapted from Fig. 1a of Xu et al.22

Discussion
The structure presented provides insight not only into the signaling conformation of the receptor ectodomain but also into the nature of insulin’s second site of interaction on the receptor surface, seen here as involving domain FnIII-1′. It is tempting to map insulin’s interaction with domain FnIII-1′ (Fig. 3a) directly to that which affords hIR higher insulin affinity than sIR, as it has always been postulated that it is the presence of such an interaction that affords hIR’s higher insulin affinity20. However, the insulin interaction seen here with domain FnIII-1′ is also present in structures sIR + 1 and sIR + 2 (or to at least the degree discernible at the disparate spatial resolution of the three structures)7, but sIR itself does not display high-affinity (i.e., sub-nM) insulin binding3,7. The source of sIR’s lower insulin affinity is thus not obvious, but it may arise from the relatively large separation of the FnIII domains ‘legs’ of apo sIR. Within apo sIR, the distance between the FnIII-2 and FnIII-2′ domains would regulate the distance between the αCT and αCT′ segments, as the latter are linked via disulfide bond(s) between the Cys682/Cys683/Cys685 triplets. We thus posit that there may be

αCT and αCT′ segments to the respective upstream residues Cys647 and Cys647′. Residues Cys647 and Cys647′ and their respective disulfide-bond connections to β-chain residues Cys860 and Cys860′ are relatively well resolved in the density maps. Within the axial density features also lie the inter-α-chain disulfide bond(s)18 at residues Cys682/Cys683/Cys685 and Cys682′/Cys683′/Cys685′; however, these latter disulfide bond(s) and their interconnection cannot be unequivocally resolved. The Fv 83-7 epitope (on domain CR) is distal to the insulin-binding sites both in the current and all prior structures of this receptor ectodomain5,7 and the Fv 83-7 itself is not here in proximity to any domain-domain interface within the insulin-bound IRΔβ-zip. The location of this moiety is such that there is no prima facie way in which it likely could have restricted or directed the conformational change associated with insulin binding, compatible with experiments that show that mAb 83-7 itself has no significant effect on insulin binding to hIR19. We conclude the structure presented here is most likely effectively identical to that which would be observed without Fv attachment.
insufficient freedom in the disulfide-linked αCT segments of sIR to allow these segments to adopt conformations consistent with high-affinity insulin complexes at either or both the respective L1 + αCT and L1′ + αCT sites, yielding instead lower insulin affinity and an associated linear Scatchard plot. Within apo IRΔβ-zip, zipper tethering results in a smaller separation of the FnIII-domain modules, allowing a single insulin bound to L1 + αCT sufficient conformational freedom to form a high-affinity complex. To test this hypothesis, we constructed a mutant sIR with four glycines inserted between Pro686 and Lys687 (location is indicated in Fig. 3b) and found that the mutant sIR (“s686G4”) bound insulin with significantly higher affinity (i.e., sub-nM) than sIR (see Methods and Fig. 5a). The affinity of hIR itself was not altered by the insertion of the four glycines (Fig. 5b). The increased affinity of s686G4 supports our hypothesis that adequate αCT conformational flexibility is necessary for high-affinity binding. Further (though indirect) support arises from the fact that the isolated ectodomain (sIGF-1R) of the holo type 1 insulin-like growth factor receptor (holo IGF-1R, a close homolog of the insulin receptor) binds insulin-like growth factors with sub-nM, holo IGF-1R-like affinity, aligning with the observed smaller separation of the FnIII-domain modules within the isolated IGF-1R ectodomain.

The above reasoning and observations may also be used to inform the source of the long-known negative cooperativity of insulin binding to hIR and (as observed here) to IRΔβ-zip. Negative cooperativity in the current context implies that when one insulin molecule binds to domains L1, CR, FnIII-1′, and αCT′ as observed here, a second insulin molecule is prevented from binding, at least not with high affinity, to domains L1′, CR′, FnIII-1′, and αCT. Our above study of the s686G4 mutant indicates that a possible source of such restriction is the disulfide linkage between the Cys682′-Cys683′-Cys685′ triplet18. Similarly, we note that, unlike that in sIR + 1, it appears here that the unliganded binding site in IRΔβ-zipInsFv is maintained in near apo-like conformation, with αCT′ located upon the L1′ domain surface and domain L1′ in contact with FnIII-2. In the presence of excess insulin, insulin could bind at this second (apo-like) site but in so doing likely perturb the already insulin-bound site via the disulfide coupling of their respective αCT elements. Such binding would thus lead to accelerated dissociation of the already bound insulin, compatible
with the classical observations demonstrating the negative cooperativity of insulin binding to hIR.

