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Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation

Jacob Piehler^{1,*}, Christoph Thomas², K. Christopher Garcia², and Gideon Schreiber^{3,*}

¹Department of Biology, University of Osnabrück, Osnabrück, Germany

²Departments of Molecular and Cellular Physiology, and Structural Biology, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA, USA

³Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel

Summary

Type I interferons (IFNs) form a network of homologous cytokines that bind to a shared, heterodimeric cell surface receptor and engage signaling pathways that activate innate and adaptive immune responses. The ability of IFNs to mediate differential responses through the same cell surface receptor has been subject of a controversial debate and has important medical implications. During the past decade, a comprehensive insight into the structure, energetics, and dynamics of IFN recognition by its two-receptor subunits, as well as detailed correlations with their functional properties on the level of signal activation, gene expression, and biological responses were obtained. All type I IFNs bind the two-receptor subunits at the same sites and form structurally very similar ternary complexes. Differential IFN activities were found to be determined by different lifetimes and ligand affinities toward the receptor subunits, which dictate assembly and dynamics of the signaling complex in the plasma membrane. We present a simple model, which explains differential IFN activities based on rapid endocytosis of signaling complexes and negative feedback mechanisms interfering with ternary complex assembly. More insight into signaling pathways as well as endosomal signaling and trafficking will be required for a comprehensive understanding, which will eventually lead to therapeutic applications of IFNs with increased efficacy.

Keywords

interferon; receptors; structure/function

The type I interferon system

The innate immune system is the first line of defense against infection by pathogens and against malignant cells. Jawed vertebrates that evolved an adaptive immune system of T and

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Correspondence to: Gideon Schreiber, Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, Tel.: +972 8 9343249, Fax: +972 8 9346095, gideon.schreiber@weizmann.ac.il.

*Jacob Piehler and Gideon Schreiber contributed equally as senior authors.

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B cells, also developed the type I interferon (IFN) cytokine family, which are secreted proteins dedicated to signal the presence of intracellular infection to surrounding cells and thus provide defense against viruses and intracellular bacteria (1). Upon binding to their cell surface receptor, these secreted cytokines activate the expression of over 1000 genes involved in a wide variety of activities, which were suggested to be required for targeting different viruses, with each virus targeted by a unique set of activities (2). The initiation of an antiviral state is indeed the most intensively studied outcome of IFN stimulation and is also the eponym of IFNs: these cytokines interfere with viral replication within host cells (3). The family of IFNs comprises sixteen members in humans, including IFN β , IFN ϵ , IFN κ , IFN ω , and 12 subtypes of IFN α . All IFNs bind to a shared cell surface receptor comprising two transmembrane subunits, IFNAR1 and IFNAR2 (4, 5). Following ternary complex assembly (Fig. 1), the Janus family kinases (JAK) tyrosine kinase 2 (Tyk2) and Jak1, which are associated with the membrane-proximal part of the cytoplasmic domains of IFNAR1 and IFNAR2, respectively, are activated by reciprocal transphosphorylation (6). Subsequently, they phosphorylate several tyrosine residues in the membrane-distal, intracellular domains of IFNAR1 and IFNAR2, which, in turn, recruit further effector proteins that propagate downstream signaling. This activates an antiviral response in the host organism, including direct cellular defense mechanisms and activation of further elements of the innate and the adaptive immune response. IFNs evoke a wide range of additional biological activities, both protective and counterprotective (7). For example, IFN β was shown to be lethal during certain bacterial infections (7), but protective against some protozoa and fungi (8, 9). Comparative studies of wildtype mice with IFN receptor knockout mice have revealed that in addition to its activities in defense against pathogens and viruses, IFNs play an important role in cancer prevention, cell differentiation, and cell-type-specific activities, such as dendritic and natural killer cell activation, T-cell proliferation and survival, B-cell antibody class switching, memory T-cell survival, apoptosis, and angiogenesis (10, 11).

A striking feature of IFN signaling is the high redundancy of ligands, which engage the same receptor. Interestingly, differential cellular responses have been observed for different IFNs. Although all IFNs potentially activate antiviral responses, more complex cellular responses requiring long-term signaling were reported to be preferentially activated by a subset of IFNs. A number of excellent recent reviews have covered many of the biological aspects of the type I IFN system and their importance in the immune system (12–19). Herein, we focus on the interplay between structure, energetics, and dynamics of IFN–receptor interactions and their role in regulating biological responses. On the basis of this highly quantitative understanding, we propose a model for differential cellular signaling using a single cell surface receptor.

Signaling pathways induced by type I IFNs

Following the formation of the IFN–receptor complex, the signal is propagated to various effector proteins (Fig. 1), many of which belong to the family of signal transducers and activators of transcription (STAT). Upon activation of the receptor-associated kinases Tyk2 and Jak1 by transphosphorylation, they phosphorylate several tyrosine residues on the membrane-distal part of both receptor subunits. These serve as docking sites for STAT

proteins (STAT1, STAT2, STAT3, STAT4, and STAT5), which in turn are phosphorylated by the JAKs on a specific tyrosine residue (pSTATs). pSTATs form homo- and heterodimers by respective intermolecular interactions of their Src homology 2 (SH2) domains with the phosphorylated tyrosine residue and subsequently translocate into the nucleus, where they directly regulate gene transcription. The hallmark of IFN signaling is a STAT1/STAT2 heterodimer, which, together with IRF9, forms the transcription factor ISGF3 that binds IFN-stimulated regulatory elements (ISREs) within the promoter of IFN-stimulated genes (ISGs). Other IFN-activated STATs have been described to bind the IFN γ activation sequence (GAS) elements present in the ISG promoters. ISGs encode for a multitude of proteins that are responsible for antiviral, antiproliferative, and immunoregulatory cellular responses, and it is believed that specificity is made possible by the preferential binding of different STAT dimers to specific sequence elements. For antiviral responses, primarily phosphorylation of STAT1 and STAT2 is required (13). In addition to members of the classical JAK–STAT pathway, other signaling factors have a role in IFN activity. These include isoforms of the protein kinase C and the multifunctional adapter protein CrkL, members of the p38 mitogen-activated protein kinase (MAPK) pathway, the phosphoinositol 3-kinase (PI3K) signaling pathway, and the extracellular signal-regulated kinase (ERK) MAPK pathway (12, 13). Notably, although the importance of these pathways in IFN signaling is well established in some systems, it seems that cell-type specificity plays a crucial role in their relevance.

Although all type I IFNs bind to the same cell surface receptor, they are functionally not fully redundant, and many instances of differential activities of IFNs have been reported (20–30). Differential activity refers to the observation that all IFNs hardly differ in their potencies of activating STAT phosphorylation and antiviral responses, but some members show strongly different gene expression patterns and elicit further cellular responses much more potently than others (Fig. 2A). In particular, IFN β has been shown to be capable to mount cellular responses, such as antiproliferative activity, for which very high concentrations of the α -subtypes are required that are probably above physiological levels (Fig. 2A). Interestingly, those responses specifically mounted by IFN β require long-term activation of cellular signaling, suggesting that negative feedback mechanisms may play an important role in dictating the differential IFN activities (31).

