Interferon- λ Is Functionally an Interferon but Structurally Related to the Interleukin-10 Family*

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Interferon- λ (IFN- λ) is an antiviral cytokine that signals through a distinct receptor complex, composed of the IFN- λ R1 and interleukin-10R2 (IL-10R2) receptor chains. We have determined the crystal structure of human IFN- λ 3 and characterized the interaction with its receptor complex through structure-based site-directed mutagenesis. The ability of IFN- λ 3 mutants to signal was determined by measuring the antiviral activity and induced STAT2 phosphorylation. In conclusion, our data show that, although IFN- λ is functionally an interferon, it is clearly structurally related to members of the IL-10 family. In particular, we found an interesting similarity between IFN- λ and IL-22, and we suggest that IFN- λ and IL-22 possess parallel functions, protecting epithelial tissue against viral and bacterial infections, respectively.

In 2003, a novel family of cytokines was discovered simultaneously by two independent research teams (1, 2). Initially, the two research teams named these novel cytokines either interferon- λ (IFN- λ)² 1, 2, and 3 or interleukin-29 (IL-29), -28A, or -28B. Throughout this report we will use the IFN- λ designation or type III IFN, when referring to all three subtypes as a group. Like type I IFN, type III IFN induces antiviral activity both *in vitro* (3, 4) and *in vivo* (5). Thus the two types of IFN seem to have similar biological effects at a cellular level. IFN- λ uses a distinct receptor complex consisting of a unique subunit, named IFN- λ R1, as well as the IL-10R2 subunit. The similar biological effects caused by type I and III IFN can be explained by activation of a highly overlapping set of transcription factors. In particular, both types of IFN lead to phosphorylation of signal transducers and activators of transcription (STAT) 1, 2, and 3, followed by assembly of the interferon-stimulated gene factor 3 transcription factor and thus target the same population of genes for induction (6–8).

The expression of type III IFN appears to be induced by the same stimuli and depend upon the same signaling pathways as type I IFN. For example, type III IFN has been shown to be

induced by a variety of viruses such as influenza A virus, herpes viruses, and Sendai virus as well as lipopolysaccharides or double-stranded RNA (9–11). However, the contribution of different cell populations to the production of IFN- λ is not yet clear. An important difference between the type I and III IFN systems is the expression pattern of their receptors. Whereas most cell types express the type I IFN receptor complex and the IL-10R2 component of the IFN- λ receptor, the expression of the IFN- λ R1 receptor subunit is highly restricted (12, 13). The exact expression pattern of the IFN- λ R1 is not yet fully established, but it is clear that cells of epithelial origin express IFN- λ R1 (13). Because type III IFN cannot signal without both receptor subunits, the response to type III IFN is restricted as well. Recent work, using IFN- λ R1 knock-out mice, demonstrated that IFN- λ is effective against influenza virus, if the virus is administered through the intranasal route, but not if it is administered in a systemic fashion (12). This agrees well with a role for IFN- λ in preventing viruses in infecting and penetrating the lung epithelia.

In addition to its antiviral activity, IFN- λ is capable of modulating the Th1/Th2 response (14, 15). IFN- λ can down-regulate IL-13 and thus suppress the Th2 response (16). Furthermore, it was reported that deficient IFN- λ expression after rhinovirus infection is linked to increased severity of asthma in patients (17). Whether the protection against asthma induced by IFN- λ is due to suppression of viral replication or to the reduced Th2 response or both is currently unknown. However, if type III IFN plays an important role in development and severity of asthma, the potential medical relevance of type III IFN is substantial.

Type III IFN is a member of the class II cytokine family. This family includes type I, II, and III interferons and the IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26). The IL-10R2 receptor subunit is shared by IL-10, IL-22, IL-24, IL-26, and IFN- λ . Although these cytokines share one subunit of their receptor complexes, they have widely different biological activities. For example, IL-10 induces immunosuppressive and immunostimulatory activities, whereas IL-22 regulates protection and reorganization of tissue cells at outer body barriers and is critical in establishing an efficient antibacterial defense in lung and gut epithelia (18, 19). It is interesting to note that both IL-22 and IFN- λ play critical roles in the innate immune defense of epithelia cells against bacterial and viral infections, respectively.

