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The EM structure of a type I interferon – receptor complex reveals a novel mechanism for cytokine signaling

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Abstract

Type I interferons have pleiotropic effects, including antiviral, antiproliferative and immunomodulatory responses. All type I interferons bind to a shared receptor consisting of the two transmembrane proteins ifnar1 and ifnar2. We used negative stain electron microscopy to calculate a three-dimensional reconstruction of the ternary complex formed by a triple mutant interferon- $\alpha 2$ with the ectodomains of ifnar1 and ifnar2. We present a model of the complex obtained by placing atomic models of the subunits into the density map of the complex. The complex of interferon- $\alpha 2$ with its receptor, a class II cytokine receptor, shows structural similarities to the complexes formed by growth hormone and erythropoietin with their receptors, members of the class I cytokine receptor family. Despite different assembly mechanisms, class I and class II cytokine receptors thus appear to initiate signaling through similar arrangements of the receptors induced by the binding of their respective ligands.

Keywords

interferon- $\alpha 2$; single particle electron microscopy; type I interferon receptor; cytokine signaling; receptor – ligands complex

INTRODUCTION

Interferons (IFNs) regulate a number of key biological functions in the innate immune response including antiviral activity and immunomodulatory as well as cell growth regulatory functions. Human IFN $\alpha 2$ belongs to the type I IFNs, a family of homologous helical cytokines that are secreted by cells of the immune system in response to challenges from foreign agents, such as viruses, bacteria, and tumor cells. To date, the family comprises 13 IFN α isotypes (and allelic forms) and single forms of IFN β , IFN κ , and IFN ω ^{1, 2}. Sequence homology between all IFN α isotypes is high (~80% identity) and the sequence identity of IFN α isotypes with IFNs β , κ , and ω sub-types ranges from 27% to 50%. Human type I IFNs have initially been identified for their antiviral effect, but have now been recognized as true pleiotropic cytokines³. Since

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human type I IFNs are the direct products of the immune system in response to foreign agents, they have been widely used in the treatment of various diseases, including viral hepatitis, multiple sclerosis and several kinds of cancers ⁴.

By definition, all type I IFNs are recognized by a common cell surface receptor that consists of two polypeptide chains, ifnar1 and ifnar2 ⁵. Ifnar1 and ifnar2 belong to the family of class II helical cytokine receptors, which possess a distinct pattern of four positionally conserved cysteine residues and lack the Trp-Ser-X-Trp-Ser motif that is conserved in class I cytokine receptors ⁶. Other members of class II cytokine receptors include the subunits of the IFN γ receptor (IFNGR1/IFNGR2), the subunits of the IL-10 family receptors (IL-10R1/IL-10R2, IL-20R1/IL-20R2, IL-22-R1 and IL-28R α), as well as IL-22 binding protein IL-22BP and tissue factor ²; ⁷. For this family, only structures of the ligands in complex with their high-affinity receptor subunits have been solved so far ⁸; ⁹, while the architecture of the complete signaling complexes have remained enigmatic. Ligand recognition by ifnar1 and ifnar2 has been studied in detail ¹⁰. Ifnar2 binds IFN ligands with much higher affinities than ifnar1 and can thus form binary ifnar2-IFN complexes without ifnar1. Signaling is, however, only initiated once IFN forms the ternary complex that includes both receptor chains. The formation of the ternary complex is thus a two-step process: first ifnar2 binds the ligand to form a binary complex, which then recruits ifnar1, forming the ternary complex that instigates signaling ¹¹. Growing evidence suggests that while ifnar2 is the major binding protein, ifnar1 is the determining factor for differential activities of various IFN ligands. In support of this notion, Jaitin et al. recently showed that a triple mutant of IFN α 2 (IFN α 2-HEQ) displays higher affinity for ifnar1, comparable to that of IFN β , and elicits biological responses that are much more similar to those initiated by IFN β ¹¹.

