Type I Interferon Signaling Is Decoupled from Specific Receptor Orientation through Lenient Requirements of the Transmembrane Domain*

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Type I interferons serve as the first line of defense against pathogen invasion. Binding of IFNs to its receptors, IFNAR1 and IFNAR2, is leading to activation of the IFN response. To determine whether structural perturbations observed during binding are propagated to the cytoplasmic domain, multiple mutations were introduced into the transmembrane helix and its surroundings. Insertion of one to five alanine residues near either the N or C terminus of the transmembrane domain (TMD) likely promotes a rotation of 100° and a translation of 1.5 Å per added residue. Surprisingly, the added alanines had little effect on the binding affinity of IFN to the cell surface receptors, STAT phosphorylation, or gene induction. Similarly, substitution of the juxtamembrane residues of the TMD with alanines, or replacement of the TMD of IFNAR1 with that of IFNAR2, did not affect IFN binding or activity. Finally, only the addition of 10 serine residues (but not 2 or 4) between the extracellular domain of IFNAR1 and the TMD had some effect on signaling. Bioinformatic analysis shows a correlation between high sequence conservation of TMDs of cytokine receptors and the ability to transmit structural signals. Sequence conservation near the TMD of IFNAR1 is low, suggesting limited functional importance for this region. Our results suggest that IFN binding to the extracellular domains of IFNAR1 and IFNAR2 promotes proximity between the intracellular domains and that differential signaling is a function of duration of activation and affinity of binding rather than specific conformational changes transmitted from the outside to the inside of the cell.

Type I interferons (IFNs) orchestrate the antiviral innate immunity in vertebrates. They consist of 16 members in humans as follows: 12 different IFNα subtypes, as well as IFNβ, IFNω, IFNε, and IFNκ. They are clustered on the short arm of chromosome 9. All type I interferons elicit their innate and adaptive immune responses after binding to the receptor and forming the IFNAR1-IFN-IFNAR2 ternary complex (1, 2), the formation of which is essential for signaling. Both IFNAR1 and IFNAR2 belong to the class II helical cytokine receptors with four fibronectin type III-like subdomains for IFNAR1 and two subdomains for IFNAR2. The extracellular domains are followed by a single helix of 21 amino acids spanning the membrane, which in turn is connected to mostly natively unstructured intracellular domains and their associated effectors (Fig. 1A) (3). Cells lacking one of the receptors lack normal IFN signaling (4, 5). Structurally different members of type I IFNs bind to the same cell surface receptor but mediate differential responses that result from differences in binding affinities, concentration of IFN, and duration of activation (5–13). Differential signaling is realized through robust (antiviral) versus tunable (antiproliferative and immunomodulatory) gene induction leading to different phenotypic outcomes (14, 15). Receptor dimerization drives the activation of cytosolic associated Janus family kinases (JAKs), which in turn initiate downstream signaling cascades that propagate the signal into the nucleus and regulate gene transcription mainly via signal transducer and activator of transcription (STAT) proteins (11, 13, 16–18). Detailed information exists on the structure function relations of the extracellular domains (5, 7), although less is known on the intracellular domains (9, 11–13).

The original model for initiation of signal transduction by cytokines involved receptor dimerization, which initiates the intracellular associated kinases to activate transcription factors (19, 20). However, over time more elaborate schemes for receptor activation have been proposed, often involving pre-formed receptors that are activated by structural rearrangements. Biochemical and computational studies have suggested that both growth hormone receptor and the EPOR exist as pre-formed dimers, where ligand binding promotes structural changes transmitted from the extracellular domain through the TMD to the intracellular domains (21–23). Different orientations and specific amino acid mutation at the TMD and juxtamembrane region of growth hormone and EPOR were suggested to modulate receptor dimerization and activation (21, 24, 25). These findings were followed by other cytokine receptors, including IL6R, EGF receptor, and VEGF receptor, where ligand-induced structural movements of the intracellular domains were shown to be important in signaling (26–34), including IFNAR (35), where pre-dimerization was suggested. However, recent work has disputed the pre-formation of IFNAR1 and IFNAR2 and has shown clear evidence for ligand-induced receptor dimerization (2, 36). Still, IFN binding clearly induces a large conformational

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This article contains supplemental Table S1 and Fig. S1.

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2 The abbreviations used are: EPO, erythropoietin receptor; TMD, transmembrane domain; EGFR, EGF receptor; qPCR, quantitative PCR; TM, transmembrane.
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shift in IFNAR1, as evident from comparing the unbound to bound structures of the complex and from fluorescence quench and FRET experiments (7, 37). Thus, although IFN is required to produce the ternary complex, the role of structural plasticity for signaling through the interferon receptor is not resolved.