We present the first crystal structure of a type III IFN and suggest a model for receptor interaction. This model is based

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² The abbreviations used are: IFN, interferon; IL, interleukin; EMCV, encephalomyocarditis virus; STAT, signal transducers and activators of transcription; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation; EC₅₀, concentration required for 50% protection.

upon structure-based site-directed mutagenesis and is further validated by quantitative measurement of the antiviral activity and STAT2 phosphorylation induced by wild-type and mutated forms of human IFN- λ 3. The crystal structure of human IFN- λ 3 reveals a surprisingly close relationship with IL-22, which is further discussed.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Expression and purification of human IFN- λ 3 were done as previously described (20). However, the buffer used for size exclusion chromatography was replaced with 150 mM NaCl, 20 mM MES, pH 6.5, and the eluted protein was used immediately for crystallization.

Crystallization, Data Collection, and Structure Determination—Initial crystallization screening was performed with the Crystal ScreenTM system (Hampton Research). Optimal crystals were grown by mixing equal volumes of protein solution with reservoir solution containing 28% w/v polyethylene glycol 4000, 200 mM ammonium sulfate, 3% v/v isopropyl alcohol, and 10 mM zinc chloride and using the sitting drop, vapor-diffusion method. The crystals were frozen in the crystallization solution plus 10% v/v glycerol, and data were collected at either the I911-3 beam line at MAX laboratory (21) or the X12 beam line at the European Molecular Biology Laboratory Hamburg. The crystals belonged to the P3₁21 space group with cell dimensions of $a = b = 91.12$ Å, $c = 150.21$ Å.

To obtain experimentally determined phases, crystals were soaked in crystallization solution plus 100 mM sodium iodide for a few minutes before being frozen. Data were collected at both high (maximal anomalous signal) and low (maximal resolution) wavelengths. The program SHELX (22) was used to find the initial iodide sites by a single wavelength anomalous dispersion approach using the high wave length dataset. The sites found using SHELX were transferred to SHARP (23), and a full single isomorphous replacement with anomalous scattering approach, using two different iodide derivatives and a native dataset, was adopted. The experimental phases from SHARP were improved using density modification procedures as implemented in RESOLVE (24). The structure was manually build using the programs O (25) and Coot (26) and refined using the programs CNS (27) and Phenix (28). The final refined structure has five residues in the non-allowed region of the Ramachandran plot (1%). These residues are predominantly found in the poorly defined regions of chain D. Root mean square deviations were calculated using the SSM routine implemented in Coot. The figures were made using the program PyMOL.³

Establishment of IFN- λ 3 Mutants and Measurement of Antiviral Activity—Selected residues of human IFN- λ 3 were mutated to alanine using the QuikChange site-directed mutagenesis kit (Stratagene), expressed, and purified the same way as the wild-type protein. The antiviral activity of human wild-type and mutant IFN- λ 3 against encephalomyocarditis virus (EMCV) in HepG2 cells was done as previously described (20).

STAT2 Phosphorylation Induced by IFN- λ 3 Mutants—HepG2 cells were treated with either wild-type IFN- λ 3 or the indicated mutants at concentrations, which correspond to the concentration required for 80% protection in the antiviral assay (EC₈₀). Cells were seeded in 6-well tissue culture plates with a density of 60,000 cells per well and incubated for 24 h to allow the cells to settle. The induction with each IFN was done in replica of three, and the induction time was 2 h. For detection of the phosphorylation status of STAT2, we used Luminex Technology. Briefly, the filter plate was washed with assay buffer, and 50 μ l of freshly vortexed antibody-conjugated beads was added to each well. The plate was washed with assay buffer, and samples and standards were added. After a brief shake (30 s at 1.100 rpm), the plate was incubated at 4 °C overnight in the dark with light shaking (300 rpm). After one washing step, 25 μ l of the detection antibody was added to each well, and the plate was shaken and incubated as above. Subsequently, the plate was washed and incubated for 30 min with 50 μ l of a streptavidin-phycoerythrin solution with shaking (30 s at 1.100 rpm, 10 min 300 rpm). Finally the plate was washed, 125 μ l of assay buffer was added to each well, and the plate was shaken for 10 s at 1100 rpm and read immediately on the Bio-Plex machine. The phosphorylation of STAT2 is expressed in arbitrary units.