The structures of several type I IFNs have been solved by NMR ¹² or x-ray crystallography ^{13–17}. The structure of the IFN binding ectodomain of ifnar2 has also been solved by NMR ¹⁸. Mutagenesis, immunoblocking, and NMR chemical shift perturbation studies identified individual residues in the binding interfaces on IFN α 2 and ifnar2. Based on these data, a preliminary model of the binary ifnar2-IFN α 2 complex was proposed ¹⁸, which was recently refined based on further NMR studies ¹⁹. Ligand binding studies with ifnar1 have shown that all three N-terminal domains are required for ligand binding ^{20–23}, suggesting an unusual architecture of the signaling complex. The lack of structural information for the ternary ifnar1–ifnar2-IFN α 2 complex has now become one of the major roadblocks for further progress in our understanding of the mechanism underlying IFN α 2 signaling.

In this study we used single particle electron microscopy (EM) to visualize a stable ternary complex formed by IFN α 2-HEQ with the ectodomains of ifnar1 (ifnar1-EC) and ifnar2 (ifnar2-EC). We calculated 2D averages of negatively stained ternary complexes formed with full-length and truncated ifnar1-EC, which allowed us to determine the organization of the complex. We also calculated a random conical tilt three-dimensional (3D) reconstruction of the ternary complex, which we could interpret by placing atomic models of the binary ifnar2-EC-IFN α 2 complex and a homology model of ifnar1-EC into the density map. The results of this study provide first insight into how IFN α 2 interacts with its receptor. We compare the structure of this first structure of a class II helical cytokine receptor–ligand complex and the mechanism of IFN α 2 signaling with those of other cytokine receptor–ligand systems.

RESULTS

Preparation of the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex

The ectodomains of the IFN receptor subunits ifnar1 (ifnar1-EC) and ifnar-2 (ifnar2-EC) were expressed in *Sf9* insect cells (ifnar1-EC) and in *E. coli* (ifnar2-EC), and purified as described before ²³; ²⁴. Purifications typically yielded 10 mg ifnar1-EC and 1 mg ifnar2-EC from 1 l

culture. IFN α 2-HEQ was expressed in *E. coli*, refolded from inclusion bodies and purified by ion exchange and size exclusion chromatography²⁵, yielding about 150 μ g from 1 l culture. The ternary complex was formed by mixing ifnar1-EC, ifnar2-EC and IFN α 2-HEQ at a molar ratio of 1:1.5:1 and subsequent fractionation by size exclusion chromatography (Fig. 1A). The complex eluted at 1.35 ml, whereas a second smaller peak at 1.65 ml represented unbound ifnar2-EC, which was present in excess. The absence of peaks representing ifnar1-EC and IFN confirmed the formation of a stable ternary complex under these conditions. The composition of the peak fraction, which was used for subsequent EM studies, was assessed by SDS PAGE analysis, which confirmed that the complex contained all three components (Fig. 1B).

Identification of residues in ifnar1-EC involved in IFN α 2 binding

The contribution of several residues in ifnar1-EC towards binding of IFN α 2 was quantitatively assessed by *in vitro* ligand binding studies. Ifnar1-EC mutants carrying individual amino acid mutations were purified to homogeneity and binding was assessed for both wild-type IFN α 2 and IFN α 2-HEQ, a triple mutant of IFN α 2 (H57A, E58A, and Q61A) that increases the lifetime of the ternary complex by a factor of 30¹¹. Binding was monitored in real time by reflectance interference spectroscopy, a label-free solid phase detection method (Fig. 2). Mutants were chosen according to previous mutational studies done with cellular binding assays^{21; 22}. For some mutants, e.g., W250A, we did not obtain monomeric protein, indicating that these residues are essential for the structural integrity of ifnar1-EC. Only mutants yielding major monomeric protein fractions similar to wild-type ifnar1-EC were taken into consideration. A comparison of binding curves for IFN α 2-HEQ binding to different ifnar1-EC mutants is shown in Figure 2B, and the binding affinities are summarized in Table 1. From these binding assays, Y70, K113, W129 and F136 could be clearly confirmed as hot spots for IFN α 2 binding. Minor contributions were found for E111, N155, and K251, while residues R76, L247, R279, D298 and N368 did not affect the interaction.