In this study we characterized the effect of changing the TMD of IFNAR1 on ligand binding and IFN signaling. A series of mutant receptors were prepared and transfected to hepatocarcinoma HUH7 cells (strain 48RC) lacking IFNAR1 and thus void of IFN-induced activity. Binding, STAT phosphorylation, and gene induction were measured for all mutated receptors. Mutants included insertion of up to five alanine residues in different positions of the TMD, replacing the TMD of IFNAR2 and insertion of up to 10 serine residues between the extracellular and TM domain of IFNAR1. The mutations are expected to affect the angular rotation of the N and C termini of the TMD and translated along the helical axis; mutations are expected to affect the angular rotation of the N and C termini of the TMD and translated along the helical axis.

Experimental Procedures

Cell Lines and Antibodies—Human HUH7 IFNAR1 knock-out cells (48RC) were kindly provided by Professor Nobuyuki Kato (University of Okayama). Monoclonal anti-IFNAR1-EC AA3 antibody was a gift from Biogene. Monoclonal anti-STAT1 and anti-pY701-STAT1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-pY689-STAT2 and anti-STAT2 antibodies were purchased from Delta Biolabs. p-AKT and AKT antibodies were purchased from Cell Signaling; P-ERK, T-ERK, P-p38, and P-STAT2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal STAT1 and anti-pY701-STAT1 antibodies were purchased from BioGenes. AA3 antibody was a gift from Biogene. Monoclonal anti-

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Cloning and Transfection—Mutations were introduced to full-length IFNAR1 in pDisplay plasmid by PCR amplification with mismatch containing primers. Details of all primers used in this study are summarized in supplementary Table S1. The human hepatocarcinoma cell line HUH7 (IFNAR1 knock-out cells 48RC strain) was cultured at 37 °C with 5% CO2 in DMEM (Sigma) supplemented with 10% fetal bovine serum (Equitech-BIO, Inc.), 1× penicillin/streptomycin/glutamine (BioLabs). The cDNA-IFNAR1 within the pDisplay plasmid was transiently transfected to HUH7 IFNAR1 knock-out cells (48RC strain) using jetPEI following the manufacturer’s standard protocol.

Gene Induction Assay—HUH7 cells transfected with IFNAR1 receptors were treated with IFN for 8–24 h. RNA was extracted with the PerfectPure RNA cultured cell kit (5-Prime) followed by cDNA preparation using high capacity cDNA reverse transcription kit (Applied Biosystems). Selected human interferon-stimulated gene expression levels were measured with the ABI Prism 7300 real time PCR system, using the SYBR Green PCR master mix (Applied Biosystems). Expression levels of selected interferon genes were examined as 2^(-ΔΔCT). The HPRT1 gene was used as internal control.

High throughput qPCR was performed using BioMark 48 × 48 and 96 × 96 Dynamic Arrays (Fluidigm Corp.) according to the manufacturer’s protocol. cDNAs (50 ng/ml) were pre-amplified with a mixture of all the primers combined. Data analysis was performed with the Fluidigm real time PCR analysis software.

In Situ Binding Assay—Wild-type IFNα2 was labeled with [125I] by PhoenixPeptide. For the competition assay, HUH7 cells were transfected with different mutants of IFNAR1. After 48 h, cells were washed once with PBS + 0.1% sodium azide (to block endocytosis) and then incubated for 10 min with the same solution. Next, cells were incubated for 1 h at room temperature with the labeled wild-type IFNα2 (200,000 cpm/well) in the presence of unlabeled YNS (YNS is interferon containing the triple H57Y/E58N/Q61S mutation exhibiting higher affinity toward IFNAR1) interferon at 10 different concentrations from 200 nM at 4-fold dilution in culture medium + 0.1% sodium azide. Cells were then washed five times in PBS on ice to remove unbound interferon. Cells were removed from wells by using 0.1 M NaOH plus 0.1% SDS and transferred into test tubes for measuring of bound, [125I]-labeled IFNα2, using a γ-counter (Packard). The experiment was done in duplicate. IC_{50} values were calculated using KaleidaGraph Synergy Software version 4.1.

Determination of STAT1, STAT2, ERK, p38, and AKT Levels—All protein levels were determined by lysing cells in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, proteas, and phosphatase inhibitors. The supernatants were collected and assayed for protein concentration using the Bradford method (Bio-Rad). Fifty micrograms of cell extracts were mixed with Laemmli sample buffer and boiled for 10 min. After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose (Amersham Biosciences) and checked for protein expression using specific antibodies. For p-AKT, p-ERK, and P-p38 cells were serum-starved for 24 h prior to addition of 1 nM IFNα2 for 30 min.