RESULTS

Structure Determination—Recombinant human IFN- λ 3 (corresponding to the mature polypeptide except residues 1–12) was expressed in *Escherichia coli* in inclusion bodies and purified by metal-ion affinity chromatography under denaturing conditions followed by renaturation. This procedure allows large scale production of fully active IFN- λ 3 (Table 2), which was used for crystallization (20). Crystals were diffracted to a maximal resolution of 2.8 Å, and the structure was determined using traditional isomorphous replacement combined with optimal use of the anomalous signal. The crystal structure of IFN- λ 3 was determined and refined to a resolution of 2.8 Å with an *R*-factor of 26% and a free *R*-factor of 31% (see Table 1 for data statistics). The asymmetric unit contains four molecules that are pair-wise linked by an intermolecular disulfide bond between Cys-48 of neighboring molecules. The final model contains residues 13–117 and 128–175. Residues 118–127 could not be modeled because of poor electron density, which is probably due to the flexibility of this region (the DE loop) and the lack of stabilizing crystal contacts.

An Overview of the Structure—Class II cytokines share a characteristic topology consisting of six secondary structure elements named A–F (29, 30). Elements A and C–F are all α -helices, whereas element B generally is flexible and adopts a number of structures. Helices A, C, D, and F form a four-helix bundle, which is the core of the structure. The structure of IFN- λ 3 follows this typical class II cytokine fold (Fig. 1). Element B is poorly ordered and adapts a flexible coil structure in the crystal structure of IFN- λ 3. Helices A, C, D, and F constitute the typical four helix bundle where helix F is bending similar to what is seen in other members of the IL-10 family.

Out of seven cysteines present in IFN- λ 3, six cysteines are engaged in intramolecular disulfide bonds, and one cysteine (Cys-48) is presumed to be free in solution. In the crystal,

³ W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.

TABLE 1
Data collection, phasing and refinement statistics

	Native	Iodine 1	Iodine 2
Data collection			
Space group	P3 ₁ 21	P3 ₁ 21	P3 ₁ 21
Cell dimensions			
<i>a</i> = <i>b</i> , <i>c</i> (Å)	91.0, 150.9	91.3, 150.9	91.2, 151.2
α , β , γ (°)	90, 90, 120		
			Near remote Far remote
Wavelength	0.907 Å	0.905 Å	1.65 Å 0.9 Å
Resolution (Å)	50–4.0	50–2.8	50–3.0 50–3.4
<i>R</i> _{sym} or <i>R</i> _{merge}	0.036 (0.07)	0.095 (0.45)	0.094 (0.41) 0.095 (0.45)
<i>I</i> / <i>σ</i> _i	45 (29.8)	15.2 (2.2)	14.7 (2.6) 15.2 (2.2)
Completeness (%)	98.8 (99.8)	99.7 (97.6)	98.8 (88.6) 99.7 (97.6)
Redundancy	8.8 (9.1)	5.1 (3.0)	5.2 (2.6) 5.1 (3.0)
Refinement			
Resolution (Å)		23.8–2.8	
No. reflections		18,112	
<i>R</i> _{work} / <i>R</i> _{free}		0.26/0.31	
No. atoms		4,511	
Protein		4,419	
Ligand/ion		35	
Water		57	
<i>B</i> -factors			
Protein		95.11	
Water		69.57	
r.m.s.d. values			
Bond lengths (Å)		0.003	
Bond angles (°)		0.662	

For the iodine derivatives, Friedel pairs were kept separated.

Cys-48 forms an intermolecular disulfide bond connecting two molecules of IFN- λ 3. This disulfide bond arose during crystallization as we failed to observe any dimeric forms of IFN- λ 3 on non-reducing SDS-PAGE prior to crystallization (data not shown). The mutation of Cys-48 to alanine did not have any effect upon the antiviral activity of IFN- λ 3 (Table 2), thus we do not believe that the intermolecular disulfide bond observed in the crystal has any biological relevance. Of the three intramolecular disulfide bonds, the first disulfide bond between Cys-16 and Cys-115 connects the N terminus to the end of helix D, the second disulfide bond between Cys-50 and Cys-148 connects the AB loop to the beginning of helix F, and the third disulfide bond between Cys-157 and Cys-164 forms a small loop at the end of helix F (Fig. 1).