A major challenge in defining the activities of IFNs is their pleiotropy, i.e., their strongly different behavior in different cell lines. Thus, STAT activation in primary immune cells shows that the potency of IFNs in inducing phosphorylation of STAT1, STAT2, STAT3, STAT4, and STAT5 are similar in CD4, CD8, and monocytes, with up to fourfold variations in EC₅₀ values being observed between the different STATs (32). Contradicting results were shown for the variation in STAT activation in B cells versus CD4⁺ T cells and monocytes. Whereas only minor differences were observed in one study (32), much lower levels of activation of pSTATs in B cells were recorded in another study (33). All tested IFNs have shown similar potencies with respect to STAT phosphorylation (32) (Fig. 2A). pSTAT activation was found to have a short half-life peaking at about 30 min after induction. pSTAT1 and pSTAT3 decline faster than pSTAT2 (26), reaching non-detectable levels after about 16 h of continuous IFN induction (34). It is still an open question whether also non-phosphorylated STATs are active as transcription factors. It was shown that IFNs stimulate a

substantial increase in the concentration of STAT1 that persists for several days. Moreover, increasing concentrations of STAT1 were shown to result in an increased expression of IFN-induced genes (35). Consistent with these findings, STAT1 was present in the nuclei of these cells, suggesting that IFN-induced expression of unphosphorylated STAT1 may act as long-term effector of IFNs, long after the phosphorylation of STATs decays.

Upon IFN treatment, cells undergo a dramatic shift in gene expression, with over 1000 genes being affected, most of them being upregulated (36, 37). Fig. 2B shows a comparison of the number of upregulated genes upon induction with IFN α 2 at 15, 300 pM, and 3 nM concentrations versus 150 pM IFN β (31, 38). The figure shows a number of interesting characteristics of IFN-induced gene expression. At 300 pM IFN α 2 (which is a concentration saturating antiviral response, while not significantly activating antiproliferative response in WISH cells), the number of activated genes peaks after 8 h. The number of genes induced is somewhat smaller when 15 pM was applied, and larger with 3 nM. This is true, independent of the fold-change threshold. The number of activated genes at 16 h increased relative to 8 h only when 3 nM IFN α 2 or 150 pM IFN β were used. These concentrations already promote antiproliferative activity in WISH cells (39). However, even at a 20-fold higher concentration of IFN α 2 versus IFN β , a substantially higher number of genes is induced by IFN β . The EC₅₀ required for activation of individual genes shows that the concentration of IFN required to activate different genes varies (Fig. 2A). In the two examples shown, 100-fold less IFN α 2 is required to induce the expression of PKR than CXCL11, whereas much lower concentrations of IFN β are required for both genes (32, 40). In summary, it appears that IFN-induced genes can be divided into two groups: (i) genes that are highly sensitive and require only pM concentrations for activation, and (ii) genes that require 100-fold higher IFN concentrations. Analysis of gene array results has shown that genes related to the IFN antiviral activity belong to the first group (such as Mx1, PKR, and OAS2), whereas the functions of genes of the second group relate to cell proliferation, chemokine activity, inflammation, and more [e.g. IL6, CXCL11, and Trail (2, 31, 32, 38, 41–44)].

How can such differences in activity of different IFNs be communicated through the same cell surface receptor? Initially, specific, additional components have been suspected to be responsible, but these have never been found. Thus, differential recognition of IFNs by the receptor subunits IFNAR1 and IFNAR2 must be responsible for differential activity, and therefore a comprehensive picture of the structure, the energetics, and the dynamics of the IFN–receptor interactions is required.

Structure of the unbound and bound components of the type I IFN system

IFNAR1 and IFNAR2 are both transmembrane proteins that belong to the class II helical cytokine receptors (19, 45). Like other receptors in this class, the ectodomain (ECD) of the high-affinity subunit IFNAR2 comprises two fibronectin type III (FNIII)-like subdomains (5) referred to as D1 and D2, respectively. The low-affinity receptor chain IFNAR1, however, consists of four FNIII-like subdomains (SD1–SD4), which most likely emerged from gene duplication (4).

The first structure of a type I IFN (murine IFN β) was reported in 1992 (46), followed by the X-ray and NMR structures of human IFN α 2b (47, 48), as well as the crystal structure of human IFN β (49). The structure of the ECD of IFNAR2 (IFNAR2-EC) was solved using NMR (49). In that and follow up studies, models of IFN α 2–IFNAR2 complexes were built using double-mutant cycle data or/and NMR transfer data as constraints for docking (51, 52). Indeed, the determined structures confirmed that double-mutant cycle and NMR determined distance constraints are valuable inputs to obtain accurate models of complexes. In addition, low-resolution structures of the ternary complexes harboring IFN α 2 and IFN β , respectively, were obtained by single particle electron microscopy. These correctly conceived the general architecture of the ternary complex (53, 54) and showed no difference in binding of these two IFNs (Fig. 3B). More recently, the structures of two heterotrimeric type I IFN receptor–ligand complexes have been determined by X-ray crystallography, in addition to the high-resolution structures of unliganded IFNAR1 and the binary IFN α 2–IFNAR2 complex (32) (Fig. 4). Thus, we now know the experimental structures of all the components of IFN–receptor complexes in the bound and unbound state. The recently solved ternary complex structures contain two different ligands with distinct physiological activities, IFN ω and a mutant of IFN α 2, called YNS, that binds IFNAR1 50-fold tighter than the wildtype protein (see below). These are the first structures of a complete signaling complex of the class II helical cytokine family. Before, only structures of class II helical cytokines, including IFN γ , IL-10, IL-22, and IFN λ , in complex with their high-affinity receptor subunits were known (55–58). Interestingly, despite the different physiological activities of the two ligands, the heterotrimeric receptor–ligand complexes share the same architecture (Fig. 3A). Upon superimposition of the two complexes, the root-mean-square deviation of C α atoms is 0.9 Å.

IFNAR1 and IFNAR2 bind on opposing sides of the IFN ligand in an almost orthogonal arrangement that is unique among cytokine–receptor complexes. The interface between IFNAR2 and the ligand buries approximately 1800 Å² of surface area and is formed between parts of helices A, E, and the A–B loop of IFN and the D1 subdomain of IFNAR2 (Fig. 4). The loop between strands 13 and 14 is the only portion of the IFNAR2-D2 subdomain that is involved in the interface. Thus, unlike most type I and II cytokine–receptor complexes, the IFN ligand does not bind at the apex of the elbow region between the D1 and D2 subdomains of IFNAR2, but its long axis shows almost parallel alignment with the β -strands of D1 (Fig. 4). The overall IFN–IFNAR2 binding mode resembles those observed in the IFN γ –IFNGR1 (58) and IFN λ –IFNLR1 (59) complexes. However, IFN γ and IFN λ bind closer to the apex of the hinge region between the two subdomains of the receptor, and the interdomain angles in IFNGR1 and IFNLR1 are wider than in IFNAR2, allowing larger portions of the C-terminal D2 subdomain to interact with the ligand. The IFNAR2 interface is characterized by the presence of several hot spot residues.

The IFNAR1–IFN interface buries approximately 2200 Å² of surface area and is formed by contacts between helices B, C, and D of the IFN molecule (52, 60) and subdomains 1–3 of IFNAR1 (32). The membrane-proximal subdomain 4 is not involved in ligand binding (61). There was no electron density for this part of IFNAR1 in the maps of both ternary complexes (32), suggesting that it is flexible. A variable location and orientation of SD4 with respect to the remaining complex has also been suggested by electron-microscopic

studies (53, 54). As the SD1–SD2 and SD3–SD4 modules most likely arose by gene duplication and are thought to share a similar architecture and interdomain angle, it was possible to determine the approximate position of SD4 by superimposing SD1 of the SD1–SD2 module onto SD3. In this modeled complex, the C-termini of SD4 of IFNAR1 and D2 of IFNAR2 are 4.5 Å apart (Fig. 5). The binding mode of IFNAR1 is unprecedented among cytokine–receptor interactions: the IFN ligand binds to IFNAR1 at the hinge between subdomains 2 and 3, and the long axis of the helical bundle lies perpendicular to the IFNAR1 receptor chain (Fig. 4). The top of the IFN molecule is capped by SD1. In contrast to type I (e.g. human growth hormone, IL-2, and erythropoietin) and other type II cytokine–receptor systems, where the ligands interact with the loops in the ‘elbow’ region of the receptor, important receptor–ligand interactions are mediated by a region of IFNAR1 that is opposing the hinge between SD2 and SD3 (Fig. 4). The binding mode of the SD1–SD2 module conforms more to the canonical ‘elbow’-mediated cytokine–receptor interaction.

