Ligand Binding Induces a Conformational Change in ifnar1 that Is Propagated to Its Membrane-Proximal Domain

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The type I interferon (IFN) receptor plays a key role in innate immunity against viral and bacterial infections. Here, we show by intramolecular Förster resonance energy transfer spectroscopy that ligand binding induces substantial conformational changes in the ectodomain of ifnar1 (ifnar1-EC). Binding of IFNα2 and IFNβ induce very similar conformations of ifnar1, which were confirmed by single-particle electron microscopy analysis of the ternary complexes formed by IFNα2 or IFNβ with the two receptor subunits ifnar1-EC and ifnar2-EC. Photo-induced electron-transfer-based fluorescence quenching and single-molecule fluorescence lifetime measurements revealed that the ligand-induced conformational change in the membrane-distal domains of ifnar1-EC is propagated to its membrane-proximal domain, which is not involved in ligand recognition but is essential for signal activation. Temperature-dependent ligand binding studies as well as stopped-flow fluorescence experiments corroborated a multistep conformational change in ifnar1 upon ligand binding. Our results thus suggest that the relatively intricate architecture of the type I IFN receptor complex is designed to propagate the ligand binding event to and possibly even across the membrane by conformational changes.

Keywords: type I interferon receptor; fluorescence spectroscopy; cytokine receptor; protein–protein interaction; conformational dynamics

Introduction

Type I interferons (IFNs) are cytokines with key functions in the innate immune response against intracellular infections and malignancies.1–3 Consequently, IFNs have substantial medical potential and are already used in the treatment of hepatitis C and multiple sclerosis as well as several kinds of cancer.4 All type I IFNs bind to the same cell surface receptor, which is composed of the two subunits ifnar1 and ifnar2.5 Strikingly, different members of the IFN family instigate different cellular responses while using the same cell-surface receptor.6–11 A better structural and functional understanding of type I IFN receptor assembly could therefore pave the road for the rational engineering of IFNs with activity patterns designed for medical applications.

Over the past years, mutational and NMR studies have systematically unraveled the recognition of IFNs...
by their two receptor subunits. IFNs bind with nanomolar affinity to an epitope on ifnar2, which shows the architecture of a typical cytokine-binding module composed of two Ig-like domains. In contrast, the three N-terminal domains of ifnar1 are required for binding of IFNs, yet ifnar1 binds IFN ligands with affinities that are 2–3 orders of magnitude lower than that of ifnar2. Mutational studies have corroborated the involvement of the three N-terminal Ig-like domains of ifnar1 in the interaction with the ligand, which was also confirmed by a recent three-dimensional reconstruction of the ternary complex by a single-particle electron microscopic analysis. The membrane-proximal Ig-like domain is not required for ligand binding, but has very specific properties required for the assembly of the ternary complex on the membrane-proximal domain of ifnar1 and ifnar2 are in close proximity. While the role of the unusual architecture of the interaction between ifnar1 and IFNs remains enigmatic, a possible explanation would be the differential use of ifnar1 by different IFNs, which may also explain the observed differences in signal activation. Interactions with ligands mediated by multiple receptor subunits and the resulting large binding interface points, however, also towards a conformational change in ifnar1. Ligand-induced conformational changes have already been suggested to play a critical role in cytokine receptor assembly and signaling, but the underlying mechanisms have so far not been explored experimentally. NMR experiments have revealed only minor conformational changes in ifnar2-EC and IFNα2 upon complex formation. Binding studies have established that the two receptor subunits interact with the ligand independently of each other. The distribution of the ligand-binding site of ifnar1 over three Ig-like domains suggests that ligand binding may cause a rearrangement in the overall structure of ifnar1. The key role of ifnar1 recognition for mediating differential responses has been demonstrated. Thus, differences in the recruitment of IFN ligand and the resulting conformational changes in ifnar1 may account for the different cellular responses to the binding of different IFNs.

Here, we used several fluorescence techniques and single-particle electron microscopy (EM) to explore the conformation of the ectodomain of ifnar1 (ifnar1-EC) in complex with ifnar2-EC and two different IFNs. We observed a substantial spatial rearrangement of the Ig-like domains of ifnar1-EC upon ligand binding. Strikingly, this conformational change is propagated from the membrane-distal domains to the membrane-proximal domain of ifnar1-EC, which is not involved in ligand binding. The conformational change in ifnar1 resulting from binding of IFNα2 and IFNβ was indistinguishable, suggesting that its propagation to the membrane-proximal domain is conserved and may play an important role for receptor assembly and transmembrane signaling.