Structural organization of the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex

To understand the organization of the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex, we visualized the complex by negative stain EM. Cryo-EM was not considered because the complex is too small (MW ~100 kDa) to generate sufficient contrast for it to be visible in vitrified ice. Images of negatively stained sample revealed a variety of particles, which made the images initially difficult to interpret (Suppl. Fig. 1A). Upon closer inspection, however, we could identify particles with comparable size and structural features (marked by circles in Suppl. Fig. 1A), which were likely to represent the ternary complex. The other particles present in the images would then result from dissociation and aggregation of the complexes.

To obtain a better understanding of the particles, we interactively selected 2,748 particles of consistent size from 56 images and classified them into 50 classes. Despite the small size of the complex, 2D averages obtained with negatively stained specimens resolved IFN α 2 and all the individual ~12.5-kDa Ig-like domains of ifnar1-EC and ifnar2-EC (see below). This result encouraged us to collect image pairs at tilt angles of 60° and 0° (Suppl. Fig. 2) needed to calculate a 3D reconstruction of the complex with the random conical tilt approach²⁶. 18,379 particle pairs were interactively selected from 66 60°/0° image pairs. The particles from the images of the untilted samples were classified into 50 classes. While the class averages showed particles with variable structural features (Suppl. Fig. 3A), a number of class averages appeared very similar (Fig. 3A, panels 1–6). For simplicity, stain-excluding domains will be referred to as densities in the following description. The averages showed particles consisting of a central density, surrounded by six additional densities; two on the left side and four on the right side of the central density. In most of the class averages the six peripheral densities are very close to each other and thus form a three-quarter circle around the central density with a one-quarter circle wide gap at the top left (Fig. 3A, panels 1–4). Some averages showed another, smaller

gap between the two densities at the bottom of the central density, which constitutes a separation of the two densities on the left side from the four densities on the right side of the central density (Fig. 3A, panels 5 and 6). We therefore interpreted the density in the center to represent IFN α 2, the densities to its left side to be the two Ig-like domains of ifnar2-EC and the densities to its right side to be the four Ig-like domains of ifnar1-EC (Fig. 3A, schematic drawing).

To test our interpretation of the 2D averages, we expressed a truncated ifnar1-EC construct (SD123), in which Ig-like domain 4 was deleted, which is not required for the formation of a ternary complex²³. Negative stain EM revealed that preparations of ternary complexes formed with truncated ifnar1-EC were similarly heterogeneous as those of ternary complexes formed with the full-length ifnar1-EC construct. Nevertheless, particles of similar size and structural features could again be discerned (marked by circles in Suppl. Fig. 1B). 15,084 of these particles from 112 images were classified into 50 classes (Suppl. Fig. 3B). A number of classes showed particles similar to those observed with the complex formed with the full-length ifnar1-EC, except that the density predicted to represent Ig-like domain 4 of ifnar1 was indeed missing (Fig. 3B, panels 1–6 and schematic drawing). This result confirmed the identities of the remaining densities representing ifnar1-EC. Because the membrane-proximal domains of ifnar1 (Ig-like domain 4) and ifnar2 (Ig-like domain 2) should be close to each other in the ternary complex, it also confirmed the identities of the densities representing the two domains of ifnar2-EC.

3D reconstruction and modeling of the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex

To calculate a random conical tilt 3D reconstruction of the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex, we chose the four classes that showed the clearest structural features from the 50 class averages obtained with the particles selected from the images of the untilted samples (Fig. 3A, panels 1–4). The density map calculated from these four classes revealed the same densities as the 2D averages (Fig. 4A). The resolution of the final density map was estimated by Fourier shell correlation (FSC) to be 32 Å with the FSC = 0.5 criterion (Suppl. Fig. 4). We also calculated a 3D reconstruction of the ternary complex formed with truncated ifnar1-EC (Suppl. Material and Suppl. Fig. 5A) at a resolution of 30 Å (Suppl. Fig. 5B). A 3D difference map between the complexes containing full-length and truncated ifnar1-EC showed a strong peak at the position of deleted Ig-like domain 4 (Suppl. Fig. 5C).