The ligand-docking mode seen in the two ternary complexes does not seem to be restricted to IFN α and IFN ω , but appears to be shared by all type I IFNs. IFN β , for example, exhibits only 30% and 33% sequence identity with IFN ω and IFN α 2, respectively. Mutational studies, single particle electron microscopy (Fig. 3B), and blocking-antibody experiments, however, suggest that IFN β shares the overall receptor-binding mode with IFN α , except for some differences in details in the interfaces (62, 63). Superimposing human IFN β [(49) PDB code 1AU1] onto the ligand in the IFN ω ternary complex structure leads to only two clashes of side chains (Tyr92 and Tyr155) with the receptors, indicating that the IFN β ligand could be easily accommodated by the receptors in a position similar to IFN ω and IFN α 2. Furthermore, the superposition of IFN β onto IFN α 2 in the IFN α 2–IFNAR2 binary complex shows that Trp22 on IFN β and Ala19 on IFN α 2 overlay onto each other and suggests a direct interaction between Trp22 on IFN β and Trp100 of IFNAR2. Trp100 of IFNAR2 has been shown to be a specific hot spot residue for IFN β binding, and double-mutant cycle analysis has demonstrated that Trp100 in IFNAR2 and Trp19 in an IFN α 2–A19W mutant interact (64), corroborating the hypothesis that Ala19 of IFN α 2 and Trp22 of IFN β occupy similar positions with respect to Trp100 of IFNAR2 in the ligand–receptor complexes. Taken together, these findings substantiate the notion that all type I IFNs share the same receptor-binding mode and form structurally highly similar ternary signaling complexes.

Conformational dynamics and its role in ligand binding and signaling

Comparison of the unbound receptor subunits with the bound forms revealed a large movement in the receptor orientation and an outward movement of IFN (Fig. 5 and <http://proteopedia.org/wiki/index.php/Journal:Cell:1>). Structural movements of receptor components upon ligand binding have previously been implicated to have an important role in signal propagation across the membrane. A mechanism that involves the transduction of a conformational change in a preformed extracellular receptor domain toward the intracellular domain and thus inducing signaling was suggested for IFN γ (65), growth hormone (66), erythropoietin (67, 68), and others (69). For the prolactin receptor, ligand-dependent conformational switch that stabilizes the dimeric state was observed, demonstrating a structural link between the WSXWS motif, hormone binding, and receptor dimerization (70).

In the case of IFNAR1, a conformational change was first observed by fluorescence resonance energy transfer (FRET) measurements (54), which showed an increase of approximately 12 Å in the distance between the C- and N-terminal domains upon ligand binding. Moreover, it was established that the ligand-induced conformational changes were propagated to the membrane-proximal Ig domain of IFNAR1, which does not interact with the ligand. This was shown by an electron transfer-sensitive fluorescence dye attached covalently to residue N349C (54, 71). Fluorescence quenching of this dye by a neighboring Trp residue (Trp347) is abrogated upon IFN binding, suggesting that the accessibility of this Trp residue is altered by a conformational change. As SD4 is not required for ligand binding (61), the observed conformational movement in SD4 suggests a transfer of signal from the IFN-binding site to the membrane-proximal domain of IFNAR1. The key role of SD4 in signaling was also shown by the inability of a chimeric IFNAR1 with SD4 being replaced with the corresponding domains of other class II cytokine receptors to form a ternary complex and to activate an IFN response (61). Both the global and the local conformational changes were observed for IFN α 2 and IFN β . Thus, although the conformational change may have important implications for the transmembrane signal activation, it is not likely that they are the basis for differential signal activation.

Energetics and dynamics of IFN recognition by IFNAR1 and IFNAR2

All IFNs bind IFNAR1 with micromolar affinity and the IFNAR2 receptor with nanomolar affinity. This highly asymmetric binding affinity is a common feature in cytokine receptors (72). However, IFN subtypes vary substantially in their respective binding affinities toward IFNAR1 and IFNAR2. A systematic analysis of all IFN α subtypes has shown that they bind IFNAR1 at affinities of 0.5–5 μ M, and IFNAR2 at affinities ranging from 0.4 to 5 nM [except for IFN α 1 – 220 nM (27, 73)]. The tightest binding IFN is IFN β , which binds IFNAR1 with 100 nM affinity and IFNAR2 with 0.1 nM affinity (74, 75). The binding affinity of IFN ω is close to that of IFN α [0.4 μ M toward IFNAR1 and 2 nM toward IFNAR2 (27)]. Comparing the product of the affinities toward both receptor subunits, as measured *in vitro* using surface plasmon resonance, to the cell surface binding affinity of a range of mutants and IFN subtypes showed a good correlation between the two (38, 73). The product of the receptor binding affinities of IFN α 2, 4, 5, 10, 17, and 21 are similar, IFN α 7, 8, and 16 have a three- to fourfold higher binding affinity, IFN ω has a fivefold higher affinity, and IFN α 6 and 14 have an eightfold higher binding affinity compared with IFN α 2. Two IFNs are clear outliers, IFN α 1 (40-fold lower affinity) and IFN β (1000-fold higher affinity) than IFN α 2.

Likewise, the differences in the kinetics of the interactions of IFNs with the receptor subunits are characteristic. All IFNs rapidly bind to IFNAR2 with association rate constants of 10^6 – 10^7 /M/s (27, 63, 73, 75), which have been shown to be enhanced by electrostatic attraction (77). In contrast, the association with IFNAR1 is relatively slow, with association rate constants of approximately 5×10^5 /M/s. Differences in binding affinities between different IFNs are mainly manifested as differences in the dissociation rate constants. Thus, the lifetime of IFN α 2 in complex with IFNAR1 is approximately 1 s (78), whereas it is approximately 100 s in case of IFN β . The lifetimes of IFN complexes with IFNAR2 range from approximately 10 s for IFN α 1 over approximately 100 s for IFN α 2 and IFN ω to 1000 s for IFN β .

To obtain a comprehensive biophysical understanding of the relations between sequence, three-dimensional structure, energetics, and function, the ligand–receptor interfaces between IFN and IFNAR1 and IFNAR2 were subjected to systematic alanine scanning mutagenesis, and the binding affinities and biological activities of the muteins were determined. The most comprehensive mutational analysis was carried out using IFN α 2 as template, but mutational studies were also done with IFN ω , IFN β , and several other IFN α subtypes (32, 62, 79). The energetic contributions of amino acid residues in the binding interfaces of IFNAR1 and IFNAR2 with IFN α 2 are summarized in Fig. 6A in an open-book representation of the ligand–receptor complexes. The two IFN α 2 representations are rotated by 180° with respect to each other. Side chains are colored according to their contribution to binding. In the high-affinity IFN α 2/IFNAR2 interface, hotspot residues are located at the center, surrounded by a ring of residues contributing less, with mutations of the outer-ring residues having only a minor effect on binding. Conversely, on the IFNAR1 binding site of IFN α 2, only a single hotspot residue (R120) was found (80). On IFNAR1, two hotspot residues located on SD2 and SD3 were identified (32). This fragmented distribution of binding energies may explain the low affinity of IFNs toward IFNAR1. The IFNAR1 binding site buries 2200 Å² of surface area, whereas the IFNAR2 binding site buries 1840 Å², showing again that the size of the interface is not related to its binding affinity.