### Results

#### Ligand-induced conformational changes in ifnar1-H10

We hypothesized that the interaction of multiple Ig-like domains of ifnar1-EC with an IFN ligand could induce a change in the spatial organization of the involved receptor domains. In order to test this idea, we first probed the conformation of ifnar1-EC by Förster resonance energy transfer (FRET) experiments. For this assay, the donor fluorophore was covalently coupled to cysteine residues, which were incorporated into ifnar1-EC by site-directed mutagenesis. The quencher fluorophore was introduced in situ by noncovalent interactions with the C-terminal decahistidine (H10) tag using the multivalent chelator tris-nitrilotriacetic acid (NTA) as schematically depicted in Fig. 1a and b. Detailed characterization and calibration of this technique, which will be published elsewhere, confirmed the FRET-based quenching of the donor fluorophore. Ifnar1-EC mutants with individual cysteines inserted into different Ig-like domains (cf. Fig. 1c) were site-specifically labeled with Oregon Green 488 (OG488) maleimide and purified to homogeneity. The Cy5 analogue fluorophore FEW646 was used as FRET acceptor and conjugated to the multivalent chelator tris-NTA (FEW646tris-NTA, Fig. 1a). It has been shown that such conjugates bind with high affinity and selectivity to His-tags introduced into proteins, thus yielding quantitative labeling with the acceptor dye.

Upon addition of FEW646tris-NTA to the OG488-labeled ifnar1-H10 cysteine mutants (OG488ifnar1-H10), a strong decrease in fluorescence intensity was observed, as shown for OG488ifnar1-H10 N23C in Fig. 1d and OG488ifnar1-H10 N349C in Fig. 1e. The extent of fluorescence quenching depended on the position where OG488 was inserted in ifnar1-H10 and correlated with the distance of the labeled cysteine residue from the C-terminal His tag. For the S147C, N286C and N349C mutants, the relative quenching amplitudes were qualitatively in agreement with the distances of the mutated residues from the C terminus as estimated from the structural model of the ternary complex (Fig. 1c). For the N23C mutant, however, the quenching amplitude was similar to those of the S147C and N286C mutants, which is not consistent with the larger distance of this residue from the C terminus compared to the other two residues (Fig. 1c). These results suggest that ifnar1-EC in the absence of a bound ligand adopts a bent conformation. The similar distances of the three residues N23, S147 and N286 from the C terminus in this conformation would explain the similar extents of fluorescence quenching observed in the FRET experiments. If free ifnar1 is bent, while extended in the ternary ifnar1-EC–ifnar2-EC–IFNα2 complex, ligand binding should cause a measurable change in fluorescence quenching with the N23C mutant. Indeed, addition of the ligand IFNα2-HEQ (which
binds ifnar1-EC with ~100 nM affinity) at saturating concentrations resulted in a substantial recovery in fluorescence (Fig. 1d). A significant recovery in fluorescence was, however, not only observed for OG488ifnar1-H10 N286C, but, surprisingly, also for OG488ifnar1-H10 N349C (Fig. 1e and f), supporting a substantial rearrangement of the molecule upon interaction with the ligand. While the fluorescence recovery pattern obtained for the mutants N23, S147 and N286 (Fig. 1f) points towards a move of the N-terminal cytokine-binding module along the linker between Ig-like domains 2 and 3, the effect on OG488ifnar1-H10 N349C was explained by the proximity to the Trp residue 347 (Fig. 1h) as demonstrated by further experiments (see below). The strongest recovery was observed for the N23C mutant, and the resulting quenching amplitudes after ligand binding were qualitatively consistent with the distances of the residues in the model of the ternary complex (Fig. 1c). Taking into account the theoretical Förster radius of ~50 Å for this FRET pair, we estimate that ligand binding increases the distance of N23 from the C terminus by ~13 Å.

The ligand-induced rearrangement of the Ig-like domains was confirmed with a different acceptor fluorophore, AT565tris-NTA, for which both stronger quenching of the donor fluorophore as well as higher recovery upon ligand binding was observed (Fig. 1g). These measurements yielded a similar increase in the distance of ~11 Å between N23 and the C terminus upon ligand binding. Using this more sensitive probe, we furthermore compared the fluorescence dequenching of OG488ifnar1-H10 N23C upon binding of IFNα2-HEQ in complex with

![Fig. 1. FRET analysis of the conformation of ifnar1-H10 in solution.](image)
ifnar2-EC, and of IFNβ in complex with ifnar2-EC. These experiments revealed that the ligand-induced conformation of ifnar1 is (i) independent of ifnar2 and (ii) independent of the type of ligand (Fig. 1g).

Corresponding architectures of the ternary complexes formed with IFNα2 and IFNβ

The results described above suggested that binding of IFNα2 and IFNβ, which cause the most different cellular responses, induces very similar conformations of ifnar1-EC. We therefore compared the architectures of the ternary signaling complexes formed by IFNα2 and IFNβ by single-particle EM analysis. The stable complex of IFNβ with ifnar1-H10 and ifnar2-H10 was isolated by size-exclusion chromatography and imaged by negative stain EM. Individual complexes could be clearly identified (Fig. 2a). A total of 14,782 particles were selected interactively from the images and classified into 50 classes (Supplementary Fig. 1). A typical class average is shown in Fig. 2b in comparison with a corresponding class average previously obtained for the ternary complex formed with IFNα2-HEQ (Fig. 2c).27 The arrangement of the Ig-like domains of ifnar1-EC in the ternary complex formed with IFNβ appeared very similar to that seen in the complex formed with IFNα2-HEQ. The relative orientation of the receptor subunits is also indistinguishable in the two IFN complexes. Remarkably, a similar relative shift between the membrane-distal domains of ifnar1-EC and ifnar2-EC can be observed in both ternary complexes. The similar mode of binding of IFNα2 and IFNβ to ifnar1 was confirmed by quantitative ligand-binding studies with ifnar1 single-amino-acid mutants using reflectance interference (RII) detection (Table 1). All residues involved in the interactions with IFNα2-HEQ were also involved in the interaction with IFNβ. These results corroborate that IFNα2 and IFNβ form ternary complexes with very similar architectures.