The second receptor-binding surface of insulin has been associated with the set of insulin residues SerA8, SerA12, LeuA13, GluA17, HisB10, GluB13, and LeuB17 that overlaps the hexamerization surface of insulin.20,23 Of these residues, only HisB10 (and possibly GluB13) are observed here—and in structure sIR+2—to interact with FnIII-1’, with insulin residues SerA12, LeuA13, GluA17, and LeuB17 being distal to that interaction and solvent exposed (Fig. 6). The role (if any) of SerA12, LeuA13, GluA17, and LeuB17 in receptor engagement thus lies elsewhere. We speculate that these residues may be involved in the initial docking of insulin to the receptor, an event postulated to precede the energetic relaxation of insulin.

Assay of insulin binding to hIR-A and IRΔβ-zip. As a source of hIR-A for binding measurements (hIR-A = full-length construct of the insulin receptor isoform A), a Flip-In CHO cell line (ThermoFisher Scientific; catalog no. R758-07) was transfected with a pcDNA5/FRT/TODest Plasmid (ThermoFisher Scientific) harboring the IR-A nucleotide sequence according to the manufacturer’s instructions. IR-A expressing cells were selected and maintained in medium containing hygromycin-B (300 µg/mL). Cells were regularly demonstrated to be free of mycoplasma contamination using a kit (Lonza, MycoAlert) exploiting with high selectivity and sensitivity mycoplasmal enzymes in the cell-culture supernatants.

Methods

Cloning and production of IRΔβ-zip. A gene encoding IRΔβ-zip (residues 1-916 of the insulin receptor IRΔ construct1 followed at its C terminus by the 33-residue GCN4 zipper sequence RMEKQLEDKVELLKIQNEVLKKLQVGER25 and inclusive here of the population variants Tyr144His, Ile421Thr, and Gln465Lys26,27) was synthesized by ATUM (USA) and then cloned into the pEE14 mammalian expression vector (Lonza) for stable expression of the protein in CHO Lec8 cells. Briefly, cells were transfected with complexes of plasmid DNA and X-tremeGENE 9 transfection agent (Roche) and then selected with 25 µM HEPES-NaOH, 100 mM NaCl, 10 mM MgSO4, 0.025% (v/v) Tween-20 and adjusted to pH 7.8, and incubated for 30 min. After a single wash, a solution of solubilized hIR-A or IRΔβ-zip (provided as conditioned media from the initial cell culture, see above) was added and then incubated for further 60 min, followed by a single washing step. Subsequently, 100 µL re-suspended IR-antibody-SPA beads (containing 2–10 µg total insulin receptor) was mixed in a 96-well plate with 50 µL [125I]-labeled insulin tracer (100 pM, [125I]-(TyrA14)-insulin, Perkin Elmer) and 1 mM HEPES-NaOH, 100 mM NaCl, 10 mM MgSO4, 0.025% (v/v) Tween-20 at 1000 nM). After incubation for 12 h at room temperature, radioactivity bound to the beads was measured in a microplate scintillation counter (Wallac Microbeta). Binding data were fitted using a nonlinear regression algorithm in GraphPad Prism7.0 (GraphPad Software Inc.).

Affinity purification of insulin-bound IRΔβ-zip. For use in IRΔβ-zip purification, bis-BOC-insulin (BBI) affinity resin3 was prepared as follows. 1 gram batches of bovine insulin (blns; Sigma catalog no. I5500) were individually suspended in 20 mL dimethyl sulfoxide (DMSO) and 1 mL triethylamine then added to raise the pH in order to dissolve the blns. 74 mg of BOC (di-tert-butyl dicarbonate) dissolved in 1 mL DMSO was then added to the bIns, upon which the solution clarified. The mixture was incubated for 30 min on a rotor mixer at room temperature and the reaction then was stopped by addition of 50 µL ethanolamine. The modified blns was collected by precipitation after addition of several drops of concentrated HCI until a precipitate formed. After incubation for 12 h at room temperature, radioactivity bound to the beads was measured in a microplate scintillation counter (Wallac Microbeta). Binding data were fitted using a nonlinear regression algorithm in GraphPad Prism7.0 (GraphPad Software Inc.).