TOXCAT Assay—The TOXCAT assay was performed as described previously (38), using the ToxR’(ErbB-TM)β-lactamase chimera containing the complete TMD of ErbB2 receptor as positive control (39). In short, the TMD of IFNAR2 (Ile-244–Leu-264) was inserted in-frame with β-lactamase generating ToxR’(IFNAR2-TM)β-lactamase chimera under the control of the toxR promoter and the ctx promoter. Plasmids encoding toxR’(IFNAR2-TM)β-lactamase were transformed into Escherichia coli 10G competent cells and plated on LB plate (ampicillin 100 μg/ml + chloramphenicol 100 μg/ml).

Results

Inserting Alanine Residues into the Transmembrane Domain of IFNAR1—All type 1 IFNs seemingly bind IFNAR1 and IFNAR2 at the same sites and form structurally very similar ternary complexes in a 1:1:1 stoichiometry, which is followed by the activation of downstream signaling (9). The extracellular domains are followed by a single helix of 21 amino acids spanning the membrane, which in turn is connected to mostly natively unstructured intracellular domains and their associated effectors (Fig. 1A) (3). There is an ongoing debate on whether ligand binding to the extracellular domains transmits a
FIGURE 1. Inserting one to five alanine residues near the N terminus of the TMD of IFNAR1 has little effect on binding and activity. A, ribbon representation of the ternary complex of IFNAR1, IFNAR2, and IFN (based on Protein Data Bank 3SE3) (7) and the location of the additional alanine residues inserted within the transmembrane domain of IFNAR1. To the left is a schematic representation of the expected effect of adding one to five alanine residues (A1–A5). B, in situ binding curves of the different IFNAR1 mutants. Signal emitted from $^{125}$I-labeled wild-type IFN was measured after competing with cold IFN-YNS at different concentrations. The y axis represents the fraction of y signal relative to the signal in the absence of cold competitor. As control, we also measured binding to non-transfected cells and to cells transfected with the IFNAR1 mutant L163C. IC$_{50}$ values were calculated by fitting the normalized data using KaleidaGraph 4.1. C, A1–A5 mutant IFNAR1 HUH7 cells were treated for 30 min with 1 nM IFN and then analyzed by Western blotting using antibodies for pSTAT1 and pSTAT2. After stripping, the blots were re-analyzed with anti-STAT1 and -STAT2 antibodies. The graph on the right shows the normalized (to total and untreated) levels of phosphorylation. D, gene expressions of transiently transfected HUH7 cells after 16 h of treatment with 0.5–1000 pM IFN. qPCR was then performed for IFI6 and MX1 genes as indicated. The data presented are the relative expression levels compared with those of untreated cells, normalized against HPRT1. The results are average and standard error of three independent experiments. E, fold change in gene expression using the Fluidigm system (see "Experimental Procedures"). Cells were treated as in D, and cDNAs (50 ng/ml) were preamplified with all the primers pooled and analyzed with the BioMark real time PCR instrument. Data are presented using the NetWalker analysis tool. The upper eight genes are tunable genes, and the lower 12 genes are robust genes (15). The colors represent the value of a given gene (rows) in a specific mutant and concentration of interferon (columns). Genes with a high $\Delta\Delta$Ct value (high expression) are in red, and genes with a low $\Delta\Delta$Ct value (low expression) are in blue. Comparisons of the fold changes were done relative to those of untreated cells. Data are representative of two independent experiments.
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In Fig. 1A, we observed that the interferon binding data on HUH7 cells suggest that there is not much difference in terms of ternary complex formation between wild-type IFNAR1 and two mutants as determined by FACS using the anti-IFNAR1 antibody AA3. The interferon binding data on HUH7 cells suggest that there is no effect on the relative orientation of the extracellular domain in relation to the intracellular domain and that a translation of up to 7.5 Å along the helical axis is expected to result in a rotation of the extracellular domain relative to the intracellular domain by 100° and a translation of 1.5 Å along the helical axis (Fig. 1A). We inserted 1–5 alanine amino acids within the transmembrane domain of IFNAR1 (A1–A5, see Fig. 1A) near the N terminus of the TMD of IFNAR1 between isoleucine 440 and valine 441. Alanine was chosen as it is helix-forming, more than any other amino acid. All the alanine insertions were done on the wild-type cDNA of IFNAR1 using site-directed mutagenesis. After subcloning the mutant receptor genes into a pDisplay vector, they were transiently transfected into HUH7 cells lacking endogenous IFNAR1. Wild-type IFNAR1 was transfected as control to compare the effects of the mutations. Levels of transfection of wild-type IFNAR1 and two mutants as determined by FACS using the anti-IFNAR1 antibody AA3 are shown in Fig. 2. Clearly, the level of surface IFNAR1 was similar for all the variants, with no IFNAR1 being detected on the non-transfected HUH7 cells.