Mutagenesis and structural studies have identified the IFNAR2 binding site of IFNs to be located on helix A, the A–B loop, and helix E, whereas the IFNAR1 binding site involves helices B, C, and D (Fig. 7A). By far the most important hotspot in the IFN–IFNAR2 binding interface is the conserved Arg33^{IFN}. Replacing this residue in IFN α 2 by alanine destabilizes binding by over four orders of magnitude, with even the conserved mutation R33K reducing the binding affinity by 1000-fold (39). This is due to five side chains to backbone interactions of Arg33^{IFN} to Ile45, Met46, Lys48, Pro49, and Glu50 on IFNAR2, in addition to the single side-chain–side-chain interaction with Thr44 (Fig. 7C). A second critical residue located on the A–B loop is L30: mutating this residue to Ala reduces binding by over 100-fold (39). Interestingly, the structures of the binary and ternary complexes do not reveal a direct strong contact between Leu30^{IFN} and IFNAR2, suggesting that this residue may stabilize the IFN-binding site rather than directly contributing to binding. Two hydrophobic-interaction clusters are present in the IFN α –IFNAR2 interface, one is formed between Leu15^{IFN}/Met16^{IFN} and Trp100^{R2}/Ile103^{R2}, and the second one comprises Leu26^{IFN}/Phe27^{IFN}/Val142^{IFN} binding Met46^{R2}/Leu52^{R2}/Val80^{R2}/Thr44^{R2}. Mutations of these residues (except Met16^{IFN}) reduce binding by 3- to 50-fold. While none of the mutations in IFN to alanine increases binding, we found that replacing the C-terminal tail of IFN α 2 with the strongly positively charged tail of IFN α 8 increases binding to IFNAR2 by 20-fold [fourfold slower dissociation and fivefold faster association (64)]. Further mutagenesis work suggested that this is a result of binding to the three negatively charged residues Glu 132–134 on IFNAR2.

Except for Arg120^{IFN}, no hotspot residue was identified on the IFNAR1 binding surface on IFN (60, 80) (Figs 6A and 7A). Surprisingly, three neighboring interface residues on IFN α 2 (His57, Glu58, and Gln61, circled in Fig. 6A, C) were identified to increase binding affinity to IFNAR1 upon mutation to alanine (31). Two of the three residues (Glu58 and Gln61) are conserved between all IFNs, whereas His57 is conserved in all but IFN β (Fig. 7A).

Selection using phage display has identified the triple mutant H57Y, E58N, Q61S to increase the binding affinity to IFNAR1 by 50-fold [to 30 nM (81)]. With an affinity threefold higher than that of IFN β , the IFN α 2-YNS mutant is currently the IFN with the highest affinity for IFNAR1. Comparing the ternary complex structure of IFN ω (which contains the original HEQ sequence) with that of IFN α 2-YNS does not reveal the underlying reason for the 50-fold stabilization of this mutant, as no apparent clashes or stabilizing effects are observed (Fig. 7B). The fact that the IFN α 2 affinity toward IFNAR1 was readily increased by individual mutations clearly suggests that weak binding to the IFNAR1 receptor chain is of biological importance, as it is conserved between the different subtypes.

Fig. 7 shows the consensus sequence of IFNs, the location of the binding interfaces, and the energetic consequence of mutations. As can be expected, most (but not all) residues that affect binding affinity are located at the interfaces; however, only five of 34 interface residues are fully conserved. Two of those are His57 and Glu58, which upon mutation into Ala increase binding to IFNAR1. There is also no direct relation between energetically important residues and their conservation. Still, three of six binding hotspots are fully conserved, and the other three are conserved in all IFNs, except for IFN β or IFN ω . The mutation data suggest that there are multiple solutions to obtain a similar binding affinity between IFN and its receptors.

Promiscuity of the receptor interface tolerates binding of the different type I IFNs

A detailed analysis of the residues involved in the IFN α 2–IFNAR2 versus IFN ω –IFNAR2 interaction highlights the complexity of IFN crossreactivity (32). An example of structural differences that affect the relative binding affinities is Arg149 in IFN α 2 and the analogous Lys152 in IFN ω and their respective energies of interaction with Glu77^{R2}. Whereas Arg149 is a hotspot in IFN α 2, Lys152 is of lesser importance for binding of IFN ω (32). Indeed, the binding affinity of IFN ω to IFNAR2 is increased by the K152R mutation. Additional examples of IFN sequence differences playing a role in ligand subtype discrimination include Leu26^{a2} (the L26A mutation causes a fivefold reduction in binding affinity), which corresponds to Pro28 in IFN ω . The IFN ω mutation P28A had no effect on receptor binding, whereas swapping Pro28 ω with Leu26^{a2} (i.e. P28L ω) reduced binding by sixfold in IFN ω . Thus, these residues have evolved distinct energetic contributions by substituting side chains. Two additional residues that differ between IFN α 2 and IFN ω in the IFNAR2 binding site are Phe152^{a2} that is a Ser in IFN ω , and Ala145^{a2} that is Met148 in IFN ω . The mutation S155F ω reduces binding by 3.5-fold, while F152A^{a2} reduces binding by 10-fold (32). Within IFN α subtypes, alanine is the consensus residue in position 145 (148 in IFN ω). Still, M148A ω reduces binding by approximately 2.5-fold. The complementary mutation of A145M in IFN α 2 reduces binding by approximately sixfold. This shows that Ala in this position is preferred for IFN α subtypes, whereas Met is preferred for IFN ω (and Val is preferred for other IFNs). These examples demonstrate again that swapping of these residues does not lead to reconstitution of the donor affinity.

On IFNAR2, the V80A mutation differentially affects IFN binding, with the contribution to IFN ω being significantly smaller than to IFN α 2 binding. Two other residues in IFNAR2, His76^{R2} and Met46^{R2}, contribute to ligand discrimination. The double mutation on IFNAR2, H76A, N98A increases the binding affinity to IFN β by 50-fold, while having no effect on binding to IFN α 2 (63). The high affinity toward IFN β makes the soluble, ECD of IFNAR2 with the 76/98 to Ala mutation an ideal carrier protein or antagonist for IFN β , while not affecting IFN α binding or activity. Two other mutations on IFNAR2 that differentially affect IFN β and IFN α 2 binding are M46A that results in a 500-fold decrease in IFN α 2 binding, but only 10-fold decrease in IFN β binding, and W100A that reduces binding to IFN α 2 by threefold and binding to IFN β by 100-fold (82).

While mutation coverage of IFNAR1 is less complete than the mutational analysis of IFNAR2, a number of residues were found that upon mutation differently affect binding to IFN subtypes. Most notable is N155T that has no effect on binding of IFN α 2 or IFN ω , but increases the affinity toward IFN β by eightfold [resulting in a nanomolar affinity interaction between these two proteins (54)]. Conversely, the IFNAR1 mutant E111A reduces the affinity toward IFN α 2 by threefold and to IFN β by >10-fold. A third residue is N242, which, upon mutation into Ala, reduces the affinity to IFN α 2 by twofold, but increases the affinity to IFN β by 50%. Overall, the mutagenesis data clearly show that in particular ligand binding to IFNAR1 but also to IFNAR2 is not optimized for high affinities. Moreover, specific amino acids on the receptors confer different energetic contributions toward different IFN subtypes. In conclusion, it appears that IFNs and their receptors have evolved to keep a certain range of binding affinities with significant variations in the absolute binding affinity as well as the ratio of binding affinity toward the receptor subunits. The IFNAR1 binding affinity of all IFNs is always much lower than the affinity for IFNAR2. With respect to the integral binding affinity toward the cell surface receptor, the range between the tightest binder IFN β and the weakest binder IFN α 1 covers more than three orders of magnitude. The IFN α subtypes, however, bind within a relatively narrow range of affinities, in between those measured for IFN α 1 and IFN β . These insights substantiate a hypothesis that rather than the structure of the signaling complex, the large differences in binding affinities and complex stabilities of IFNs are responsible for differential activity.