To interpret the 3D reconstruction, we placed atomic models of the individual components into the density map following our assignment of individual domains to densities in the 2D averages (Fig. 3). For the binary ifnar2-EC–IFN α 2 complex we used the model obtained by NMR (pdb accession code 2HYM)¹⁹, which we placed first into the density map without any modifications (Fig. 4B). For ifnar1-EC we generated a homology model based on the ectodomain of the IFN γ receptor (pdb accession code 1FYH)²⁷. The ectodomain of the IFN γ receptor consists only of two domains, and the additional two domains in ifnar1-EC are thought to have arisen from gene duplication². We therefore generated separate homology models for the first two and the second two domains of ifnar1-EC, conserving the angle between the two domains in each fragment. These two models were placed into the EM map individually (Fig. 4B). When placing the two fragments, we made sure that the C terminus of the N-terminal ifnar1-EC fragment was in close proximity to the N terminus of the C-terminal fragment. In addition, we chose the orientation of the two fragments thus that residues of ifnar1-EC identified by mutational analysis to be important for ligand binding (Table 1) were in close proximity to the IFN α 2 molecule (Suppl. Fig. 6). We placed the atomic models into the density map to give the best visual fit, but because of the limited resolution of the density map, we did not perform any further refinement of their positions by computational algorithms to avoid the impression that our model is more accurate than what our data warrant. The model we created

in this way is only an approximation of the structure of the ternary complex and is not precise in its atomic details. It is, however, consistent with the available structural data on the complex and should accurately reflect the general organization of the ternary ifnar1-EC-ifnar2-EC-IFN α 2 complex, the first structural insight into a class II receptor-ligand complex.

DISCUSSION

Wild-type IFN α 2 does not form a stable ternary complex with its two receptor subunits, ifnar1 and ifnar2. While the higher affinity of ifnar2 for IFN α 2 leads to the formation of a stable binary complex, ifnar1 only transiently associates with the ifnar2-IFN α 2 complex^{24; 28}. Recently, Jaitin et al. reported that the IFN α 2 triple mutant, IFN α 2-HEQ (H57A, E58A, and Q61A), increases the lifetime of the ternary complex by a factor of 30¹¹. We took advantage of this more stable complex to analyze its structure by molecular EM. Because of its small size (~100 kDa) and the remaining tendency of ifnar1 to dissociate from the binary ifnar2-IFN α 2 complex, the ternary complex is a challenging specimen for current EM technology. Even though we prepared the complex by negative staining, which generates higher image contrast than specimen vitrification, it remained very difficult to select the corresponding particles from the 0°/60° image pairs. Nevertheless, we were able to calculate 2D averages (Fig. 3) and to reconstruct a 3D density map (Fig. 4A), which clearly resolved the individual domains of the complex. Although the resolution of the 3D reconstruction is low, it was sufficient to place atomic models of the components into the density map and thus to generate a first view of how IFN α 2 forms a complex with its receptor.

It was straightforward to place the model of the ifnar2-EC-IFN α 2 complex¹⁹ into the 3D reconstruction, demonstrating that our density map is consistent with the NMR-based model of the binary complex (Fig. 4B). Since we did not have to modify the structure to obtain a good fit with the density map, our placement also suggests that ifnar1 binding does not induce any major changes in the way ifnar2 interacts with IFN α 2. This result is in line with the independent interaction of the ligand with the receptor subunits, which has been observed in detailed ligand binding studies²⁴. The structure of ifnar1-EC has not yet been determined and so we used homology models for the first two and the second two ifnar1 domains, which were based on the crystal structure of the ectodomain of the IFN γ receptor. We could fit these two fragments into the density map without changing the angles between the two domains in each fragment (Fig. 4B). The resulting model for the ternary complex is strikingly similar to a hypothetical model of the complex that was previously proposed by Anglistter and co-workers¹⁸.