Binding affinity is a major determinant of IFN activity. To evaluate the effects of mutations at the TMD on binding to the cell surface receptors, 125I-labeled IFNα2 was mixed with increasing concentrations of cold IFN-YN5 (a mutant with 50-fold higher affinity toward IFNAR1 (40)). After a 1-h equilibration, residual 125I-labeled IFNα2 counts were determined. Non-transfected cells (lacking IFNAR1) or cells transfected with the non-active IFNAR1 mutant L163C did not show significant binding of 125I-labeled IFNα2 (Fig. 1B), suggesting that the binding affinity of IFNα2 toward IFNAR2 alone is too low to be detected with the 125I-labeled IFN used in this experiment. EC50 values of 0.03–0.06 nM were determined for wild-type IFNAR1 and the A1–A5 mutants, values that are within the experimental uncertainty for these measurements (Fig. 1B). The interferon binding data on HUH7 cells suggest that there is no change in rotation and translation caused by the insertions of 1–5 alanine residues are directed toward the intracellular domain of IFNAR1 and thus do not affect binding at the extracellular domain or that the insertions are absorbed by changes in the regions surrounding the TMD. These could be a result of twisting or bending of the transmembrane helix or of the inherent flexibility of the unstructured regions surrounding the TMD.

Receptor activation upon interferon binding results in the rapid phosphorylation of STATs, particularly STAT1 and STAT2. Thus, we next determined IFN-induced signaling for the IFNAR1 containing A1–A5 mutant receptors. The effects on phosphorylation of STAT1 and STAT2 were determined by Western blot after treatment with 1 nM IFNα2 for 30 min using specific antibodies against pSTAT1 and pSTAT2 and total STAT1 and STAT2 (total STAT levels do not change at this short time of induction). Cells expressing A1, A2, A3, A4, or A5 showed similar levels of pSTAT1 and pSTAT2 induction as cells expressing the wild-type IFNAR1 gene (Fig. 1C). These results show that STAT activation is not dependent on the relative orientation of the extracellular domain in relation to the intracellular domain and that a translation of up to 7.5 Å along the helical axis (for the A5 mutant) also has no effect.

STAT activation results in gene induction of a wide set of interferon-induced genes. To evaluate the effect of A1–A5 mutations on gene induction, we measured gene induction in HUH7 cells transfected with wild-type and mutant IFNAR1s. Cells were treated with 0.5, 10, and 100 pM and 1 nM IFNα2 for 16 h followed by real time qPCR to evaluate the levels of expression of MX1 and IFI6 genes (Fig. 1D). No difference in levels of gene expression was observed between the mutant IFNAR1 and between them and the wild type. Moreover, also the level of IFN required to obtain maximum induction was 100 pM IFNα2 for all the IFNAR1 clones. These results demonstrate that the different IFNAR1 mutations activate the expression of IFI6 and MX1 to the same extent and that the efficacy of the receptors for activation is the same.

IFI6 and MX1 are robust genes, which are highly sensitive to interferon induction (15). To evaluate a wider range of interferon-stimulated genes, including both robust and tunable genes (which are cell type-specific and require much higher IFN concentration for induction), we used the Fluidigm technology, where up to 96 different genes from 96 different biological samples can be evaluated simultaneously. In Fig. 1E, we show the level of gene induction (ΔΔC_T relative to non-treated) of a representative set of previously defined robust and tunable genes (15). Tunable genes were only slightly up-regulated in HUH7 cells, even at the highest IFNα2 concentration (for example, CXCL10 levels were only 2–3-fold higher after induction with 1000 pM IFNα2). Conversely, robust genes showed very high levels of induction (up to 1000-fold). The gene induction profile of HUH7 cells is similar to that of other cell lines that do not undergo antiproliferative activity upon IFN treatment (such as T47D (15)). Overall, all the genes exhibited comparable fold-change upon stimulus with different concentrations of interferon, independent of the IFNAR1 TMD mutant. Taken together, systematic insertion of alanine residues close to the N...
terminus of the TMD of IFNAR1 does not produce any detectable change in either IFN binding or signaling relative to the wild-type receptor. As the insertion of 1–5 alanine residues close to the N terminus of the TMD did not result in any detectable changes, we next added 1–4 alanines at the C terminus of the TMD (between Ala-457 and Lys-458, which is the first residue of the intracellular domain, Fig. 3A). Assuming that these additional alanines will form a helix as well (as is predicted), they will result in a relative rotation and translation of the intracellular domain of IFNAR1 relative to that of IFNAR2 (see Fig. 3A). The four insertions, called T1, T2, T3, and T4, were subjected to the