IFN- λ Is a Functional Interferon with an IL-10/IL-22-like Structure—A comparison between the structure of IFN- λ 3 and structures of other class II cytokines (IFN- α 2, IFN- β , IL-10, IL-19, and IL-22, Protein Data Bank accession codes 1rh2, 1au1, 2ilk, 1n1f, and 1m4r, respectively) shows that IFN- λ 3 is structurally more similar to members of the IL-10 family, especially IL-22, than to type I IFNs (Fig. 2). IFN- λ 3 and IFN- α 2 or IFN- λ 3 and IFN- β have a root mean square deviation (r.m.s.d.) for C- α atoms of 4.2 and 3.7 Å, respectively, whereas IFN- λ 3 and IL-19 or IFN- λ 3 and IL-22 have an r.m.s.d. of 3.1 and 2.7 Å. IL-10 differs from the rest of the IL-10 family given that it is the only member so far that is known to form intertwined dimers, where helices E and F from one molecule pack with helices A, B, C, and D from another molecule and *vice versa*. However, if one ignores this and compares IFN- λ with an artificial IL-10 molecule composed of helices A, B, C, and D from one molecule (residues 12–117) and the intertwined helices E and F from another molecule (residues 118–156), the similarity between IL-10 and IFN- λ 3 is relatively high with an r.m.s.d. of 2.9 Å.

In contrast, sequence alignment of the amino acid sequences of these proteins does not show the same pattern of similarities.

FIGURE 1. The crystal structure of human IFN- λ 3. Upper panel, front view of the final model of IFN- λ 3. Disulfide bonds are shown as sticks in yellow. Lower panel, electron density (final σ_A -weighted $2F_o - F_c$) for the molecule contoured at 1.0 σ and presented as blue mesh. Two iodide ions are shown in magenta.

The sequence similarity between IFN- λ 3 and IFN- α 2 or IFN- β is 33 and 31%, respectively, whereas the similarity between IFN- λ 3 and IL-10, IL-19, and IL-22 is 23, 22, and 22%, respectively. Thus with respect to sequence, IFN- λ 3 is more similar to type I IFNs than the IL-10 family. The pattern of disulfide bonds in IFN- λ 3 is also similar to that of type I IFNs. The Cys-16 to Cys-115 disulfide bond in IFN- λ 3 is conserved in type I and type III IFNs (except IFN- β and IFN- ϵ) as well as the IL-10 family. In contrast, the Cys-50 to Cys-148 disulfide bond in IFN- λ 3 is only found in type I and type III IFNs and not in any members of the IL-10 family. The last disulfide bond, Cys-157 to Cys-164, is a unique feature found in all type III IFNs described so far except human IFN- λ 1.

Interaction of IFN- λ 3 with the IFN- λ R1/IL-10R2 Receptor Complex—The structure of IL-10 in complex with IL-10R1 showed that IL-10 interacts with its receptor via an area primarily composed of helices A, B, and F as well as the AB loop. The recent structure of IL-22 in complex with IL-22R1 showed that IL-22 likewise interacts with its receptor through an area composed of the same helices. We designed a series of alanine