Conformational changes are propagated from the membrane-distal domains to the membrane-proximal domain of ifnar1-EC

A very surprising outcome of the fluorescence quenching assays shown in Fig. 1 was the rather strong dequenching observed for OG488ifnar1-H10 N349C (Fig. 1e). In case of this mutant, OG488 is attached to the C-terminal Ig-like domain, which carries the His tag and thus the quencher dye. Furthermore, binding studies with various deletion and chimeric constructs23 as well as the structure of the ternary complex obtained by single-particle EM27 suggested that this domain is not involved in interactions with ligands. Furthermore, the ligand-induced fluorescence dequenching of OG488ifnar1-H10 N349C was largely independent on the quencher attached to the His tag (Supplementary Fig. 2).

To explore the conformation of the C-terminal Ig-like domain in more detail, we attached the fluorophore ATTO 655 to ifnar1-H10 N349C (AT655ifnar1-H10 N349C). ATTO 655 is an oxazine-based dye that is very potently quenched by photo-induced electron transfer to tryptophan.43 We speculated that W347, a tryptophan residue in the vicinity of N349, may be able to quench the covalently attached fluorophore.
Indeed, a more than twofold increase in fluorescence of AT655ifnar1-H10 N349C was observed upon ligand binding, which is typical for quenching of this dye by tryptophan. The fluorescence quenching of ATTO 655 was shown to be due to interaction with W347, as no ligand-induced increase in fluorescence was observed for the W347F, N349C double mutant of AT655ifnar1-H10 (Fig. 3b). Unchanged ligand-binding activity of ifnar1-H10 W347F, N349C was confirmed by solid-phase binding assays using Rif detection (data not shown). Moreover, ligand-induced dequenching of AT655ifnar1-H10 N349C was monitored in real time by simultaneous total internal reflection fluorescence correlation spectroscopy (TIRFS)–Rif detection (Fig. 3c and d). The fluorescence and mass-sensitive signals were in excellent agreement (Fig. 3d).

With this very sensitive probe for conformational changes in hand, we analyzed the influence of ifnar2-EC on the conformation of the binary ifnar1-EC/IFNα2 complex and the potential differences between IFNα2 and IFNβ (Fig. 3e and Supplementary Fig. 2). The dequenching amplitudes showed no statistically significant differences for IFNα2, IFNβ in complex with ifnar2-EC, and IFNβ in complex with ifnar2-EC. Since the membrane-proximal domain is not involved in ligand binding, these results suggest that the ligand-induced rearrangement of the three N-terminal Ig-like domains of ifnar1 is propagated to the membrane-proximal domain. Furthermore, binding of IFNα2 and IFNβ results in a very similar conformational change of the membrane proximal domain of ifnar1-EC, confirming that ifnar1 adopts very similar conformations in complex with IFNα2 and IFNβ.

**Accessibility of W347 is drastically reduced upon ligand binding**

Although the temperature dependence of the fluorescence intensity of AT655ifnar1-H10 N349C was negligible (Fig. 3f), indicating that conformational flexibility contributed little to fluorescence dequenching, these measurements did not completely rule out the possibility that loss of conformational flexibility may play a role in dequenching. Therefore, we further explored the nature of the conformational change in the membrane-proximal domain of ifnar1 by time-resolved fluorescence measurements. Ensemble fluorescence lifetime measurements of AT655ifnar1-H10 N349C in the absence and presence of IFNα2-HEQ indicated only little change of the fluorescence lifetime of ATTO 655 (Fig. 4a), confirming a fast transition of the fluorophore between a fully quenched and an unquenched state as expected for quenching processes based on electron transfer. The transition dynamics of the fluorophore were further characterized by fluorescence correlation spectroscopy. For this purpose, the fluorescence of ~1 nM AT655ifnar1-H10 N349C was monitored in the absence and presence of the ligand IFNα2-HEQ by time-correlated single-photon counting. The autocorrelation functions obtained for lag time τ ranged from 25 ns to 1 s (Fig. 4b and c). Without ligand, two decay times could be clearly distinguished in the autocorrelation function: one in the millisecond range, which corresponds to the diffusion time, and one in the microsecond range, which is attributed to the dynamic quenching process, which requires the fluorophore to come into very close proximity to the tryptophan residue. Strikingly, ligand binding almost completely eliminated the decay of the autocorrelation in the microsecond range (Fig. 4b). The same measurements were carried out with the AT655ifnar1-H10 double mutant W347F, N349C. The absence of the fast decay in the microsecond range in the absence of W347 (Fig. 4c) confirmed that this tryptophan residue was responsible for the dynamic quenching of AT655ifnar1-H10 N349C. These results thus corroborated that ligand binding causes a shift of the membrane-proximal domain of ifnar1. The fast autocorrelation time of ~5 μs corresponds well with the autocorrelation time measured for a flexible tryptophan-containing peptide linked to an analogous dye, suggesting that the quenching dynamics are determined by the conformational dynamics of the linker between the dye and the protein. The fact that the fast autocorrelation almost completely disappeared upon ligand binding indicates that the accessibility of W347 is substantially changed. These single-molecule measurements therefore support a ligand-induced conformational change rather than a loss in flexibility.