FIGURE 3. Inserting one to four alanine residues near the C terminus of the TMD of IFNAR1 (T1 to T4) has little effect on binding and activity. A, the location of the inserted alanine mutations. B, in situ binding curves of the different IFNAR1 mutants. C, analyzing pSTAT1, pSTAT2, STAT1, and STAT2 levels upon 1 nM IFNα2 induction for 30 min using specific antibodies. D, gene expressions for IFI6 and MX1 upon IFN induction at the given concentrations. E, fold change in gene expression for a set of tunable and robust genes upon IFN induction. Further experimental details are provided in the legend of Fig. 1.
same evaluations as described for A1–A5 (Fig. 3, B–E). Again, as in the case of A1–A5, we did not detect any significant changes in binding of IFN to the cell surface receptor (Fig. 3B), levels of STAT phosphorylation (Fig. 3C), and expression of downstream genes (Fig. 3, D and E) upon T1–T4 insertions. Together, the results here clearly show that signal transduction...
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from the outside to the inside of IFNAR1 is not dependent on transduced structural changes.

Alanine Substitutions and Additions at the Juxtamembrane Junctions—Lys-458 in IFNAR1 is predicted to be the first residue of the intracellular domain. Mutations at the transmembrane-cytosolic junctions, which link the TMD and the intracellular domain, were shown to play important roles in receptor activation by a variety of protein-protein and protein-lipid interactions (24). We then asked whether the specific sequence at the juxtamembrane between the transmembrane and intracellular domain in IFNAR1 has a role in downstream signaling. For this, we mutated lysine 458 and valine 459 at the juxtamembrane to alanine (Lys to Ala and Val to Ala, respectively, see Fig. 4A). As done in the previous sections, the two mutant IFNAR1 constructs were transiently transfected to HUH7 cells, and their activity was determined. Subjecting these two mutants to the same tests as described above showed that these mutations do not promote any significant changes in binding of IFN to the cell surface receptor (Fig. 4B), levels of STAT phosphorylation (Fig. 4C), and activation of the expression of downstream genes (Fig. 4D and E). Thus, alanine substitution at the juxtamembrane between the transmembrane and intracellular domain of IFNAR1 does not produce any significant change in position of insertion of IFNAR1 into the membrane as validated by its capacity to bind IFN and induce the same biological outputs as wild-type IFNAR1. These data are fundamentally different from the ones obtained for the thrombopoietin receptor (24).

At this stage, we already realized that mild mutations in the transmembrane domain or its proximity do not produce any observed changes in either IFN binding or signal transduction. Therefore, we decided to see what would happen if we introduced a larger change in the location of the extracellular domain of IFNAR1 by inserting 2, 4, and 10 serine residues (S2, S4, and S10) between Ser-435 and Lys-436 (Fig. 4A). Already, the cell surface binding measurements with $^{125}$I IFN showed that only S10 promoted a significant change in the EC$_{50}$ which was ~5-fold lower in comparison with the other IFNAR1 variants (Fig. 4B). The weaker binding is translated into weaker STAT phosphorylation (Fig. 4C) in comparison with all other variants. This in turn resulted in lower gene activation, both in terms of overall fold induction and a higher IFNα2 concentration required for gene activation (Fig. 4D). These data show that although the two receptors have flexibility in accommodating IFN, there is a limit to the inherent flexibility, which seems to be reached when adding 10 serine residues.

Substituting the TMD of IFNAR1 by that of IFNAR2—A still open question in cytokine research is whether the TMDs are interacting with one another and thereby facilitating the receptor dimerization in the case of heterodimeric receptors. To directly address this question, we substituted the TMD of IFNAR1 with that found on IFNAR2, producing a homodimeric TMD (Fig. 4A, right panel). The IFNAR1 harboring the IFNAR2 TMD is denoted TMR1R2. Again, as in most former experiments, IFN surface binding was not significantly affected by the TMR1R2, nor was STAT1 and STAT2 activation or $\text{IFI6}$ gene activation (both qualitatively and quantitatively). These results suggest little direct involvement of the TMD sequence in bringing IFNAR1 and IFNAR2 together.

To evaluate the tendency of the TMD of IFNAR2 in the TMR1R2 to dimerize, we performed a TOXCAT assay (38, 41), where the TMD of IFNAR2 is inserted between toxR' and $\beta$-lactamase. Upon expression, the TMD-driven dimerization results in toxR activation and transcription of the cat gene, which results in chloramphenicol resistance. As seen in Fig. 5A, only the control and not the IFNAR2 TMD drives chloramphenicol resistance, suggesting that the TMD of IFNAR2 has no intrinsic tendency to dimerize, and thus it is not contributing toward the interferon response observed for this variant. As the interferon response for TMR1R2 is equal to wild type, one can conclude that a potential interaction of the TMDs of IFNAR1 and IFNAR2 is not expected to significantly contribute toward IFN signaling.