Systematic correlation of binding affinities with IFN activities

The detailed energetic information on the binding interfaces and the possibility to systematically reduce and increase IFNs affinities toward IFNAR1 and IFNAR2 allowed exploring the role of receptor binding affinity in activating biological activities. Using protein engineering, the complete range of naturally occurring binding affinities of the different IFNs was reconstructed on the template of IFN α 2 (38–40, 60, 81). Using these muteins allowed investigating the relations between affinities to either one of the receptors and the seemingly differential biological activities of the different IFNs. To this end, the relative inverse EC₅₀ values of antiviral and antiproliferative dose–response analyses were compared with the product of the binding affinities toward the two-receptor subunits (Fig. 8A). A very good linear relation between relative binding affinity and antiproliferative potency is observed spanning five orders of magnitude (Fig. 8A). Fig. 8B shows that the binding affinities measured for the different IFN α subtypes also correlate with their

antiproliferative potencies. This strict correlation clearly demonstrates that antiproliferative activity is foremost determined by ligand affinity. In contrast, the antiviral activity correlates with the affinity only for low binding affinities (Fig. 8A). For IFNs with binding affinities higher than IFN α 2, only a marginal increase in the antiviral activity is observed. Moreover, the slope of the linear affinity–activity correlation for mutants with lower binding affinity than wildtype IFN α 2 is below 1, suggesting that binding affinity is not the sole factor dictating antiviral potency (39). Thus, rather than the higher antiproliferative activity of IFN β compared with IFN α 2, which can be explained by its higher binding affinity, the very similar antiviral activity of these two IFNs is a puzzling factor responsible for their differential activity.

Consistent with the mutant data, significant variations between receptor binding affinity and antiviral potency were observed also between the different IFN α subtypes and between the different viruses and cell lines used (Fig. 8C). The low activity of IFN α 1 is consistent with its low receptor binding affinity, whereas the relative high antiviral activity of IFN α 17 is surprising (83), as its binding affinity is similar to IFN α 2. Yamamoto *et al.* (79) selected by phage display IFN α 8 variants that differ by 100-fold in their antiviral potency on amnionic FL challenged with Sindbis virus versus LS 174 T cells challenged with VSV. This finding suggests that yet unknown factors tune the specific antiviral potency (in addition to binding affinity). One suggestion was that structural changes in the C-helix of IFN α alter the ability of IFN to limit retroviral activity and reduce toxicity (84). Classifying IFN α proteins by their ability to induce specific genes showed that this is a good marker for their ability to confer cellular protection against pathogens, independent of the target or cellular background. Differences in IFN activity were only observed at subsaturating levels. The divergent potencies of IFN α s and the cell-type-specific regulation of target genes may result in the tuning of the cellular defense (10). Another way to analyze the importance of individual IFN subtypes is by monitoring their divergence in human population. Type II and type III IFNs as well as some type I variants have evolved under strong purifying selection, whereas other type I variants have more relaxed selective constraints, in agreement to the degree of importance in immunity to infection (85).

Systematic modulation of binding affinities toward IFNAR1 and IFNAR2 allowed addressing the question whether higher binding to either of the two-receptor subunits dictates a specific activity (38). Increasing binding to IFNAR1 while decreasing binding to IFNAR2 affected the antiproliferative potency consistent with the change in the integral affinity toward both receptor subunits, independent on the identity of the high-affinity receptor. A similar result was also obtained for the antiviral activity; however, the relation between affinity and activity was much weaker as described above (Fig. 8A). Another critical difference between the activation of an antiviral state and of an antiproliferative response is the duration of IFN induction. To initiate antiviral activity requires a few hours of IFN, whereas antiproliferative activity requires days of constant IFN induction.

The number of surface receptors is another important variable in signaling, which differs between individual cells (76). Manipulating receptor expression by siRNA concentrations reduced the fraction of responsive cells independent of the IFN used. A correlation between receptor numbers, STAT activation, and gene induction was observed. Our data suggest that

for a given cell the response is binary ($\pm$) and dependent on the stochastic expression level of the receptor subunits on an individual cell. All of our data suggest that the antiviral activity of IFN is a robust feature, requiring very low ligand and receptor concentrations over a short time and is common to all cells. Conversely, antiproliferative activity (and other IFN-related activities requiring signaling over longer time periods) requires high IFN and receptor concentrations as well as tight binding over a prolonged time and thus is a tunable function of IFN that strongly differs between different cells and different IFNs (40). Indeed, it was shown that IFN-receptor expression regulates the antiproliferative effects of IFNs on cancer cells and solid tumors, suggesting that IFN-receptor expression can play a major role in determining the clinical outcome of IFN-based cancer therapeutics (86).

These systematic correlations of binding affinities with functional properties of IFNs in different cellular context clearly established the key role of receptor binding affinity for differential IFN activity. For the proof-of-concept studies, an engineered IFN α 2 variant containing the mutations H57A, E58A, and Q61A (IFN α 2-HEQ) was used, which binds IFNAR1 with a similar affinity as IFN β (31). Strikingly, this IFN α 2 mutant already very closely mimicked the functional properties of IFN β (Fig. 9), whereas STAT activation and antiviral activity by IFN α 2-HEQ increased only slightly compared with wildtype IFN α 2, a dramatic increase in the antiproliferative activity was obtained (Fig. 9A). IFN α 2-HEQ also reproduced the gene activation pattern of IFN β with high fidelity, which was not observed for wildtype IFN α 2 even at strongly elevated concentrations (Fig. 9B). By further optimization of the binding affinity toward IFNAR1 (IFN α 2-YNS), the differential activity compared with wildtype IFN α 2 was even further increased (81).

The dynamics of receptor assembly on artificial membranes and in living cells

How can differential binding affinities modulate cellular response patterns? To answer this question, a quantitative mechanistic understanding is required on the role of differential binding affinities and rate constants for the formation and the dynamics of the ternary IFN-receptor signaling complex on the plasma membrane. To this end, extensive studies were performed *in vitro* using artificial membranes. To mimic membrane anchoring of the receptor subunits, the extracellular domains of IFNAR1 and IFNAR2 were site-specifically tethered onto solid-supported membranes through their C-terminal His-tag (75, 87, 88). For probing IFN binding and ternary complex assembly in a quantitative manner, real-time detection by simultaneous total internal reflection fluorescence spectroscopy and reflectance interference was used (87). By using this approach, the surface concentration of the receptor subunits could be readily controlled and quantified and the kinetics of interactions could be probed in a highly versatile manner (89).

No interaction between the receptor subunits was detectable in the absence of IFN and a two-step assembly of the ternary complex was observed (Fig. 10): after IFN binding to the high-affinity subunit IFNAR2, IFNAR1 is subsequently recruited on the membrane. The sequence of binding events is determined by the substantially faster binding of IFN to IFNAR2 than to IFNAR1 (k_a^B) rather than the higher equilibrium binding affinity (K_D^B). This