**Non-Arrhenius temperature-dependent ligand-binding kinetics of ifnar1-EC**

In order to explore the mechanistic implications of the ligand-induced conformational change, we measured the temperature dependency of the rate constants of complex formation for ifnar1-H10 in comparison to ifnar2-H10. The proteins were captured onto NTA-functionalized gold surfaces, and binding of IFNα2 and IFNα2-HEQ was monitored at various temperatures between 5 and 40 °C by surface
plasmon resonance (full binding curves at selected temperatures are shown in Supplementary Fig. 3, and the rate constants are summarized in Table 2). For ifnar2, the rate constants increased substantially with higher temperatures, which is particularly apparent in the dissociation curves (Fig. 5a). The association and dissociation rate constants obtained from these curves are fully consistent with the Arrhenius law (Fig. 5c and d), yielding activation energies of 60 kJ mol$^{-1}$ for the association kinetics and 85 kJ mol$^{-1}$ for the dissociation kinetics. The same results were obtained for IFN$\alpha$2-HEQ binding to ifnar2-H10 (data not shown). In contrast, a decrease in the dissociation rate constant was observed for ligand binding to immobilized ifnar1-H10 (Fig. 5b). The dissociation rate constant only
began to increase again at a temperature above 30 °C. In the Arrhenius plots, both association and dissociation kinetics behaved in a nonlinear fashion (Fig. 5c and d). The slope of the Arrhenius plot for the association rate constants changed significantly between 20 and 25 °C, but the dissociation rate constants showed a positive slope between 5 and 30 °C, yielding an apparent negative activation energy in this temperature range. Very similar results were obtained with ifnar1-EC without the membrane-proximal Ig-like domain (H10-SD123). In protein folding, apparent negative activation energies have been attributed to heat-capacity changes in the activated complex or to temperature-induced changes of the ground state. For the interaction of folded proteins, however, apparent negative activation energies have been explained by changes in the rate-limiting step of multistep reactions. The temperature dependence of the kinetics of the ifnar1–IFNα2 complex formation indicates a transition of the rate-limiting step between 20 and 30 °C. To our knowledge, negative activation energies have only been reported for the association of macromolecular complexes that cause large conformational changes. Here, the dissociation is clearly dominated by a two-step process, suggesting that thermal energy is required for maintaining a conformational change needed to stabilize the complex.

Strongly negative changes in heat capacity for the ifnar1–IFNα2 complex formation

Analysis of the temperature-dependent equilibrium constants yielded a similar picture. For the binary IFNα2/ifnar2-H10 complex, the equilibrium dissociation constant changed very little with increasing temperature (Fig. 6a), yielding a linear correlation of $\Delta G^0$ with temperature (Fig. 6c) with a temperature-independent $\Delta S^0$ of 63 ± 9 J mol$^{-1}$K$^{-1}$ and $\Delta H^0$ of $-27 \pm 3$ kJ mol$^{-1}$ (Table 3). For ligand binding to ifnar1, however, the binding affinity increased substantially when the temperature was increased from 5 and 25 °C (Fig. 6b), resulting in a nonlinear correlation of $\Delta G^0$ with temperature (Fig. 6c). This result indicates that substantial changes in heat capacity $\Delta C_p^0$ upon complex formation cause $\Delta H^0$ and $\Delta S^0$ to be temperature-dependent. Therefore, this curve was fitted by an extended Gibbs–Helmholtz equation, yielding a $\Delta H^0$ of 30 ± 5 kJ mol$^{-1}$.

Table 2. Rate and equilibrium constants obtained with solid-phase binding assays at different temperatures

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<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
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* Association rate constant (average standard deviation of ~15%).
* Dissociation rate constant (average standard deviation of ~10%).
* Equilibrium dissociation constant determined from the rate constants (average standard deviation of ~17%).

Fig. 4. Conformational changes of ifnar1-EC as monitored by time-resolved fluorescence. (a) Ensemble fluorescence lifetime measurements of ATTO 655/ifnar1-H10 N349C in the absence and presence of the ligand in comparison to free ATTO 655 dye. (b) Autocorrelation curves for AT655/ifnar1-H10 N349C in the absence and presence of the ligand. (c) Autocorrelation curves for AT655/ifnar1-H10 W347F, N349C in the absence and presence of the ligand.
and a $\Delta S^0$ of 230±20 J mol$^{-1}$K$^{-1}$ at 25 °C with a change in heat capacity of −6.7±0.7 kJ mol$^{-1}$ K$^{-1}$.