Ifnar1 has much weaker affinity for IFN α 2 than ifnar2 and only transiently associates with the binary ifnar2-IFN α 2 complex^{11; 25; 28; 29}. In addition, a recent study found no direct interaction between ifnar1-EC and ifnar2-EC in the ternary complex²⁴. Since ifnar1 is thus only loosely associated with IFN α 2 and ifnar2, one might expect slight differences in the arrangement of the ifnar1 domains in the complex. This can indeed be observed in the 2D averages (Fig. 3A). In particular, domain 4 seems to be variable in its location and orientation with respect to the remaining complex. Considering the small size of the complex and the technical limitations of negative stain EM, it is possible that the observed structural variations may simply reflect differences in the stain embedding of the molecules. However, several observations strengthen the notion that domain 4 of ifnar1 may only be loosely attached to the IFN α 2 ligand. First, our attempts to include particles showing open complexes (e.g., Fig. 3A, panels 5 and 6) in the 3D reconstruction resulted in the loss of density mostly in this domain. Second, deletion of domain 4 had no effect on the interaction with the ligand and the formation of a ternary complex in solution²³. Third, the mutations in IFN α 2-HEQ that increase the IFN α 2 affinity for ifnar1 (magenta in Suppl. Fig. 6) are clustered in a region that is close to domain 2 of ifnar1, but far away from domain 4. Fourth, most of the residues in ifnar1-EC identified to be important for IFN α 2 binding are localized in domains 1 to 3, but none in domain

4 (Suppl. Fig. 6). Thus, the variations in position and orientation of domain 4 of ifnar1 in our 2D averages is in agreement with biochemical results that only domains 1 to 3 of ifnar1 contribute to IFN α 2 binding²³, whereas domain 4 does not appear to be involved in ligand binding nor in productive interactions with the membrane-proximal domain of ifnar2.

The paradigm for cytokine receptor activation is that cytokines induce signaling by bringing the cytoplasmic domains of two cell surface receptor subunits into close proximity, which instigates signaling through receptor cross-phosphorylation and recruitment of downstream proteins in the signaling pathways³⁰. The ways in which cytokines bring the cytoplasmic domains of their receptor subunits together differ among different ligand–receptor systems.

The human erythropoietin receptor (EpoR) and the human growth hormone receptor (GHR) belong to the short variety of class I hematopoietic cytokine receptor family. Their ectodomains consist only of the two domains forming the cytokine receptor homology region (CHR). Both receptor subunits pre-associate on the cell surface by contacts between the transmembrane domains^{31; 32}, but probably also other parts of the proteins³³. A crystal structure of the ectodomains of unliganded EpoR showed that the C-terminal, membrane-proximal CHR domains (D2) of the two EpoRs were about 73 Å apart³⁴. In contrast, in a crystal structure of the liganded complex the D2 domains were only about 30 Å apart³⁵ (Fig. 5A), suggesting that the ligand brings the transmembrane and cytoplasmic domains together by initiating a rearrangement of the CHRs in the preformed receptor dimer. Although different in nature, a change in the relative orientation of the receptor CHRs upon ligand binding has also been proposed to activate human GHR³² and EpoR³⁶ (Fig. 5C).

In contrast to the short receptors, tall hematopoietic cytokine receptors, such as gp130, contain three additional membrane-proximal fibronectin-III (FNIII) domains between the CHR and the transmembrane region³⁷. Gp130 is a promiscuous shared signaling receptor, which recognizes and assembles with binary complexes between cytokines and specific α and/or β receptor subunits³⁷. Recently the crystal structure of a hexameric complex was solved, which was formed by two IL-6 molecules, two IL-6R α constructs containing the CHR domains (D2 and D3) and an additional Ig-like domain (D1), and two gp130 constructs also containing the CHR domains (D2 and D3) and an additional Ig-like domain (D1)³⁸. In the crystal structure the D3 domains of gp130 were separated by about 70 Å, too far apart to allow juxtaposition of their transmembrane domains. A subsequent EM structure of the complex containing the entire ectodomain of gp130 (D1 to D6) showed that the legs of gp130 (FNIII domains D4 to D6) bridge the gap between the two receptor subunits and come together close to the tip³⁹. This finding suggested that affinity between the two membrane-proximal D6 domains plays an important role in bringing the cytoplasmic domains together. Similarly, it has recently been shown that binding of vascular endothelial growth factor (VEGF) to the membrane-distal Ig-like domains D2 and D3 of its receptor VEGFR-2 is not sufficient for stable receptor dimerization, which also requires specific interactions between the membrane-proximal D7 domains⁴⁰.