Fig. 4 Comparison of the cryo-EM structure of IRΔβ-zipInsFv with the insulin-complexed cryo-EM structures sIR+2 and sIR+1. a Schematic illustrating the similar environments of insulin, based on an overlay of the common L1-CR modules of IRΔβ-zipInsFv and sIR+2 (shown as surface for IRΔβ-zipInsFv and omitted for sIR+2). Remaining domains are shown as colored coil for IRΔβ-zipInsFv and black coil for sIR+1. b Schematic illustrating the altered disposition of the respective unliganded L1-CR modules in IRΔβ-zipInsFv and in sIR+1. Overlay is based on common FnIII-1/FnIII-1’ modules. Domains are shown as colored coil for IRΔβ-zipInsFv and black coil for sIR+1.
**Fast Flow (GE Healthcare Life Sciences) and equilibrated with 1.5 M acetic acid, 7 M urea, pH adjusted to 3.0 with HCl (buffer A). BOC-modified bIns pellets were dissolved in 50 mL buffer A and after loading onto the column in several portions, variously-BOC-modified bIns fractions were eluted with gradients of buffer A to buffer B (1.5 M acetic acid, 7 M urea, 0.5 M NaCl, pH adjusted to 3.0 with HCl) as follows. The column was pre-loaded with 0.25 column volumes (CVs) of buffer A followed by injection of 9 mL BOC-modified bIns. The column was then washed with 1 CV of buffer A, followed by gradients of 0 to 10% buffer B (0.25 CV), 10 to 25% B (4 CV), and 25 to 100% B (0.2 CV). The volume of the peak fractions putatively containing BBI (as opposed to mono- and tris-BOC bIns; putative peak identification kindly provided by Mr John Bentley (CSIRO; Parkville, Australia)29, see below for final mass spectrometry verification) was then reduced by pooling and binding again to the column and eluting as follows. Approximately 3 L of fractions containing BBI were diluted 1:1 v/v with water, loaded in portions on the column and re-eluted with a single gradient of 200 mL 0 to 100% buffer B. Approximately 370 mL of combined BBI fractions were dialysed in two 40 cm long 54 mm diameter 3.5 kDa cut-off dialysis bags against 10 L pure water with more than twenty changes. BBI precipitated by dialysis was collected by centrifugation. Sedimented BBI pellets were dissolved in minimum volumes of 0.1% TFA/80% acetonitrile or 10 mM HCl and were freeze-dried. Total dry weight was 1.3 gm. N-terminal sequence analysis (Australian Proteome Analysis Facility; Sydney, Australia) revealed a major signal for the B-chain N terminus and a minor signal (13%) for the A-chain N terminus indicating that most of the A-chain N-terminal residues in the sample were BOC modified, as required28. Finally matrix-assisted laser desorption ionization time-of-flight mass spectrometry confirmed that most of the sample was of mass corresponding to BBI, with only small quantities of mono- and tris-BOC bIns present. Two 50 mL batches of Mini Leak Medium activated resin (divinyl-sulfone-activated agarose; Kem-En-Tec, Denmark) were then washed extensively on a sintered glass funnel with water and then with 100 mM sodium bicarbonate-sodium hydroxide, pH 8.5. The resin was transferred to a Schott bottle containing 60 mL of 100 mM sodium bicarbonate-sodium hydroxide, pH 8.5. One gram of BBI dissolved in DMSO was dropped slowly with frequent mixing into the resin slurry in an ice-water bath. The combined resin and protein mix was rocked in the Schott bottle at 32 °C for 6 days. Unreacted divinyl-sulfone-reactive groups were reacted with 200 mM ethanolamine-HCl (pH 9.0), after removal of BBI solution from settled resin. The resin was washed with 50% DMSO, then water and finally with tri-buffered saline plus azide (TBSA; 24.8 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl and 0.02% sodium azide) and finally stored in 20% ethanol for future use.