The strong temperature dependencies of $\Delta H^0$ and $\Delta S^0$ are shown in Fig. 6d, illustrating the transition from an endothermic to an exothermic interaction between 25 and 37 °C. Such large negative changes in heat capacity upon complex formation have been observed mainly for protein–DNA complexes and have been ascribed to large structural adaptations upon complex formation. These results thus confirm a substantial conformational rearrangement of ifnar1-EC upon ligand binding, accompanied by a large loss in hydrophobic surface area. From the $\Delta S^0$ and $\Delta C_p^0$ values, a rearrangement of 80 amino acid residues can be estimated. Very similar results were obtained for IFNα2-HEQ binding to H10-SD123, suggesting that the major rearrangement occurs in the three N-terminal Ig-like domains of ifnar1-EC.

**A multistep ligand-induced conformational change in ifnar1**

We further explored the kinetics of the ligand-induced conformational change in ifnar1 by performing stopped-flow experiments employing the fluorescence assays described above. AT655ifnar1-H10 N349C was rapidly mixed with IFNα2 E58A at different concentrations while monitoring the increase in fluorescence. Since IFNα2 E58A binds ifnar1-EC with approximately five times higher affinity than wild-type IFNα2 and since it can be produced in much higher yields than IFNα2-HEQ, IFNα2E 5 8 A was used for these experiments. The fluorescence increased rapidly upon complex formation with very little deviation from a monoexponential function (Fig. 7a). A strong increase in curvature was observed with increasing ligand concentrations. The fit of the concentration-dependence of the apparent rate constants (Fig. 7c) yielded an association rate constant of $k_a = (9±2) \times 10^5$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant of $k_d = (1.2±0.3)$ s$^{-1}$. The dissociation rate constant was confirmed by chasing AT655ifnar1-H10 N349C in complex with IFNα2 E58A with unlabeled ifnar1-H10 (Fig. 7d), yielding a $k_d = (1.1±0.1)$ s$^{-1}$. While both $k_a$ and $k_d$ are approximately five times higher than the rate constants determined by surface-sensitive detection, the equilibrium dissociation constant $K_D = 1.3$ μM is in good agreement with the $K_D = 0.8$ μM determined by surface-sensitive detection (data not shown).
The same set of experiments was carried out with OG488ifnar1-H10 N23C in complex with AT565tris-NTA in order to probe the kinetics of the rearrangement of the Ig-like domains (Fig. 7b). In this case, the increase in fluorescence was not monoexponential for all ligand concentrations. In a control experiment with OG488ifnar2-H10 in complex with AT565tris-NTA mixed with 20 μM IFNα2 E58A, no significant change in fluorescence was observed (Fig. 7b), confirming the specificity of the signal characteristics. The curves were approximated over a time interval of 120 s by a triexponential fit, in which two of the three rate constants were fit globally for all concentrations (Fig. 7a). The variable rate constant was dependent on the concentration of IFNα2 E58A with a slight deviation from a linear correlation (Fig. 7c). Two concentration-independent rate constants of $k_2 = 0.3 \text{ s}^{-1}$ and $k_3 = 0.03 \text{ s}^{-1}$ were obtained from the fit. The relative signal amplitude corresponding to $k_2$ was 35–50% of the signal amplitude associated with $k_1$ for all concentrations, supporting the significance of this rate constant. The significance of the rate constant $k_3$ with a signal amplitude of $\sim 20\%$ of the signal amplitude associated with $k_1$, however, is not clear. The same behavior was observed for OG488ifnar1-H10 S147C, and very similar rate constants of the triexponential fit as well as similar relative signal amplitudes were obtained (Fig. 7c).

Similar multiexponential binding kinetics was observed for OG488ifnar1-H10 N286C, but the total signal amplitudes were too low for a detailed assessment of the rate constants. These experiments clearly

**Table 3.** Thermodynamic parameters at 25 °C

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<th>Ifnar1-H10/IFNα2</th>
<th>Ifnar1-H10/IFNα2-HEQ</th>
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established a two- or multistep ligand-induced conformational change in ifnar1-EC.

Discussion

The molecular mechanisms underlying signal activation by cytokine receptors are currently under debate. While the classic model is based on ligand-induced receptor assembly,49 the current view for a number of class I cytokine receptors favors receptor preassembly and subsequent activation by ligand-induced conformational changes.29,30,36,37,50,51 Also for the IFNγ receptor—a member of the class II cytokine receptor family—preassembly and ligand-induced conformational changes have been demonstrated in live cells.35,52 These studies emphasized that the receptor conformation and conformational changes upon ligand binding play key roles in signal activation. The architecture of the extracellular domain of ifnar1 has puzzled researchers for several years until its important role in differential signal activation emerged.39 While the three N-terminal domains of ifnar1 are involved in ligand binding, the EM structure of the ternary complex revealed that the C-terminal cytokine-binding module of ifnar1 adopts a similar arrangement with respect to ifnar2 as seen in other class I cytokine receptor complexes.27 Here, we could show that the simultaneous interaction of the three N-terminal domains of ifnar1 with the ligand results in a substantial ligand-induced rearrangement of the Ig-like domains of ifnar1. Using site-specific fluorescence labeling in situ through the C-terminal His tag, we demonstrate by intramolecular FRET experiments that ligand binding induces a stretching of ifnar1 by more than 10 Å. Using an electron-transfer-based fluorescence assay, we furthermore established that the ligand-induced conformational changes are propagated to the membrane-proximal Ig domain of ifnar1, which does not interact with the ligand. The key importance of this domain for receptor assembly and signaling has previously been shown by chimeras with the corresponding domain of other class II cytokine receptors. Propagation of conformational changes was probably impaired in these chimeras, suggesting