mechanism entails a dynamic equilibrium between binary and ternary complexes on the plasma membrane, which is determined by (i) the affinity of IFN toward IFNAR1 (K_D^T), and (ii) the concentration of the receptor subunits. Both, the concentrations of the receptor subunits and K_D^T refer to area (e.g., molecules/ μm^2) rather than to volume (e.g., μM) concentrations because the receptor subunits are anchored in the membrane. Thus, K_D^T cannot be readily inferred from the equilibrium constant of the binary complex as determined by standard-binding assays. However, the equilibrium between binary and ternary complex can be assessed by ligand-binding experiments (78). As ligand dissociation from the ternary complex is much slower than from the binary complex, the equilibrium between binary and ternary complexes also affects the total ligand-binding affinity to the cell surface receptor (Fig. 10B). Thus, the decrease in ligand dissociation kinetics compared with the binary IFN–IFNAR2 interaction can be used as a measure for ternary complex formation (87). Based on this approach, K_D^T was determined for the ligand IFN α 2 to be approximately 40 molecules/ μm^2 , which in solution binds IFNAR1 with a K_D^B of approximately 5 μM . This K_D^T is surprisingly high compared with the concentration of the IFNAR1 and IFNAR2 in the plasma membrane, for which typically only a few hundred copies per cell are found (i.e., 0.1–1 molecule/ μm^2). Thus, the formation of ternary complexes by IFN α 2 and other IFN α subtypes, which all have very similar binding affinities toward IFNAR1, is probably limited by the physiological cell surface concentrations of the receptor subunits. Under these conditions, two interdependent equilibria have to be considered: (i) IFN in solution and bound to the cell surface receptor, and (ii) binary and ternary complexes. In contrast, the approximately 30-fold higher binding affinity of IFN β toward IFNAR1 (see above) probably ensures efficient ternary complex formation at physiologically relevant receptor surface concentrations. It is important to note that the limitation of ternary complex formation by K_D^T cannot be compensated by increasing the IFN concentration, as the cell surface concentration of IFN α 2 bound to IFNAR2 is the determining parameter. Thus, differential efficacy in the recruitment of IFNAR1 into the ternary complex achieved by IFN α subtypes and IFN β would be a good candidate to explain the biophysical basis of differential activities.

A dynamic equilibrium between binary and ternary complexes results in a constant exchange of receptor subunits. This may play a critical role for signaling, as the lifetime of individual signaling complexes is given by K_d^T . Again, the 2D rate constants K_d^T and K_d^T cannot be readily inferred from the corresponding 3D rate constants obtained by classic-binding assays, as membrane anchoring also changes the energy landscape and thus the reaction coordinate. By exploiting the experimental flexibility of the model system described above, the rate constant K_d^T of individual ternary complexes was probed by FRET and ligand chasing experiments (78, 88, 89). These experiments yielded a complex stability that is three to fivefold higher compared with the same interaction measured in 3D. From K_d^T and K_D^T , also K_a^T was estimated. Interestingly, collisions on the membrane are 10- to 100-fold more productive with respect to complex formation when compared with the

interaction in solution. At the same time, the number of collisions is lower on the membrane because of the reduced diffusion constant.

These results obtained with artificial, fully homogeneous membranes, which allow free diffusion of the tethered receptor subunits, suggest that the much more spatial organization and diffusion of the receptor subunits in the plasma membrane (90) may play an important role for the dynamics of individual ternary complexes. A detailed, quantitative picture of assembly and dynamics of the IFN signaling complex in the plasma membrane is currently not available, but some important features are emerging. For several homodimeric class I cytokine receptors, pre-association of the receptor subunits has been observed (66–68), and this has also been suggested to hold true for the IFN γ receptor (64), which belongs to the class II cytokine-receptor family. For IFNAR1 and IFNAR2, pre-association of the receptor subunits has not been detected in living cells (6). However, the more than 10-fold increased ligand-binding affinity compared with binding to IFNAR2 only – even at endogenous receptor levels (6) – suggests approximately 100-fold more efficient recruitment of IFNAR1 than predicted by the studies described above (Fig. 10B). As the concentration of the receptor subunits plays a critical role for the equilibrium between binary and ternary complexes, studies with cells overexpressing IFNAR1 and IFNAR2 as required for fluorescence imaging techniques are of limited use. Imaging techniques with single molecule sensitivity are required for resolving receptor assembly at physiological expression levels. Tracking of individual IFNAR1 and IFNAR2 yielded strongly inhomogeneous diffusion behavior with average diffusion constants of 0.05–0.08 $\mu\text{m}^2/\text{s}$ (6, 91), which can be ascribed to corraling by the cortical actin skeleton (92). Pre-assembly or clustering of IFNAR1 and IFNAR2 was not observed in absence of the ligand (JP, unpublished results). IFN stimulates very rapid formation of ternary complexes, which dynamically form and dissociate as suggested by the artificial membrane system described above (JP, unpublished results). However, not only the efficiency of ternary complex formation but also the lifetime of individual complexes is substantially higher in the plasma membrane of living cells compared with the artificial membrane (JP, unpublished results). These results suggest that further organization principles such as lipid microdomains and/or the membrane-proximal actin corrals may contribute to the receptor assembly on the cell surface (90).

The importance of the lifetime of individual complexes (determined by K_d^T) for signaling is not clear. Short-term signal activation measured as STAT phosphorylation levels does not significantly differ for stimulation by IFN α_2 or IFN β despite the strong differences in affinity of the interaction with IFNAR1 and the resulting long lifetime of individual ternary complexes. Upon a further decrease in the stability of the interaction of IFN α_2 with IFNAR1, a strong loss in STAT phosphorylation is observed (60, 80), but this is probably due to the loss in ternary complex formation. The increased lifetime of ternary complexes formed with IFN β compared with IFN α_2 , however, could be responsible for more efficient activation of other, STAT-independent signaling pathways and thus explain differential IFN activities. So far, this has not been experimentally demonstrated.

Receptor endocytosis and negative feedback mechanisms and their possible role for differential signaling

The numbers of IFNAR1 and IFNAR2 on the cell surface are notoriously low, between 100 and a few 1000 copies/cell, dependent on the cell type (76). Cell surface expression and endocytosis of the IFN-receptor subunits is regulated on different levels and endocytic trafficking is not fully resolved. The cytoplasmic domain of IFNAR1 contains an endocytic motif mediating recruitment of the AP2 complex, which is responsible for stimulation-independent endocytosis and degradation of IFNAR1 (93). Interestingly, this motif is masked by the JAK kinase Tyk2, which is constitutively associated with IFNAR1, thus stabilizing cell surface expression of IFNAR1 (93). Upon IFN-induced receptor activation, ubiquitination of IFNAR1 is induced via specific phosphoserine residues on the cytoplasmic domain of IFNAR1 (94). These serve as docking sites for an ubiquitin E3 ligase, which in turn ubiquitinates IFNAR1 on several lysine residues (95). The ligase is recruited to IFNAR1 upon its degron phosphorylation by PKD2, the inhibition of which (and subsequent increase in IFNAR levels) increases the sensitivity of cells to IFN (96). Interestingly, ubiquitination results into a conformational change, by which the constitutive endocytic motif is unmasked (97), leading to endocytosis of the activated signaling complex. Endocytosis of the activated IFN signaling complex was confirmed to follow a classic clathrin –dynamin pathway (98). The non-catalytic role of Tyk2 in sustaining the steady-state IFNAR1 level at the plasma membrane is well documented (99). Recently, it was shown that SOCS1 acts in sequestering the IFN signal by directly interacting with Tyk2. However, the SOCS1 inhibition of Tyk2 does not only inhibit Tyk2 kinase-mediated STAT signaling but also negatively impacts IFNAR1 surface expression, which is stabilized by Tyk2, and thus provides an additional level of regulation (100). Analysis of the endosomal compartment after IFN stimulation revealed ubiquitinated IFNAR1 and a small amount of IFNAR2 as well as tyrosine-phosphorylated Tyk2 and Jak1, suggesting continuous signaling during endocytosis, which may relate to the ability of IFN to induce a multitude of signals (101). Despite the extensive work showing the importance and mechanism of IFNAR1 ubiquitination and its importance in endocytosis and subsequent sorting, no clear evidence exists to show that ubiquitination has a positive role in differential signaling by IFNs (102).