TABLE 2

Antiviral activity of IFN- λ 3 mutants

	EC ₅₀	95% CI	Slope	95% CI	Efficacy	95% CI	-Fold increase
WT	0.6	0.6–0.7	0.9	0.8–1.0	88.9	87.4–90.5	
Gln-27 → Ala	6.1	4.1–8.9	0.8	0.6–0.9	110.7	100.8–120.6	10
Gln-30 → Ala	1.5	1.2–1.9	0.7	0.6–0.8	104.5	99.9–109.0	2
Lys-33 → Ala	23.2	19.2–28.0	1.0	0.9–1.2	68.5	64.4–72.6	38
Arg-34 → Ala	20.4	17.6–23.6	1.0	0.9–1.1	78.2	74.6–81.8	33
Lys-36 → Ala	31.2	12.0–48.8	0.8	0.6–1.0	91.9	79.9–103.9	51
Asp-37 → Ala	26.0	17.3–39.1	0.7	0.6–0.8	102.7	91.6–113.7	43
Leu-44 → Ala	7.1	5.2–9.6	0.9	0.7–1.1	106.7	98.7–114.7	12
Cys-48 → Ala	0.4	0.3–0.5	0.7	0.6–0.9	81.7	78.7–84.6	1
Arg-51 → Ala	0.7	0.5–0.9	0.7	0.6–0.8	82.0	77.8–86.2	1
Arg-53 → Ala	4.3	3.7–5.0	1.4	1.2–1.6	82.0	78.8–85.1	7
Leu-54 → Ala	3.5	2.9–4.2	1.0	0.9–1.2	82.3	78.9–85.6	6
Asp-96 → Ala	2.2	1.8–2.7	0.8	0.7–0.8	111.4	106.9–115.8	4
Val-97 → Ala	41.5	32.3–53.4	1.1	0.9–1.2	76.1	69.3–82.9	68
Gln-100 → Ala	27.8	23.7–32.6	1.4	1.1–1.6	51.7	48.7–54.7	46
Phe-155 → Ala	24.5	20.2–29.6	0.8	0.7–0.9	93.4	88.4–98.4	40
Phe-158 → Ala	>400						>650

mutants of IFN- λ 3, which cover many of the surface-exposed residues in helix A, D, and F as well as the AB loop and the part of element B that is close to helix A. All the mutated proteins were purified using size-exclusion chromatography, which served to confirm the structural integrity of the mutated proteins. Next, we determined the ability of these mutants and wild-type IFN- λ 3 to induce antiviral activity against EMCV. The antiviral activities are given as the half-maximal effective concentrations (EC₅₀), the slope, and the efficacy (Fig. 3A and Table 2).

The mutagenesis data show a cluster of eight residues at the center of helices A, D, and F, which is highly sensitive to alanine substitution (EC₅₀ values is increased by a minimum of 30-fold). This cluster is composed of Lys-33, Arg-34, Lys-36, and Asp-37 on the A helix, Val-97 and Gln-100 on the D helix, and, finally, Phe-155 and Phe-158 on the F helix (Fig. 3B). Notably, the mutation of Phe-158 to alanine completely abolishes antiviral activity by IFN- λ 3. These residues are positioned close to each other and presumably constitute the core area for receptor binding. This hotspot is surrounded by a number of residues with a significant, yet less dramatic effect upon the antiviral activity. These residues are found in element B (Arg-53 and Leu-54), the N-terminal and C-terminal ends of helix A (Gln-27 and Leu-44, respectively). On the contrary, mutations in the AB-loop (Cys-48 and Arg-51) had little or no effect upon the antiviral activity.

STAT2 Phosphorylation Correlates with Antiviral Activity—It is often assumed that STAT2 phosphorylation is driving the antiviral effect of both type I and III IFN. Using the Luminex system, we performed measurements of the amount of phosphorylated STAT2 found in HepG2 cells after treatment with different doses of IFN- λ 3. The experiment was performed using IFN- λ 3 concentrations corresponding to 25, 50, 75, and 95% protection of cells against the cytopathic effect of EMCV virus. The data revealed a correlation between the dose of IFN- λ 3 used and the amount of phosphorylated STAT2 found (Fig. 4a).

To test the direct effect of the introduced mutations upon the signaling, we treated HepG2 cells with either wild-type IFN- λ 3 or the indicated mutants at concentrations, which correspond to the concentration required for 80% protection in the antiviral assay (EC₈₀). Subsequently, we measured the amount of

phosphorylated STAT2. As seen in Fig. 4b, the various mutants and wild-type IFN- λ 3 induce comparable levels of STAT2 phosphorylation when used at concentrations corresponding to their EC₈₀ values, thus there is a direct correlation between the antiviral activity of the mutants and their ability to induced phosphorylation of STAT2.