**Cloning and expression of Fv 83-7.** Fv 83-7 was prepared using a *Brevibacillus* expression system using protocols identical to those described previously23. Briefly,
codon- and expression-optimised DNA corresponding to murine monoclonal antibodies 9E10 and M2, respectively, was used to inoculate 2 mL of 2SY broth containing 10 μg/mL neomycin sulfate (Sigma-Aldrich, USA) (2SYnm), followed by incubation overnight at 30 °C at 120 rpm. A volume of 0.2 mL of these respective cultures were then used to inoculate a further 20 mL of 2SYnm broth and, once sufficiently grown, 5 mL of this inoculum was used to inoculate a further 83 mL of 2SY broth in TBSA buffer. The sample was then incubated at 37 °C for 4 h and the reaction stopped by the addition of 1 mM phenylmethylsulfonyl fluoride and by incubation on ice. The sample was then purified by means of a Superdex 75 column (GE Healthcare Life Sciences, USA). SEC and SDS-PAGE estimates of molecular weight agreed with the expected masses.

Assembly and purification of Fv 83-7. A volume of 700 mL of conditioned medium containing the c-myc-tagged 83-7 VH domain was combined with 850 mL of conditioned medium containing FLAG-tagged 83-7 VL domain and incubated for 30 min at room temperature, followed by addition of 3 M Tris HCl (pH 8.5) at the ratio of 5 mL of combined media. This process was repeated to give a slight excess of VL monomers in the VL/VH mixture. The pH-adjusted combined media would then be run through a 9E10 Mini-Lect high affinity column (Kem-En-Tec, Denmark)30 and the desired Fv 83-7 eluted with c-myc peptide (decameric form) prepared in Tris-buffered saline plus azide (24.8 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 μM KCl plus 0.02% NaN3; 'TSBA'). Fractions were combined with one tablet of Complete protease inhibitor cocktail (Roche, Switzerland). Fractions from Superdex 200 10/300 SEC (GE Healthcare Life Sciences) examined by SDS-PAGE showed the presence of two bands of molecular weight 14 and 16 kDa, respectively, indicating a correctly-formed Fv eluting at 22 kDa. The c-myc tag was then removed from the 83-7 VH domain of the Fv 83-7 as follows. The Fv 83-7 was used to inoculate a further 83 mL of 2SY broth in TBSA buffer. The sample was incubated at 37 °C for 4 h and the reaction stopped by the addition of 1 mM phenylmethylsulfonyl fluoride and by incubation on ice. The sample was then re-purified by means of a Superdex 75 column (GE Healthcare Life Sciences, USA). SEC and SDS-PAGE estimates of molecular weight agreed with the expected masses.

Production of hIR-A and sIR-A 686G4 mutants. Holo-receptor (hIR-A, residues 1-1434) and ectodomain (sIR-A, residues 1-917) sequences of the A receptor. The degree of deglycosylation was assessed by SDS-PAGE analysis. The IR-A-zipFv sample was then centrifuged and run on a Superdex 2000 10/300 SEC column (GE Healthcare Life Sciences, USA) in TBSA buffer.