A rapid decrease in both IFNAR1 and IFNAR2 levels on the cell surface is observed after IFN stimulation (31, 32, 95, 103). Whereas transient downregulation was observed also when applying low-IFN concentrations, a tight correlation was observed between the ability of IFNs to continuously induce endocytosis of IFNAR1 and IFNAR2 and their antiproliferative potency (81), with tighter binding IFNs (such as IFN β or IFN α 2-YNS) promoting an increased receptor downregulation (32). However, tight binding to only one of the receptor subunits, without binding to the second receptor subunit, will neither promote antiproliferative activity nor cause receptor downregulation, and thus will act as an antagonist (80). Moreover, irreversible IFN uptake is observed, confirming endocytosis of the entire signaling complex. Interestingly, the K152R mutation on IFN ω , which specifically increases binding to IFNAR2 by fivefold, dramatically increases IFNAR2 endocytosis (32). Thus, also the interaction with IFNAR2 seems to play a critical role for regulating endocytosis or endocytic trafficking.

Several implications of IFN-receptor endocytosis for (differential) signaling have been suggested. (i) The resulting decrease in surface concentration of IFNAR1 and IFNAR2 on the cell surface probably shifts the equilibrium from ternary to binary complex (31). Indeed, the concentrations of IFNAR1 and IFNAR2 in the plasma membrane play a critical role for their sensitivity toward IFNs. While in cells with native receptor numbers IFN β was more potent than IFN α 2 in antiproliferative activity, IFN α 2 matched IFN β in cells with highly increased receptor numbers (76). Decreasing the cell surface expression of IFNAR subunits has been shown to affect responsiveness toward IFN α 2 stronger than toward IFN β (40). This can be explained by more efficient recruitment of IFNAR1 by IFN β due to its higher affinity. These observations could explain why long-term signaling is possible by IFN β but not IFN α 2. (ii) It is very likely that entire signaling complexes are taken up by endocytosis. Signaling may proceed in early endosomes, and notably, even a critical role of endocytosis for signaling has been suggested (98). In any case, endocytosis could explain why the potency of IFNs with respect to very early cellular responses does not increase with affinity above a certain threshold (Fig. 8A): an increase in binding affinity is typically accompanied by an increase in complex stability; if reversible ternary complex formation is followed by irreversible endocytosis, an increase in complex stability pays off only up to a certain threshold, above which complexes are faster endocytosed than they dissociate (Fig. 11).

Specific functions of IFN β compared with IFN α subtypes require long-term activation of the signaling complex. Thus, negative feedback mechanisms on the level of gene transcription are likely to play a critical role in regulating differential cellular responses. An important negative feedback mechanism is based on IFN-induced expression of the isopeptidase USP18 (UBP43). USP18 specifically removes the ubiquitin-like protein ISG15 from target proteins (104). Protein ISGylation has complex regulatory functions in IFN signaling (105). Interestingly, USP18-deficient mice are hypersensitive to IFN, suggesting a critical role in negative feedback regulation (106). Rather than by its enzymatic activity, USP18 was shown to interfere with IFN signaling by binding to the membrane-proximal region of the cytoplasmic domain of IFNAR2 (107). Interestingly, USP18 regulates signal activation on the level of the assembly of the signaling complex, as binding and uptake of IFN α 2 is strongly reduced in cells expressing USP18 (108). Binding of USP18 to the cytoplasmic domain of IFNAR2 does not affect the binding affinity of the extracellular domain, which binds IFNs independent of the membrane and the transmembrane domain (JP, unpublished results). Rather, it appears that USP18 interferes with the assembly of the ternary complex by increasing K_D^T substantially above the cell surface concentration of the receptor subunits. This hypothesis is consistent with the observation that expression of USP18 results in a strong loss of sensitivity toward IFN α 2 but not toward IFN β (108). This can be explained by the substantially higher binding affinity of IFN β toward IFNAR1, corroborating the notion that binding of USP18 to the cytoplasmic domain of IFNAR2 interferes with ternary complex formation. However, the important consequence of differential negative feedback by USP18 is that signaling by IFN α subtypes but not by IFN β is abrogated by the first wave of cellular responses. This could explain why IFN β can more efficiently elicit responses, which require maintaining signaling over extended time periods.

Concluding remarks

In this review, we have highlighted the comprehensive insight into the structure, energetics, and dynamics of IFN recognition by its receptor subunits, and how this is translated into gene induction and biological activities (briefly summarized in Fig. 11A). One of the challenges of studying type I IFNs is that they are produced by and act on all nucleated cells and activate a wide range of activities. The common denominator for all type I IFNs is their ability to induce an antiviral state as part of the innate immunity. More cell-type-specific activities include the promotion/inhibition of cell differentiation, antiproliferation (cell cycle arrest and apoptosis), and others. The differential activities of IFNs are manifested as different affinity–activity relationships for these different cellular responses. One cannot expect to obtain a simple yet comprehensive model to explain IFN action in all cell types, particularly as signal transduction cascades may vary in different backgrounds. However, based on the conceptual understanding of receptor assembly and dynamics and its regulation by endocytosis and USP18, we can put forward a basic model, how differential IFN activity can be mediated through a shared receptor (Fig. 11B). In naïve cells, all type I IFNs apparently bind the same two receptor subunits at the same orientation but with varied affinities. Still, at physiological receptor densities, IFN binding to the cell surface receptor rapidly induces formation of the ternary signaling complex with similar efficiency for all IFNs. Binding affinities even of the IFN α subtypes toward IFNAR1 (K_D^T) and the receptor surface concentrations are probably adjusted to a ratio, which allows efficient recruitment of IFNAR1 into the ternary complex (Fig. 11B, bottom-right insert). After signal activation, signaling complexes are rapidly endocytosed. Thus, the rate of endocytosis and degradation/recycling rather than the ligand dissociation kinetics dictates the decomposition of the signaling complex. This could explain why similar activities can be observed for all IFNs except IFN α 1 in the early STAT phosphorylation and the immediate antiviral responses. For IFN α 2 mutants with decreased complex lifetimes (as well as for IFN α 1), endocytosis competes with ligand dissociation. Thus, the activities of these IFNs decrease with decreasing receptor binding affinity. Differences in potencies of IFN α subtypes may be explained also by differences in endocytic trafficking (i.e. rates of degradation and recycling), an issue requiring further investigation. IFN α 2 mutants with lower binding affinity dissociate more rapidly than they are endocytosed and, for this reason, show reduced activity compared with wildtype IFN α 2.

After the first wave of gene transcription/translation, negative feedback by USP18 reduces IFN affinity to the cell surface receptor, probably caused by increasing K_D^T toward IFNAR1 ('primed cells' in Fig. 11B). As the lifetime of the ternary complex is reduced, ligand dissociation competes with endocytosis and the activity of IFN α 2 in primed cells decreases. Owing to its much higher binding affinity, IFN β (as well as IFN α 2 mutants with increased binding affinities) can even in the presence of USP18 form highly stable ternary complexes, which are probably endocytosed prior to dissociation. Thus, the responsiveness of primed cells is substantially more reduced for IFN α 2 compared with IFN β , and cellular responses requiring prolonged signaling can be sustained more efficiently by IFN β compared with the IFN α subtypes. This model implicates that the low binding affinity of IFN α subtypes toward IFNAR1 relative to the receptor cell surface expression level is evolutionary

optimized to a level that allows selective tuning by the negative feedback regulator USP18. For cells with an increased level of cell surface receptor expression, this regulatory mechanism is not viable, which is consistent with the observation that differential activity is observed only for cells with low receptor expression levels. This model explains why receptor numbers affect biological outcome (Fig. 11C). Cells with very high receptor numbers will be able to initiate an antiproliferative response even with lower concentration of IFN α , whereas cells with very low receptor numbers will not be able to initiate such response even with high IFN β . Conversely, the antiviral response is robust and will be initiated both with high and low receptor numbers.

