The structural origin for the negative cooperativity in the binding of insulin to IR

Our structural work explores the basis of negative cooperativity in the insulin binding to IR[25]. In both the **Γ**- or ***T***-shaped asymmetric IR dimers, the two disulfide-linked α-CT dimers adopt a rigid and elongated conformation (**Fig. 6e, f**). In this structural configuration, the conformational flexibility of the unliganded α-CT, which is required for another insulin binding, is restricted. Thus, the unliganded α-CT in the asymmetric IR dimer is retained in a position that reduces a capability of the binding of a second insulin, suggesting the negative cooperativity between two sites-1s. A similar molecular mechanism underlying the negative cooperativity in the binding of IGF1 to IGF1R has been proposed previously [24]. Furthermore, in the middle region of the ***T***-shaped IR dimer with two insulin bound, sites-1 and-2 are in close proximity to each other, and largely overlapped (**Fig. 6c, d**). Therefore, the binding of insulin at site-1 might hinder the binding another insulin to site 2, or conversely, the binding of insulin at site-2 will affect the binding of another insulin to site 1. This structural observation indicates the potential negative cooperativity between sites-1 and -2 in the asymmetric conformation, further increasing the complexity in binding of multiple insulins to the IR. Future binding assays using site-1 or site-2 insulin mutants are required to define the source of negative cooperativity in the binding of insulin to IR.

The conformational differences of the membrane proximal domains between asymmetric and symmetric IR dimers

The membrane proximal regions from both protomers in the asymmetric and symmetric IR dimer are brought together to enable trans-autophosphorylation and activation. However, our cellular and *in vivo* functional assays using insulin mutants and previous study using site-2 IR mutants [14] demonstrate that the symmetric IR has higher activity than asymmetric IR. This raises interesting question why the asymmetric IR dimer is partially active. Notably, the relative position and orientation between the two membrane proximal stalk domains are significantly different between asymmetric and symmetric IR dimer (**Extended Data Fig. 10d, e**). (1) In the **Γ**-shaped asymmetric IR with a single insulin bound, the unliganded L1 domain bridges the two stalks of the IR dimer into close proximity, and these two stalks are arranged asymmetrically (**Extended Data Fig. 10d**). (2) In the ***T***-shaped asymmetric IR with two insulins bound, the L1 domain no longer mediates the two stalks. Instead, the proximity of the two stalks is mediated by residues 647-653 in an extended loop of the FnIII-2 domain. As a result, the FnIII-3 domains in this ***T***-shaped IR dimer is more widely separated than the **Γ**-shaped IR dimer (**Extended Data Fig. 10e**). (3) In the **T**-shaped symmetric IR dimer with two or four insulins bound, the two stalks are arranged in a symmetric

manner, and their interaction is mediated by the homotypic contact between the two extended loops in FnIII-2 domains (**Extended Data Fig. 10d**).

Because the FnIII-3 domain is connected to the TM domain through a short linker (4 residues), it is tempting to speculate that extracellular, TM and intracellular domains are coupled allosterically, and that the differences in the arrangements of the membrane proximal regions in the asymmetric and symmetric IR dimer may cause differential dimeric assembly of TM and intracellular domains. Similar mechanism for kinase activation was proposed for epidermal growth factor receptor (EGFR) in response to different ligands [26]. Such structural coupling partially explains our functional results that the **T**-shaped symmetric IR exhibits higher activity than the asymmetric IR dimer. As the IR may have differential insulin binding occupancy under varying insulin concentrations, such a unique multi-sites system allows the IR to respond differently to a wide range of insulin concentrations at different metabolic states. Revealing the structure of TM and kinase domains, in the context of the entire full-length IR/insulin complex, is needed to further test this hypothesis.

The major differences between the activation mechanisms of IR and IGF1R

In contrast to the maximum 2:4 stoichiometry of active IR/insulin complex, binding of only one IGF1 molecule to the IGF1R dimer is sufficient for full receptor activation [24]. We speculate that the predominant 2:1 stoichiometry in the IGF1R/IGF1 structure is mediated by two factors: (1) IGF1 is unable to bind the site-2 of IGF1R; (2) there is a strong negative cooperativity between the two sites 1s of IGF1R. Because the insulin site-2 mutant cannot bind IR site-2, it should in principle behave similarly to IGF1 in inducing receptor activation. Nevertheless, most of the asymmetric IR/insulin site-2 mutants particles exhibit two insulins bound at both site-1s. These data suggest that the structure of a single IGF1 bound IGF1R might be more stable than that of a single insulin bound IR. Indeed, the key interface residues in IGF1R that are important for maintaining the structure of a single IGF1 bound IGF1R dimer, such as K163 and Y173, are not conserved in IR [24]. Taken together, our new and published structural results show that insulin and IGF1 can differentially modulate the structure of the extracellular module of IR and IGF1R, respectively. This also partially explains how insulin and IGF1 generate biased signaling (metabolic versus mitogenic) through closely related receptor tyrosine kinases.