Fig. 7. Ligand-induced conformational change probed by stopped-flow fluorescence measurements. (a) Increase in fluorescence upon mixing 50 nM \( {\text{AG655ifnar1-H10}} \) with IFNα2 E58A at different concentrations (red, 1 μM; blue, 10 μM). (b) Increase in fluorescence upon mixing 50 nM \( {\text{AG655ifnar1-H10 N23C}} \) in complex with \( {\text{AT655tris-NTA}} \) with IFNα2 E58A at different concentrations (red, 1 μM; blue, 10 μM). As a control, the same experiment was carried out with \( {\text{OG488ifnar2-H10}} \) in complex with \( {\text{AT655tris-NTA}} \) with 10 μM IFNα2 E58A. (c) Comparison of the concentration-dependent rate constant obtained for \( {\text{AG655ifnar1-H10 N23C, OG488ifnar1-H10 SI47C and AT655ifnar1-H10}} \). (d) Decrease in fluorescence upon mixing 25 nM \( {\text{AG655ifnar1-H10}} \) preincubated with 1 μM IFNα2 E58A with 10 μM unlabeled ifnar1-H10.
that the ligand-induced conformational changes play a critical role for signal activation. Temperature-dependent binding studies confirmed large conformational rearrangements during ligand recognition and furthermore indicated that complex formation occurs in more than one step. This hypothesis was verified by stopped-flow fluorescence experiments, which suggested that a further rearrangement of the Ig-like domains takes place in a second step after ligand recognition. Taken together with the two-step recruitment of the receptor subunits observed on solid-supported membranes,\textsuperscript{36,53} a multistep assembly mechanism as schematically depicted in Fig. 8 emerges.

Interestingly, no differences in the conformational changes in ifnar1 were observed upon binding of IFNa2 and IFNb, and EM averages revealed that the two ternary complexes also have very similar architectures. While differential receptor assembly by IFNs has been previously proposed to be responsible for differential signal activation, our results do not support this hypothesis. Even on the level of rather small conformational changes in the membrane-proximal domains probed by photo-induced electron transfer, we could not detect differences in the ifnar1 conformations in the ternary complexes formed with IFNa2 and IFNb. Recently, the different stabilities of the interaction of IFNa2 and IFNb with ifnar1 have been shown to play a critical role for their differential response patterns.\textsuperscript{39,40} This finding is interesting in the context of the rather slow rate constant (0.3 s\textsuperscript{-1}) we have observed for the conformational change after ligand binding. Since the lifetime of the ifnar1-IFNa2 interaction in the ternary complex on the membrane is in the same order of magnitude ($k_d = 0.3 \text{ s}^{-1}$),\textsuperscript{53} the probability for the conformational change to happen during a binding event is much lower than for IFNb, for which the lifetime of the complex with ifnar1 is 40 times longer. If the conformational change is important for signal activation, this difference between IFNa2 and IFNb could be responsible for a higher potency of IFNb compared to IFNa2 observed under conditions of prolonged stimulation.

The exact structural consequences of the ligand-induced conformational changes remain to be elucidated. However, we have identified a tryptophan residue close to the membrane (W347) with a strong loss in accessibility upon ligand binding, which is probably due to a switch between an exposed to a buried state upon ligand binding. These results are to some extent consistent with a generic mechanism, which has recently been proposed for ligand-induced activation of class I cytokine receptors.\textsuperscript{28} This mechanism is based on a ligand-induced rearrangement of the WSXWS motif, which is found in the class I cytokine receptor family. Weidemann et al.\textsuperscript{28} postulated that the accessibility of the tryptophan residues is changed by a ligand-induced conformational change, thus modulating the interaction of the receptor ectodomains with the membrane. While ifnar1, as a member of the class II cytokine receptor family, does not contain the above-mentioned WSXWS motif, it is conceivable that ligand binding also modulates the interaction with the membrane in this receptor. Strikingly, the respective tryptophan residue is conserved throughout the low-affinity subunits of the class II cytokine receptor subunits (IL-10R2, IFN-γR2 and IL-20R2), but not in the high-affinity subunits with the single exception of IFN-λR1.

Taken together, our results provide a first glimpse into the intricate structural reorganization during the ligand-induced assembly of the type I interferon receptor. It is very likely that these conformational changes are essential for signal activation, highlighting the fine-balanced molecular mechanisms that are involved in cytokine receptor signaling.