The IFN α 2–receptor complex seems to utilize yet another mechanism to instigate signaling. The two receptor subunits seem not to pre-associate on the cell surface such as EpoR and GHR, but they also do not have membrane-proximal domains with affinity for each other such as gp130 and VEGFR. Instead, binding to ifnar2 brings IFN α 2 into the appropriate distance from the membrane to be recognized by ifnar1. The membrane-proximal domain D4 of ifnar1 does not bind to IFN α 2 nor does it have a significant affinity for the membrane-proximal domain D2 of ifnar2^{23; 24}. However, because domains 1 to 3 of ifnar1 engage in complex formation with IFN α 2, the position of domain D4 is held in close proximity to membrane-proximal domain D2 of ifnar2, which may suffice to bring the cytoplasmic domains of ifnar1 and ifnar2 together for cross-phosphorylation and initiation of signaling. Thus, the structure of the ternary

complex is in line with a model for differential signal activation by type I IFNs recently proposed by Jaitin and co-workers, posing that differential ligand affinities towards ifnar1 result in differential stabilities of the ternary complex, which requires independent interactions of the ligand with the receptor subunits¹¹.

For cytokine signaling to occur, receptor dimerization is not sufficient. In addition, the receptor domains appear to have to assume specific positions and orientations with respect to each other^{32; 35}. The conformation of the receptor domains is modified by the binding of the cytokine ligands, which have two binding sites with different affinities for the receptor subunits. Ligand binding thus induces asymmetry even in homodimeric receptors, which can clearly be seen in the crystal structures of the EpoR–Epo complex³⁵ (Fig. 5A) and the GHR–GH complex⁴¹ (Fig. 5C). In both cases, the ectodomain of the receptor bound to the higher affinity site on the cytokine is further removed from the membrane than that of the receptor bound to the lower affinity site. The same arrangement is seen in our model of the ternary complex, in which the higher affinity ifnar2-EC is further removed from the membrane than ifnar1-EC (Fig. 5B). Although using a different mechanism and a heterodimeric receptor, the ternary ifnar1-EC–ifnar2-EC–IFN α 2 complex ends up with a similar arrangement of the membrane-proximal domains, suggesting a similar mechanism for activation of the cytoplasmic kinases as in the EpoR–Epo and GHR–GH signaling complexes.

The major difference of the type I interferon receptor compared to other short cytokine receptors is the presence of two additional Ig-like domains in ifnar1. Three of the Ig-like domains of ifnar1 interact simultaneously with the ligand, thus creating a larger ligand-recognition surface than those in other short cytokine receptors. Unlike other short cytokine receptors, the type I interferon receptor recognizes several interferon ligands, which elicit differential cellular responses. The larger ligand interface may be the reason why ifnar1 can recognize and discriminate between different type I interferons and/or may cause ifnar1 to adopt different conformations upon binding of different type I interferons, which could be responsible for the differential activation of IFN-dependent signaling pathways.