The experiments for the different IFNAR1 mutants were performed at a single time point. To verify that the small effect of TMD mutations reflects a general phenomena, we repeated the measurements for STAT1 and STAT2 activation also 1 h
after administering IFN (instead of 30 min as in Figs. 1, 3, 4). In addition, we measured the level of gene induction also at 8 and 24 h (in addition to 16 h as done in Figs. 1, 3, and 4). As shown previously, pSTAT1 levels are slightly higher at 30 min, although the pSTAT2 levels were the same (42). However, the different IFNAR1 variants had no differential effect on STAT activation at the different time points as well (Fig. 6A). The same is also true for differential levels of gene transcription, although the pSTAT2 levels were the same (42). However, the different IFNAR1 variants had no differential effect on STAT activation at the different time points as well (Fig. 6A). The same is also true for differential levels of gene transcription.
which are the same at 8 and 24 h, using 0.5 and 10 pm IFNα2 (Fig. 6B).

Activation of Alternative IFN-induced Signaling Pathways—In addition to STAT activation, IFN was suggested to activate also ERK, p38 MAPK, and AKT (43, 44). To determine whether the different TMD variants of IFNAR1 have a more pronounced effect on the activation of these genes, we determined their levels of phosphorylation 30 min after activation by 1 nm IFNα2 (Fig. 5B). As control, we used HeLa cells, where the activation of these three genes has been shown. In HUH7 cells, one does not observe p38 and AKT activation (conversely to HeLa cells) (45). ERK activation is seen in both HUH7 and HeLa cells to a similar degree, with the level of activation being the same for the different IFNAR1 variants except for S10, where ERK is activated to a lesser degree, as also observed for STAT activation.

Sequence Conservation of TMDs and Their Surroundings—To analyze the effects of the mutations on the TMD, we submitted the different sequences to the TOPCONS server, which predicts the location and stability of transmembrane domains (46). The predicted outcomes show only minor changes in the location of the transmembrane helix (±2 amino acids (Fig. 7A)), suggesting that A1–A5 protrude from the N-terminal side, whereas T1–T5 protrude from the C-terminal side of the transmembrane helix. This conclusion was further verified by modeling the structures of various TMD mutations using PREDDIMER (47). No large differences in the structure or helix-helix orientation were observed between the wild type, A5, and T4 mutants or IFNAR2 homodimer for the top two scoring TMD structures (Fig. 7B). It was shown for other transmembrane helices that the experimentally determined conformation is among the top-scoring models for a given dimer (47). The FSCORE values of the three highest ranking TMDs for the wild-type receptors were 1.1, 0.96, and 0.92, respectively, much below the predicted threshold for TMD interactions in PREDDIMER (47), suggesting that the TMDs of IFNAR1 and IFNAR2 do not interact. Interestingly, the only TMD with higher FSCORE values is for the IFNAR2 homodimer (2.4), which we have experimentally shown not to interact (Fig. 5A). One should, however, stress that neither this nor any other computer program can predict with certainty that TMD helices will indeed bind.

Conservation of specific sequences suggests their functional importance. To evaluate the sequence conservation of regions on either the N- or C-terminal sides of the TMD, we performed sequence alignment of IFNAR1 (Fig. 7C) and IFNAR2 (Fig. 7D) at the appropriate region. At least for IFNAR1, it is very clear that the TMD and its immediate surrounding are between the less conserved regions of this gene. Strong conservation is observed N-terminal of the TMD, at the last strand of the extracellular domain, and C-terminal of the TMD from Tyr-466 onward. Tyr-466 is an important phosphorylation site involved in receptor ubiquitination, followed by the Tyk2-binding site (48, 49).
Sequence conservation is related to functional significance (50). To obtain a more general perspective on sequence conservation and the functional role of TMDs for single helix-spanning cytokine receptors, we analyzed sequence conservation for 13 different cytokine receptors using ConSurf (50). Fig. 8A shows the sequence conservation of these receptors, ordered according to the degree of conservation of the TMD. The TMD of IFNAR1 is the least conserved, while TMDs of VEGFR2, IL-6R1, and EPOR are the most conserved. Fig. 8B shows the conservation score (ConSurf gives a score of 1–9 per residues) plotted against the sequence position, with the genes ordered as in Fig. 8A. On the low extreme, one finds the TMDs of IFNAR1, EGFR, and IFNyR1, and the highest conservation is observed for EPOR, IL6R2, and VEGFR2. Sequence conservation of the TMD of the first group is between the lowest among the proteins, and for the second group it is between the highest.