**Materials and Methods**

**Materials**

FEW647 tris-NTA and AT565 tris-NTA conjugates were synthesized and stoichiometrically loaded with Ni(II) ions as described previously.\textsuperscript{41} ATTO 655 maleimide was purchased from ATTO-Tec (Siegen, Germany), OG488
maleimide from Invitrogen (Karlsruhe, Germany). The extracellular domains of ifnar1-H10, ifnar1-H10 mutants and a fragment containing the three N-terminal domains of ifnar1 fused to an N-terminal decahistidine tag (H10-SD123) were expressed in S9 insect cells using baculovirus infection and purified by immobilized metal-chelating chromatography as previously described.17,23 Pooled fractions of ifnar1-H10 were incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) on ice and further purified by size-exclusion chromatography (Superdex 200, 16-60; 20 mM Tris, pH 8.0, 200 mM sodium chloride). Pooled fractions of the cysteine mutants were incubated with 1 mM EDTA and the free cysteines were subsequently reduced by incubation with 100 μM dithiothreitol (for the ifnar1-H10 mutants S147C, N286C and N349C) or 3 mM tricarboxyethylphosphine (for ifnar1-H10 N23C) for 1 h on ice. The reducing agent and misfolded/aggregated proteins were removed by size-exclusion chromatography (Superdex 200, 20 mM Tris, pH 7.5, 50 mM sodium chloride). A threefold molar excess of maleimide-functionalized fluorophore was added to the pooled protein fractions and incubated overnight at 4 °C. The solution was diluted fivefold with 5 mM Tris, pH 8.5, and loaded onto an anion-exchange column (Sepharose Q, Amersham Biosciences). Nonlabeled and multilabeled species were separated by elution with a gradient of 0 to 500 mM sodium chloride in 5 mM Tris, pH 8.5. Prior to use, the labeled proteins were run over a Superdex 200 size-exclusion chromatography column in Hepes-buffered sodium chloride (HBS; 20 mM Heps, pH 7.5, 150 mM sodium chloride). Typical labeling degrees were 0.5–0.7 fluorophores per ifnar1-H10 molecule for the purified proteins. In order to confirm the specificity of the labeling reaction under these conditions, wild-type ifnar1-H10 was subjected to the same procedure, which did not yield significant quantities of labeled protein after purification. Ifnar1-EC without a His tag was generated by proteolytic cleavage of an ifnar1-H10 variant carrying a factor Xa cleavage site upstream of the C-terminal His tag. IFNα2 and IFNα2-HEQ were expressed in Escherichia coli and purified from inclusion bodies as described previously.34 Metal-binding contaminants were subsequently removed by affinity chromatography using a Ni–NTA resin (Qiagen, Hilden, Germany). Ifnar2 with a C-terminal decahistidine tag (ifnar2-H10) and tag-less ifnar2-EC (ifnar2-tl) were expressed in E. coli and refolded from inclusion bodies as described before.34

Fluorescence quenching assays in solution

Spectroscopic fluorescence measurements in solution were carried out with a Cary Eclipse (Varian) at 20 °C. In a 120-μl cuvette (Hellma), 100 nM of fluorescence-labeled ifnar1-EC was diluted in HBS supplemented with 1 mg/ml bovine serum albumin. OG488 was excited at 470 nm and the emission spectra were recorded between 490 to 600 nm. Subsequently, 250 nM FEW647-tris-NTA or ATTO655-tris-NTA loaded with Nif2− was added and carefully mixed, and the fluorescence spectra were recorded until the quenching was equilibrated (~15 min). Subsequently, the binding partner (1 μM IFNα2-HEQ or 300 nM IFNβ) in complex with ifnar2-tl) was added and carefully mixed and the spectra recorded. Complexes of IFNα with ifnar2-tl were prepared by adding a 20% molar excess of ifnar2-tl. To compete out the ligand, 10 μM of unlabeled ifnar1-tl was added, carefully mixed and the spectra recorded until no further change in fluorescence was detectable. ATTO655-labeled proteins were excited at 640 nm and the emission between 660 and 800 nm was recorded. Fluorescence lifetime measurements of ATTO655 were recorded with a FluoTime 200 instrument (Picoquant GmbH, Berlin, Germany) using a 640-nm LED (Picoquant GmbH, Berlin, Germany) pulsed with a 200-ps pulse width and 20-MHz repetition rate for excitation.

Electron microscopy and image processing

Samples were prepared by conventional negative staining with 0.75% (w/v) uranyl formate as described previously.35 Images were collected with a Tecnai T12 electron microscope equipped with a LaB6 filament and operated at an acceleration voltage of 120 kV (FEL Hillsboro, OR). Images were recorded on imaging plates at a magnification of 67,000× and a defocus value of ~1.5 μm. Imaging plates were read out with a Ditalbis microm imaging plate scanner (Ditalbis 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%. Pixels (2 × 2) were averaged to yield a pixel size of 4.2 Å on the specimen level. Particles were selected interactively from the images using BOXER, the display program associated with the EMAN software package.36 The selected particles (18,379 particles from 66 images for the ternary complex formed with IFNα2-HEQ and 14,782 particles from 112 images for the ternary complex formed with IFNβ) were windowed into 64 × 64 pixel images. The particle images were rotationally and translationally aligned and subjected to 10 cycles of multireference alignment using the SPIDER software package.37 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.