Our model explains why the low binding affinity of IFN α subtypes appears to be evolutionary conserved, i.e. optimized for ensuring that cellular responsiveness is abrogated by the early cellular response. However, other features of differential IFN signaling remain enigmatic. For example, signaling leading to IFN-induced antiproliferative activity that requires the continuous presence of IFN at concentrations that saturate the surface receptors for a prolonged time cannot be explained by STAT signaling alone. Moreover, pSTATs are not detectable during long-term stimulation that is a requirement for this activity. Also the molecular mechanism supporting induction of transcription of many antiviral genes by picomolar IFN concentrations, whereas other genes require 100-fold higher concentration is not resolved. The linear relation between antiproliferative potency and binding affinity and the higher number of activated surface receptors required for the induction of antiproliferative activity suggest that this activity may be dominated by alternative signaling cascades. It is possible that the higher lifetime of individual ternary complexes formed by IFN β compared with IFN α allows more efficient activation of alternative signaling pathways that are not related to the antiviral activity. In this context, the role of endosomal signaling, sorting, and trafficking of the IFN–receptor complex remains to be explored in more detail. Answers to these questions may hold the promise to apply natural and engineered IFNs in more efficacious ways to combat diseases, such as cancer, multiple sclerosis, viral infections, and others.

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Fig. 1. The type I interferon (IFN) signaling network

By simultaneous interaction of IFNs with the two-receptor subunits IFNAR1 and IFNAR2, the active signaling complex is formed. Subsequently, the tyrosine kinase 2 (Tyk2) and Janus family kinases (Jak1) associated with IFNAR1 and IFNAR2, respectively, transphosphorylate each other, and phosphorylate-specific tyrosine residues of IFNAR1 and IFNAR2 (indicated as red dots). These serve as docking sites for effector proteins of the signal transducers and activators of transcription (STAT) family. Upon phosphorylation,

STAT1 and STAT2 form homo- and heterodimers, which translocate into the nucleus to activate transcription.

Fig. 2. Differential activities of interferon α 2 (IFN α 2) and interferon β (IFN β) in WISH cells (A) Comparison of the potencies of IFN α 2 (blue) and IFN β (pink) in signal transducers and activators of transcription (STAT) phosphorylation (pSTAT), gene expression [protein kinase R (PKR) and CXCL11], as well as antiviral (AV) and antiproliferative (AP) activity. (B) Gene induction after activation at different concentrations and times (levels of gene induction: blue, >2-fold; red, >3-fold; green, >5-fold; violet, >10-fold).

Fig. 3. Architectures of the ternary complex observed for different interferons (IFNs)
 (A) Overlay of the X-ray structures of IFN α 2 (red) and IFN ω (blue) in complex with IFNAR1 and IFNAR2. (B) Averaged single particle electron microscopy (EM) images of the ternary complex with IFN α 2 (left) and IFN β (right).

Fig. 4. X-ray structure of IFN ω (brown) in complex with IFNAR1-ectodomain (ECD; blue) and IFNAR2-ECD (green)

The helices of IFN ω are labeled with the letters (A–E). The FcIII-like domains of IFNAR1 (SD) and IFNAR2 (D) are numbered starting from the N-terminus.

Fig. 5. Ligand-induced conformational changes in IFNAR (based on a comparison of unbound and bound structures)

The bound conformation is in blue. SD4 of IFNAR1 was not visible in the X-ray structures and was modeled for clarity.

Fig. 6. Functional characterization of the interferon-receptor complex

Interferon alpha 2 (IFN α 2) is pictured with its IFNAR1 binding site (left) and IFNAR2 binding site (right). (A) Changes in affinity upon mutation: red, >10-fold reduction; orange, 2- to 10-fold reduction; blue, no change; magenta, increase in binding affinity upon mutation. (B) Electrostatic potential of the proteins. (C) Residue conservation between IFN α 2, IFN α 8, IFN β , and IFN ω and the location of IFN interfaces. The shading from dark to light red marks the degree of conservation, with residues colored dark red being conserved in all four IFNs (note that these are the minority, even in the binding site). The circle marks the location of the HEQ residues [colored in magenta in (A)].

Fig. 7. Sequence alignment of several interferons (IFNs)

(A) Colored in gray-blue is the IFNAR1 binding site, and in pink the IFNAR2 binding site. The consensus relates to all IFNs, and not just to the four subtypes displayed. Binding relates to change in binding affinity upon mutation. Two dots are for hotspots (over 10-fold change upon mutation). ^denotes mutations to Ala that result in increase in binding. (B) Overlay of the binding site between IFN (numbers in black) and IFNAR1 (numbers in red) of the site of the YNS tight-binding mutation (green) and the consensus HEQ sequence (cyan). (C) The binding site between R33 of IFN α 2 (numbered in black) and IFNAR2

(residues in cyan, numbers in red). Side-chain to main-chain hydrogen bonds are marked by arrows.

Fig. 8. Correlation with affinities and activities of interferon (IFN) subtypes and mutants

(A) Antiviral (AV) and antiproliferative (AP) potencies of IFN α 2 mutants as a function of the product of the binding affinities toward IFNAR1 and IFNAR2. All the values are relative to those determined for IFN α 2. (B) Antiproliferative activities and (C) antiviral protection of all IFN α subtypes as well as IFN β and IFN ω in different cell lines (WISH, OVCAR, and A549) in comparison to the product of the binding affinity ($R1 \times R2$). The viruses used were vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV).

Fig. 9. Similar and differential activities by type I interferons

(A) EC₅₀ values of activation of the antiviral and antiproliferative responses and of IFI6-16 gene induction. (B) Gene induction after different treatments with interferons as determined by gene array.

Fig. 10. Receptor assembly and dynamics in the plasma membrane

(A) Two-step assembly of the ternary interferon(IFN)-receptor complex in the plasma membrane (orange, IFN; blue, IFNAR2; green, IFNAR1): rapid and high-affinity binding of IFN to IFNAR2 is followed by recruitment of IFNAR1 into the ternary complex. The dynamic equilibrium between binary and ternary complexes depends on the 2D dissociation constant K_D^T and 2D concentrations of the receptor subunits. (B) Implications of this mechanism for ligand-binding affinity and kinetics: normalized ligand dissociation kinetics at different cell surface concentrations of the receptor subunits compared with the

dissociation from IFNAR2 only (red curve). The half-life of IFN binding to the cell surface receptor (indicated by dotted lines) increases with increasing receptor concentrations. The orange curve approximately corresponds to the affinity observed in cell surface binding experiments.

Fig. 11. A basic model to explain differential activity of interferon (IFN), i.e. diverging affinity–activity relationships for different cellular responses

(A) The bar-graphs show similarities and differences in response to IFN α 2 and the tight binding variant IFN α 2-YNS. (B) Shows a model that explains how differential activation (highlighted by the arrows in the bottom-left inset) can be rationalized. In naïve cells (left), reversible IFN binding and ternary complex formation is followed by endocytosis of the signaling complex. If the rate constant of endocytosis k_e significantly exceeds the rate constant of complex dissociation k_d , ternary complex formation can be considered

irreversible, independent on the IFN binding affinity. While signaling probably proceeds in early endosomes, endocytosis also leads to receptor degradation. Thus, similar activities for IFN α 2 and IFN β are observed in naïve cells. Cells activated with IFNs over extended time periods (primed cells) express the negative feedback regulator USP18, which interferes with ternary complex formation by interacting membrane proximal with the intracellular region of IFNAR. Owing to the lower affinity of IFN α 2 toward IFNAR1, a further reduction by USP18 drastically reduces ternary complex formation for this ligand at the typically low receptor surface concentrations (bottom-left inset). Thus, signaling by IFN α 2 is abrogated, while this effect is overcome by the much higher affinity of IFN β toward IFNAR1 and IFNAR2. At increased receptor levels, only minor desensitization is observed. (C) Low occupancy of few receptors is sufficient to initiate an antiviral state, whereas only high occupancy of many receptors will initiate the antiproliferative response. Therefore, cells with reduced receptor numbers will not initiate an antiproliferative response, whereas cells with increased receptor numbers will induce an antiproliferative response even at lower IFN concentration.