Structure prediction using the IUPred server (supplemental Fig. S1) (51) shows that many of the intracellular regions of these cytokine receptors are natively unstructured. However, relating sequence conservation to structure predictions around the TMD did not provide further information, as one does not see a particular relation between these two traits. The very low sequence conservation for IFNAR1 is in line with the findings of this study that the TMD and its immediate surroundings serve merely as a bridge between the extracellular and cytoplasmic domain that does not convey a structural signal. This is fundamentally different from the suggested mechanism of EPOR, IL6R2, and VEGFR2 activation, where ligand-induced structural perturbations of the intracellular domains were suggested to play a key role in signaling (26, 28, 32).

**Discussion**

Two basic mechanisms for signal activation upon ligand binding of the extracellular domains have been proposed. The simpler of the two is that ligand binding of the extracellular domains results in proximity of the intracellular domains and their associated kinases, which then cross-activate each other. The second mechanism is ligand-induced spatial perturbations of receptor orientation, which is relayed as conformational changes through the membrane-promoting cross-activation. A combination of these two mechanisms would provide additional safety against random or nonspecific signaling.

It has been shown that specific rotations of the transmembrane domain of cytokine receptors can activate kinases inside the cell, thereby activating the downstream signaling. Evidence for such a mechanism has been shown for EPOR, growth hormone receptor, and thrombopoietin receptor (21, 22, 25, 52, 53). For the type I interferon receptor, the situation is less clear. Ligand binding to IFNAR1 induces a conformational change that is propagated toward the membrane proximal domain (54). Comparing the unbound to the bound structures of both IFNAR1 and IFNAR2 shows conformational changes upon IFN binding (7). Krause et al. (35) suggested that IFNAR1 and IFNAR2 are pre-associated even in the absence of IFN, binding of which promotes a conformational change that activates signaling. In contrast, Piehler and co-workers (2, 9) showed convincing evidence that the two receptors dimerize only upon IFN binding, but this leaves open the question of whether a conformational change is necessary for signaling.

Our primary goal in this study was to determine whether propagation of conformational changes from the outside of the cell to the cytoplasmic domains upon ligand binding plays a role in IFN signaling. These conformational changes can be propagated either through rotation or translation. Similarly to the work done on growth hormone receptor (21), we systematically inserted alanine residues within the transmembrane domain of IFNAR1. Close to the juxtamembrane junction with the extracellular domain, we inserted one to five alanine residues, and close to the junction with the intracellular domain, we inserted one to four alanine residues. Each additional alanine results in a rotation of 100° of the helix termini and translation of 1.5 Å along the helical axis. After transfection to HUH7 cells lacking endogenous IFNAR1, we measured IFN binding. STAT1, STAT2, ERK, p38, and AKT phosphorylation and robust and tunable gene induction. The main conclusion from the results is that insertion of alanine residues within the TMD does not produce any detectable qualitative or quantitative change in downstream signaling. Assuming that Ala insertions result in rotation of the N- or C-terminal edge of the α-helical TMD of IFNAR1, or translation of the edges, these movements had no effect on the biological activity of the receptor compared with wild type. It is possible, however, that these insertions are absorbed by twisting or bending of the transmembrane helix and that the lack of observed changes in signaling is a result of maintaining the hydrophobic matching between the TMDs of IFNAR1 and IFNAR2. Although we do not have experimental evidence for either scenario, computational simulations of the TMDs did not show any evidence for such behavior (Fig. 7, A and B), suggesting the helices to be intact and the rotation angle and translation of the edges to be affected. A caveat in this study is that HUH7 cells produce a weak tunable response and no antiproliferative response; thus we cannot exclude the possibility that receptor orientation will be found to have a role in activating the tunable responses in other systems.