Single-particle cryo-electron microscopy. 3.6 μL aliquots of the IR-A-zipFv protein complex diluted to 340 nM in HEPES buffer (pH 7.5) were applied to holey carbon grids (C-flat 300 mesh R2/2; Protochips). glow discharged in a Polco EasyGlow device. Grids were blotted and flash-frozen in liquid ethane using a Vitrobot mark IV (ThermoFisher) with a blotting time of 1 s at 10 °C and 100% humidity. Data acquisition was performed on a Titan Krios microscope (ThermoFisher) operated at 300 kV, through a Gatan Quantum 967 LS energy filter using a 0.5 e−/Å2 dose rate. The data set was recorded using K2-Summit direct electron detector operated in super-resolution mode, at a calibrated magnification of ×130000 (resulting in a super-resolution pixel size of 0.52 Å on the object scale) with a defocus range of 1.2–2.5 μm. The movies were recorded in 20 frames for the 16 sec exposures with a dose rate of 2.5 e−/physicalpix/s, accumulating a total dose of 37 e−/Å2 at the sample level. Data collection was performed fully automatically on a single grid during a 48 h session using SerialEM32. After visual inspection of the micrographs, 2287 images were selected for further processing. The movie frames were aligned and dose-compensated with MotionCor233 using patch-based alignment (5 × 5) and Fourier space cropping (by a factor 2), resulting in a pixel size of 1.04 Å. Contrast transfer function parameters for the micrographs were estimated using Gctf34. Particles were picked automatically using RELION35, resulting in 747,074 raw particles. All 2D- and 3D classifications and refinements were performed using RELION35–37. After one round of 2D classification, 306,594 particles remained in the data set, in which only classes displaying “clean” complexes and high signal-to-noise ratio were selected. This particle data set was then submitted to 3D classification and sorted into eight classes, using as an initial model the symmetrized domains L1, CR, and L2 bound to Fv 83-7 from the crystal structure of the human insulin receptor ectodomain (PDB: 4ZXB)38 low-pass filtered to 60 Å. Two of the classes, representing 213,867 particles (42% of the data set) showed clear features of “closed” FnII domains and were used for further 3D refinement. The resulting map allowed ready identification of the constituent domains of IR-A-zipFv. The “part” of the map (encompassing domains L1, CR, L2, FnII-1, L2’, FnIII-1’, αCT, and insulin) was further refined with a focused 3D refinement step using a soft mask encompassing this volume, excluding the “lower” part (encompassing domains FnII-2, FnIII-1’, L1’, L2 and FnIII-2) and the pellets were counted in a gamma-counter. For competition experiments, the concentration of receptor was adjusted to yield less than 20% binding when no

Real-space refinement. Domains were identified by visual inspection of the cryo-EM map. Model-building was initiated by rigid-body docking of the individual crystal-structure-derived domains of IR-A (obtained from “Model S1” within ref 16, an extended version of Protein Data Bank entry 4ZXB). The hIR-A domain was used as a reference for the IR-A-zipFv. The “part” of the map was submitted to real-space refinement using the gold-standard Fourier shell correlation (FSC) 0.143 criterion39 and was corrected for the effects of a soft mask on the FSC curve by using high-resolution noise substitution40. The final density map was corrected for the modulation transfer function of the detector and sharpened by applying a negative 6 Å defocus that was previously determined41. The “lower” part of the map showed clear signs of heterogeneity, especially in the FnII domains conformation. A subsequent, focused 3D classification step was performed after partial signal subtraction of the “upper” part42. Two of the classes, representing 98,481 particles, displayed a “closed” and homogeneous state of the FnII domains and were used for further focused 3D refinement using a soft mask encompassing the “lower” part, excluding the “upper” part and Fv modules, leading to an overall resolution of 4.2 Å.

Affinity analysis of hIR-A and sIR-A 686G4 mutants. Competition binding on insulin binding to 686G4 mutated insulin receptor was performed via a polyethylene glycol (PEG) precipitation assay. Receptor samples were incubated in 1.5 mL Eppendorf tubes for 16 h at 4 °C in a total volume of 100 μL with 30 μM [125I]-Insulin (Perkin Elmer). The reaction mixture was centrifuged at 1000 g for 10 min at 4 °C and the supernatant was collected. The pellets were counted in a gamma-counter. For competition experiments, the concentration of receptor was adjusted to yield less than 20% binding when no
competing insulin was added. Binding data were fitted using a nonlinear regression algorithm in GraphPad Prism7.0 (GraphPad Software Inc.; see Supplementary Figure 5).

Data availability

The coordinates of the refined models have been deposited in the Protein Data Bank ("upper" domains: PDB entry 6HNS; "lower" domains: PDB entry 6HN4) and the corresponding B-factor sharpened cryoEM maps from which they were derived in the Electron Microscopy Data Bank ("upper" domains: EMDB entry EMD-0247; "lower" domains: EMDB entry EMD-0246). Other data are available from the corresponding authors upon reasonable request.

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**Additional information**

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