Real-time solid-phase detection by TIRFS and Rif

Ligand binding to ifnar1-EC mutants was probed in real time by using label-free Rif detection in combination with TIRFS. These measurements were performed with a home-built setup as described earlier38 using the 488-nm line of an argon ion laser for the excitation of OG488 and a 633-nm He/Ne laser for excitation of ATTO655. Proteins were tethered onto solid-supported membranes containing 5% bis-NTA lipids, which were obtained by vesicle fusion onto the silica substrate of the Rif transducer layer25 or immobilized on a polyethylene glycol polymer brush functionalized with tris-NTA.39 All measurements were carried out in HBS buffer as described in more detail before.17,23,38

Surface plasmon resonance

Label-free binding assays by surface plasmon resonance were conducted with a BIACore T100 (Biacore AB, Uppsala, Sweden). All measurements were carried out in HBS buffer supplemented with 0.05% surfactant P20. Ifnar1-H10, H10-SD123 and Ifnar2-H10 were immobilized on different NTA-functionalized surfaces (either commercial Sensor Chip NTA from Biacore or SIA Kit Au Chips treated with a bis-NTA thiol)40. The ligand was injected at a flow rate of 30 μl/min. Binding to the immobilized receptor subunit was followed in real time. Binding to ifnar1-H10 and H10-SD123 was probed by injecting IFNα2-HEQ. Binding to ifnar2-H10 was probed with
both wild-type IFNα2 and IFNα2-HEQ. Ligand-binding assays were carried out at temperatures between 5 and 40 °C with an increment of 5 °C. For each measurement, the chip was regenerated by sequential injections of 500 mM imidazole in HBS, 200 mM EDTA in HBS, and 20 mM NiSO₄ in HBS and then reloaded with the Histagged proteins for the next cycle. The association ($k_a$) and dissociation rate constants ($k_d$) were determined by individual curve fitting with the BIAssay evaluation software (version 3.1) using a 1:1 binding model (monoequilibrium) with variable $R_0$. Data analysis of temperature-dependent binding assays was done according to established methods 26–28 as described in detail in the Supplementary Data.

**Fluorescence correlation spectroscopy**

Single-molecule experiments were carried out with a home-built setup that was described previously. The excitation laser used was a pulsed diode laser at 640-nm wavelength (LDH 635, PicooQuant GmbH, Germany) generating pulses with ca 100-ps pulse width and 40-MHz repetition rate. The laser light was sent through a single-mode glass fiber and subsequently collimated to form a beam with Gaussian beam profile of ca 5-mm beam waist radius. The beam was then focused through an apochromatic water-immersion objective (UPLSAPO 60×, N.A. 1.2 w, Olympus) into the sample solution. The same objective collected the fluorescence emission (epifluorescence setup) that was separated from the excitation light by a dichroic mirror (650DRLP, Omega Optical). A tube lens focused it onto a circular pinhole with 100-μm diameter. After the pinhole, the light was split into two channels and refocused onto two single-photon avalanche diodes (PDM-50-C, Micro Photon Devices) after passing a band-pass filter (690DF40, Omega Optical). Fast electronics (PicoHarp 300, PicooQuant) were used for recording the detected photons in time-correlated time-tagged recording mode. From these raw data, the autocorrelation curves were calculated by cross-correlating photons from the two different single-photon avalanche diodes. Binding experiments were carried out with 1 nM ATG85 ifnar1-H10 mixed with 100 nM unlabelled ifnar1-H10 in HBS complemented with 1 mg/ml bovine serum albumin. The complex with the ligand was formed by incubating the mixture with 1 μM IFNα2-HEQ for 5 min prior to measurement. Data were acquired over 30 min.

**Stopped-flow fluorescence measurements**

Stopped-flow measurements were carried out with an SF-61, DX2 Double Mixing Stopped-Flow System (Hi_Tech Scientific) at 26 °C in HBS, pH 7.5, supplemented with 1 mg/ml bovine serum albumin. ATTO655-labeled ifnar1-H10 N349C was excited with a He/Ne laser (633 nm), and emission was detected through a band-pass filter (640–750 nm). ATTO655 ifnar1-H10 N349C (50 nM) was mixed with increasing concentrations of IFNα2 E85A (2, 8, 12.5 and 20 μM). Dissociation was measured by mixing 23 nM ATTO655 ifnar1-H10 N349C preassociated with 1 μM IFNα2 E85A with 10 μM unlabelled ifnar1-H10. OG488 ifnar1-H10 mutants were excited with a xenon lamp at 480 nm, and fluorescence was detected through a band-pass filter (500–570 nm). OG488 (50 nM)-labelled ifnar1-H10 mutants were preincubated with 100 nM ATTO655 trisNTA for 30 min and rapidly mixed with increasing concentrations of IFNα2 E85A at a final concentration between 1 and 10 μM. As a control, measurements were also carried out for OG488 ifnar2-H10 S35C as described above. Data analysis is described in detail in the Supplementary Data.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.01.017

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