For the thrombopoietin receptor, it has been shown that a mutation of tryptophan at the transmembrane-cytosolic junction modulates dimerization and affects activation (24). To establish whether a similar effect is observed also for the interferon receptor, we mutated the residues at the transmembrane-cytosolic junction (lysine 458 and valine 459) to alanine. However, for IFNAR1, such mutations again fail to demonstrate any effect (either quantitative or qualitative) in either binding or biological readouts. On the N-terminal side of the TMD we
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added 2, 4, or 10 serine residues, affecting the location of the extracellular domain of IFNAR1 in relation to IFNAR2. The addition of 2 and 4 serine residues had no affect on IFN binding or signaling. However, adding 10 serine residues weakened IFN binding by ~5-fold. As we did not observe IFN binding in the absence of IFNAR1, we can assume that IFN is promoting the complexation also for the S10 IFNAR1 mutant but with a weaker binding affinity. The weaker binding affinity is translated to reduced induction of STAT1 and STAT2 phosphorylation and IFI6 and MX1 gene expression. Finally, we wanted to see whether the sequence of the TMD of IFNAR1 is at all important for signaling. Therefore, we replaced the TMD of IFNAR1 with that of IFNAR2, resulting in the same TMD on both receptors. Again, this had no effect on either IFN binding or signaling. We further show that the TMD of IFNAR2 has no tendency to self-dimerize (as opposed to EPOR and ERBB2). As TMR1R2 transmits the same activity as wild type, this suggests no role for such TM dimerization for IFNAR.

Sequence conservation is one of the most powerful predictors to identify functionally important residues (50, 55). Analyzing the sequence conservation of IFNAR1 at the TMD and its immediate vicinity shows this region to be the least conserved, including the GXXA motif that may enhance IFNAR oligomerization. These results are in line with the functional neutrality observed in this study upon manipulating the TMD. Low sequence conservation is observed also for EGFR, where it remains unclear whether the asymmetry in the extracellular region of this singly-ligated dimer has a fixed relationship to that in the intracellular region, as the linker surrounding the TMD is highly flexible (56). The TXXAG and LXXXLXXXL motifs of the TMD of EGFR were suggested to be important for its dimerization; interestingly, they are not conserved. One should note that the paradigm of sequence motifs dictating interactions between TM helices seems now to be an over-simplification of the complex nature of interactions in the membrane (57). Mutating residues in the TMD of EGFR did affect clustering and basal and EGF-induced endocytosis but not EGF-induced kinase activity (58). Interestingly, an NMR structure of the TMD and flanking regions (59) suggests the helix-helix packing to be mediated by the N-terminal TM helix sequence motif, which is not conserved in the TMD of EGFR. Good evidence for a structural connection between ligand binding and conformational movements of the intracellular domains exists for the VEGF, IL6, and EPORs (3, 60–62). For VEGFR2, precise TM orientation is mandatory for kinase activation (63), which relies on a precise ligand-induced TMD conformation that is different from the ligand-free conformation. As a result, in VEGFR2 mutating the TMD affects kinase activity, and in EGFR the effect of mutations was limited to endocytosis and did not affect kinase activity. For EPOR the orientation of the transmembrane domains seems to be important for signaling (61, 64). Still, using diabodies has shown that these orientation requirements are liberal, absorbing large changes in receptor conformation while maintaining signaling (65).

Although it is difficult to draw definite conclusions from sequence conservation on the functional importance of TMDs, it still provides an interesting additional angle on this highly debated problem. It is also interesting to note that subunits of the same receptor (IFNAR1 and IFNAR2, IFNγR1 and IFNγR2, IL10R1 and IL10R2, and IL6R1 and IL6R2) have very similar conservation scores of their TMDs (Fig. 8A). Moreover, even human growth hormone receptor and prolactin receptor, which are 32% homologous (but not matched in the ConSurf analysis), have basically the same conservation score of their TMDs. These results are expected if indeed the transmembrane domains of a receptor are either transmitting or not transmitting a structural signal.

We usually strive to find mutations that will affect a measured activity. In this sense, this study is a disappointment, as we did not observe any effect resulting from most of the mutations tested. These findings are surprising, as they clearly demonstrate that the relative orientation and translation of both the intra- and extracellular domains of IFNAR are of no importance for signaling. Basically, this comprehensive study suggests that the role of interferon is to bring IFNAR1 and IFNAR2 into proximity for a given time and that this proximity is sufficient to drive signaling. This simplistic view of events is in line with our previous findings that binding affinity of interferons, number of cell surface receptors, and the duration of IFN treatment are the main determinants that dictate differential activities of type I IFNs (8–10, 15). This view was recently further promoted by the work of Wilmes et al. (2), who showed that interferon is required for receptor dimerization and that the affinity of the ligand is the main determinant that explains how signaling by the tighter binding IFNβ is maintained over longer periods of time in comparison with IFNα2. Clearly, for other receptor systems a very different mechanism of activation is possible, as was indeed suggested. As IFNs serve as the first line of defense against pathogens, such a simple and robust model of activation may be an advantage in the constant battle against obstructing pathogens.

Author Contributions—N. S. conducted most of the experiments, analyzed the results, and wrote much of the paper. G. L. conducted experiments on cloning and mutagenesis of some of the constructs and performed some of the experiments. G. S. conceived the idea of the project, analyzed the results, and wrote the paper together with N. S.

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