DISCUSSION

Although IFN- λ is functionally an interferon, our data show that it is structurally closer related to the IL-10 family than to type I IFNs. When one compares the different structures of the IL-10 family members available (IL-19, IL-22, and IL-10) to that of IFN- λ 3, it is obvious that they share a common fold. The similarity is most pronounced between IFN- λ 3 and IL-22 as well as IL-19, which are all monomers. However, even in the case of IL-10, which forms intertwined dimers, where the E and F helices switches position, the fold is still very similar to IFN- λ 3. Not only do IL-22 and IFN- λ share a high degree of structural similarity, they are also both part of the innate immune system in epithelial cells. Here IL-22 confers protection against bacterial infections, whereas IFN- λ protects against viral infection. Thus, it is tempting to speculate that IL-22 and IFN- λ (and possibly also IL-19 and -20) arose as a group of cytokines responsible for the regulation of innate immunity in tissues that suffer from a high risk of infections, unlike type I IFNs, which function systemically. This would explain why two separate IFN systems (type I and III) have been maintained throughout vertebrate evolution, even though they regulate the same subset of genes.

IFN- λ signals through the heterodimeric receptor complex consisting of the IFN- λ R1 and IL-10R2 receptor chains. Thus the similarity in structure between IFN- λ and members of the IL-10 family probably reflects a common evolutionary origin as well as the evolutionary restraint caused by the shared IL-10R2 receptor chain. Despite the similarities of this receptor complex to the IL-10 receptor complex, the resulting signal is very different and essentially identical to the one induced by type I IFN. The binding of IL-10 to its receptor complex, IL-10R1·IL-10R2, has been characterized through the crystal structure of the IL-10·IL-10R1 complex (31) as well as surface plasmon resonance and site-directed mutagenesis (32). The recent crystal structure of the IL-22·IL-22R1 complex has also helped to shed

FIGURE 3. Characterization of the receptor binding site of IFN- λ 3. *A*, antiviral activity of wild-type IFN- λ 3 and selected mutants in HepG2 cells challenged with EMCV. Only mutants with EC_{50} values increasing >25-fold as compared with wild-type IFN- λ 3 are shown. HepG2 cells were incubated with serial dilutions of IFN- λ 3 before being challenged with EMCV. The data shown for wild-type IFN- λ 3 represent four separate plates with each concentration done in replicates of four. Data shown for the mutants represent one plate with each concentration done in replicates of four. Mean $\pm$ S.D. from four replicates are shown. *B*, location of the mutated residues on the structure of IFN- λ 3. Residues, which have been mutated to alanine, are shown as sticks. Mutations with EC_{50} values increasing <5-fold are shown in blue, whereas mutations with EC_{50} values increasing between 5- and 25-fold are shown in magenta, and, finally, mutations with EC_{50} values increasing >25-fold are shown in red.

light on the binding of IL-22 to its receptor (33, 34). The binding area of IL-10 and IL-22 to their respective private receptor chains, IL-10R1 and IL-22R1, is often described in terms of two coupled binding sites, named 1A and 1B. The first binding site,

FIGURE 2. Comparison of structures of class II cytokines. *A*, superimposition of IFN- α 2 onto IFN- λ 3. Ribbons of IFN- α and IFN- λ 3 are colored green and blue, respectively. The two molecules have an r.m.s.d. of 4.1 Å. *B*, superimposition of IL-10 onto IFN- λ 3. Ribbons of IL-10 and IFN- λ 3 are colored magenta and blue, respectively. The two molecules have an r.m.s.d. of 2.9 Å. *C*, superimposition of IL-22 onto IFN- λ 3. Ribbons of IL-22 and IFN- λ 3 are colored brown and blue, respectively. The two molecules have an r.m.s.d. of 2.7 Å. Disulfide bonds are shown as sticks in yellow.

FIGURE 4. STAT2 phosphorylation induced by wild-type IFN- λ 3 and selected mutants in HepG2 cells correlates with antiviral activity. *a*, antiviral activity of wild-type IFN- λ 3 in HepG2 cells challenged with EMCV correlates with STAT2 phosphorylation. The data shown for antiviral activity corresponds to the data shown in Fig. 3A. STAT2 phosphorylation was measured using the Luminex method at four different concentrations of IFN- λ 3 corresponding to the EC_{25} , EC_{50} , EC_{75} , and EC_{95} . Mean $\pm$ S.D. from three replicates are shown. *b*, STAT2 phosphorylation induced by wild-type IFN- λ 3 and selected mutants at EC_{80} concentrations in HepG2 cells. Mean $\pm$ S.D. from three replicates are shown.