MATERIALS AND METHODS

Molecular biology, protein purification and preparation of the ternary complex

The full ectodomain of ifnar1 fused to a C-terminal His-tag (ifnar1-EC), and a construct containing the three N-terminal Ig-like domains fused to a N-terminal His-tag (SD123) were expressed in *Sf9* insect cells and purified by immobilized metal ion affinity chromatography and size exclusion chromatography as described before^{23; 24}. Wild-type IFN α 2, IFN α 2-HEQ and the ectodomain of ifnar2 were expressed in *E. coli* and refolded from inclusion bodies as reported before^{25; 42}. The ternary complex was prepared by mixing ifnar1-EC, ifnar2-EC and IFN α 2-HEQ at a molar ratio of 1:1.5:1. The proteins were concentrated to get a final concentration of the ternary complex of 30 μ M and subsequently purified by size exclusion chromatography in HBS (20 mM HEPES, pH 7.5, 150 mM NaCl) on a Superdex 200 PC 3.2/30 column (Amersham Biosciences). Site directed mutagenesis of ifnar1-EC was carried out using the ligase chain reaction. The mutants were expressed and purified like the wild-type protein.

Binding studies

Label-free binding studies were performed by reflectance interference spectroscopy (RIfS)⁴³. All experiments were carried out under flow-through. Wild-type ifnar1-EC and mutants were site-specifically immobilized onto the transducer surface through their C-terminal His-tags using multivalent chelator heads²⁴. Subsequently, wild-type IFN α 2 or IFN α 2-HEQ was injected at different concentrations, and binding was monitored in real time. For comparison

of binding affinities, the equilibrium signals were normalized to the amount of receptor loaded onto the surface.

Electron microscopy

Samples were prepared by conventional negative staining with 0.75% (w/v) uranyl formate as described before⁴⁴. Images were collected with a Tecnai T12 electron microscope (FEI, Hillsboro, OR) equipped with an LaB₆ filament and operated at an acceleration voltage of 120 kV. Images were recorded at a magnification of 67,000x and a defocus value of $-1.5\ \mu\text{m}$ using low dose procedures. For both ternary complexes containing full-length (Ig-like domains 1 to 4) and truncated ifnar1-EC (Ig-like domains 1 to 3), images were collected from untilted specimens. For the ternary ifnar1-EC-ifnar2-EC-IFN α 2 complex containing the full-length ifnar1-EC, also image pairs were recorded of the same specimen areas at tilt angles of 60° and 0°. All images were recorded on imaging plates. Imaging plates were read out with a DITABIS micron imaging plate scanner (DITABIS Digital Biomedical Imaging System AG, Pforzheim, Germany) using a step size of 15 μm , a gain setting of 20,000 and a laser power setting of 30%. 2x2 pixels were averaged to yield a pixel size of 4.5 Å on the specimen level.

Image processing

Using BOXER, the display program associated with the EMAN software package⁴⁵, 2,748 particles were interactively selected from 56 images of the ternary complex containing full-length ifnar1-EC and 15,048 particles from 112 images of the ternary complex containing truncated ifnar1-EC. The particles were windowed into 64x64 pixel images. Using the SPIDER software package⁴⁶, the particles were rotationally and translationally aligned and subjected to 10 cycles of multi-reference alignment. Each round of multireference alignment was followed by principal component analysis and K-means classification specifying 50 output classes. The references used for the first multireference alignment were randomly chosen from the raw images.

For 3D reconstruction of the ternary ifnar1-EC-ifnar2-EC-IFN α 2 complex containing full-length ifnar1-EC, 18,379 pairs of particles were selected interactively from both the images of the untilted and 60° tilted sample (66 image pairs) using WEB, the display program associated with the SPIDER package. The selected particles were windowed into 64 x 64 pixel images, and the particles from the untilted specimens classified into 50 classes as described above. Particle images from the four classes that showed the most similar 2D averages were combined (1,122 particles) and used for 3D reconstruction with the random conical tilt technique²⁶ implemented in the SPIDER package. The particles selected from the images of the tilted specimens of these classes were used to calculate an initial 3D reconstruction by backprojection, backprojection refinement, and angular refinement. 10% of the particles selected from the images of the untilted specimens in these classes were included in the data set (total of 1,234 particle images) and angular refinement was repeated. The resulting density map was used as input model for FREALIGN⁴⁷, which was used for further refinement of the orientation parameters of the individual particles and to correct for the contrast transfer function of each particle image according to its defocus value. The correct defocus value for each particle image was deduced from the position of each particle in the image and the tilt angles and defocus values of the images, which were determined with CTFTILT⁴⁸. The resolution of the final 3D reconstruction was estimated using Fourier shell correlation (FSC) and was 32 Å using the FSC = 0.5 criterion.