1A, is mainly located on the B element, part of the AB loop, and the F helix (Fig. 5). This binding site is spatially but not sequentially conserved between IL-10 and IL-22. Our data suggest that, although several residues in the corresponding 1A site in IFN- λ 3 (Arg-53 and Leu-54 on the B element but not Cys-48 and Arg-51 on the AB loop) are involved in receptor binding with increases in EC_{50} values from 5- to 20-fold (Table 2), they do not play a major role. The second binding site, 1B, is located on helix A and helix F. Especially the A helix plays a major role in the binding of IL-10 to its receptor, whereas the 1B site in IL-22 appears to play a lesser role in receptor binding. We found a cluster of six residues (Lys-33, Arg-34, Lys-36, and Asp-37 on helix A and Phe-155 and Phe-158 on helix F) at the center of the A and F helices in IFN- λ 3, corresponding to the 1B site in IL-10 and IL-22, which play a major role in receptor binding for IFN- λ 3. Mutation of any of these residues had a profound impact on the antiviral activity with increases of EC_{50} values from 30- to >500-fold.

The binding site of IL-10 and IL-22 to the second receptor chain, IL-10R2, is located on the A and D helices (32, 35, 36). It is assumed for class II cytokines that receptor binding and activation are sequential processes where the cytokine binds the first receptor chain enabling the cytokine/receptor chain complex to bind the second subunit eventually leading to activation of signaling. According to this model, mutations affecting the binding to the second receptor chain should result in a lower

FIGURE 5. Comparison of the receptor binding sites on IFN- λ 3, IL-22, and IL-10. *A*, the receptor binding site of IL-10. Residues involved in binding to IL-10R1 and IL-10R2 as determined by crystallography and surface plasmon resonance, are shown in green and blue, respectively. The approximate location of the binding sites 1A and 1B are shown by circles. *B*, the receptor binding site of IL-22. Residues involved in binding to IL-22R1 and IL-10R2 as determined by crystallography and surface plasmon resonance, are shown in green and blue, respectively. The approximate location of the binding sites 1A and 1B are shown by circles. *C*, the receptor binding site of IFN- λ 3. Residues involved in binding to its receptor, as determined by mutagenesis and antiviral activity, are shown in red. All molecules are shown from the same angle.

efficacy as well as an increase in EC_{50} values. Mutation of Gln-100 resulted in a significant reduction of the efficacy as well as an increased EC_{50} value. Mutation of the neighboring residues, Arg-34 and Val-97, also resulted in decreased efficacy, albeit less significant. Thus we suggest that the IL-10R2 receptor chain contacts IFN- λ 3 in the vicinity of Gln-100, with the interface of the IFN- λ R1 and IL-10R2 binding sites located along helix A.

In principle, the mutations that we introduced in IFN- λ 3 could interfere either with receptor binding or with the subse-

quent activation of the receptor complex or possibly with both. We investigated the STAT2 phosphorylation induced by both wild-type IFN- λ 3 and selected mutants assumed to be involved in interactions with the IFN- λ R1. In doing so, we chose to use concentrations that induced similar levels of antiviral protection (EC_{80} concentrations). This experiment revealed a dose response for STAT2 phosphorylation similar to that seen for the antiviral effect for wild-type IFN- λ 3. Furthermore, the levels of STAT2 activation were similar for different mutants at the same level of antiviral activity, demonstrating a direct correlation between the STAT2 phosphorylation and antiviral activity. Thus, it appears unlikely that these mutations have impaired the ability of the cytokine-receptor complex to signal. The STAT2 phosphorylation data rather support the sequential model mentioned above, where weakened interaction between IFN- λ 3 and IFN- λ R1 by site-directed mutagenesis leads to a lower affinity but identical signaling at saturating concentrations.

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