Placing atomic models into the EM density map

Structures used to place into the EM density map were the NMR-based model of the ifnar2-EC-IFN α 2 complex¹⁹ (pdb accession code 2HYM) and a homology model of ifnar1-EC created with the program SwissPdbViewer⁴⁹. Homology models were generated individually

for the first two and second two ifnar1-EC domains using the structure of the ectodomain of the IFN γ receptor ²⁷ (pdb accession code 1FYH) as the template. The ifnar2-EC-IFN α 2 complex was placed into the density map without any modifications. The homology models for the two ifnar1-EC fragments (domains 1 and 2 and domains 3 and 4) were placed individually into the density map. The program UCSF chimera ⁵⁰ was used to interactively place the structures into the density map to give visually the best fit. Because of the limited resolution of the density map, no attempts were made to computationally refine the placements of the structures into the density map.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Purification of the ternary ifnar1-EC-ifnar2-EC-IFN α 2 complex by size exclusion chromatography

(A) Chromatogram of the purified ternary complex in comparison to the elution profiles of the individual proteins and the binary complex. (B) Coomassie-stained SDS-PAGE gel of the fraction used for single particle analysis (shown in grey in panel a).

Figure 2. Mapping the binding site of ifnar1-EC for IFN α 2 by *in vitro* binding studies
 (A) Binding of wild-type IFN α 2 at different concentrations (1, 3 and 10 μ M). (B) Binding of IFN α 2-HEQ (300 nM) to various mutants of ifnar1-EC immobilized onto the sensor surface. The binding signals were normalized to the amount of ifnar1-EC on the surface.

Figure 3. Class averages of ternary complexes formed with the full-length (Ig-like domains 1–4) and truncated (Ig-like domains 1–3) ectodomain of ifnar1

(A) Class averages of the ternary complexes formed with full-length ifnar1-EC. (B) Class averages of the ternary complexes formed with truncated ifnar1-EC. The schematic drawings to the left depict the domain organization in the ternary complex. Scale bar is 5 nm.

Figure 4. 3D reconstruction of the ternary ifnar1-EC-ifnar2-EC-IFN α 2 complex in negative stain (A) Density map of the complex obtained by random conical tilt reconstruction. (B) Density map with the fit atomic models of the components (blue: ifnar1-EC; turquoise: ifnar2-EC; yellow: IFN α 2)

Figure 5. Comparison of the ternary ifnar1-EC-ifnar2-EC-IFNα2 complex with the human erythropoietin (Epo)-receptor and human growth hormone (GH)- receptor complexes
 (A) Crystal structure of the complex formed by human Epo with two copies of the Epo receptor (EpoR) ectodomain (pdb accession code: 1EER)³⁵. (B) Model of the ternary ifnar1-EC-ifnar2-EC-IFNα2 complex. (C) Crystal structure of the complex formed by human GH with two copies of the ectodomain of its receptor (GHR) (pdb accession code: 3HHR)⁴¹. The receptors that form the higher affinity interactions are shown in turquoise and the receptors that form the lower affinity interactions in blue.

Table 1
Binding affinities of ifnar1-EC mutants for IFN α 2-HEQ

Mutant	K_D (IFN α 2-HEQ)
wild-type	70 $\pm$ 20 nM
Y70A	> 3 μ M
R76A	like wild-type
E111A	340 $\pm$ 150 nM
K113A	> 3 μ M
W129A	> 3 μ M
F136A	> 3 μ M
N155T	200 $\pm$ 40 nM
L247A	like wild-type
W250A	-
K251A	130 $\pm$ 20 nM
R279A	like wild-type
D298A	like wild-type
N368D	like wild-type