

Mutational and Structural Analysis of the Binding Interface between Type I Interferons and their Receptor Ifnar2

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Type I interferons (IFN) exert pleiotropic activities through binding to two cell surface receptors, ifnar1 and ifnar2. We are investigating the biophysical basis of IFN signaling by characterizing the complex of the extra-cellular domain of ifnar2 (ifnar2-EC) with IFNs on the level of purified recombinant proteins *in vitro*. Here, we present a detailed mutational study on the functional epitopes on both IFN and ifnar2. Kinetic and thermodynamic parameters were determined by label-free heterogeneous phase detection. On IFN α 2, a relatively small functional epitope comprising ten amino acid residues was localized, which is nearly entirely formed by residues on the AB loop. Two hot-spot residues, L30 and R33, account for two-thirds of the total interaction energy. Comparing the anti-viral potency of the various mutants to the binding affinity towards ifnar2 revealed a proportional correlation between the two, suggesting a rate-limiting role of ifnar2 binding in IFN signaling. On ifnar2, residues T46, I47 and M48 were identified as hot-spots in the interaction with IFN α 2. For another ten residues on ifnar2, significant contribution of interaction energy was determined. Based on these data, the functional epitope on ifnar2 was defined according to a homology model based on other members of the class II hCR family in good agreement with the complementary functional epitope on IFN α 2. Although IFN α 2 and IFN β bind competitively to the same functional epitope, mutational analysis revealed distinct centers of binding for these IFNs on ifnar2. This small shift of the binding site may result in different angular orientation, which can be critically coupled to cytoplasmic signaling.

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Keywords: type I interferon receptor; protein-protein interaction; kinetics; structure-function; mutational analysis

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Introduction

Type I interferons (IFN) are a family of homologous cytokines which very potently elicit an antiviral and anti-proliferative state in cells. These pleiotropic properties of type I IFNs have found considerable attention since their discovery more than 40 years ago (Isaacs & Lindenmann, 1957). During the last decade, some basic principles of interferon signaling pathway have been unveiled.

Abbreviations used: EC, extra-cellular; IFN, interferon; ifnar, type I IFN receptor; R, receptor; hC, helical cytokine; GH, growth hormone; TF, tissue factor; RIFS, reflectometric interference spectroscopy; VSV, vesicular stomatitis virus.

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All human type I IFNs (IFN α , β , ω and τ) bind to a cell surface receptor consisting of two trans-membrane proteins, ifnar1 (Uze *et al.*, 1990) and ifnar2 (Novick *et al.*, 1994). These receptor components associate upon binding to IFN (Cohen *et al.*, 1995), leading to phosphorylation by kinases associated to the cytoplasmic domains of ifnar1 (Tyk2) and ifnar2 (JAK1) (Uze *et al.*, 1995; Velasquez *et al.*, 1995). While ifnar2 binds IFNs with moderate affinity ($K_D \sim 2$ -10 nM for IFN α 2), the intrinsic affinity of human ifnar1 to IFNs is low ($K_D > 100$ nM), stabilizing the ternary complex by approximately 10 to 20-fold (Cohen *et al.*, 1995). Although signaling to all type I IFNs is mediated through this cell surface receptor, considerable differences in the response to different interferons have been observed (e.g. Rani *et al.*, 1996). Particularly IFN β exerts specific functions which might be caused by

differences in signal transduction at the receptor level (Abramowich *et al.*, 1994; Velasquez *et al.*, 1995; Domanski *et al.*, 1998; Lewerenz *et al.*, 1998; Lu *et al.*, 1998). Several models for the unique interaction of IFN β with the receptor have been proposed (Lewerenz *et al.*, 1998; Russell-Harde *et al.*, 1999).

Type I interferons belong to the class of helical cytokines (Sprang & Bazan, 1993) and are built by five helices. The structures of several type I IFNs have been solved, such as murine IFN β (Senda *et al.*, 1992), human IFN α 2 (Radhakrishnan *et al.*, 1996; Klaus *et al.*, 1997), human IFM β (Karpusas *et al.*, 1997) and ovine IFN τ (Radhakrishnan *et al.*, 1998). Numerous mutational studies have revealed functional important residues on IFNs (Mitsui *et al.*, 1993; Runkel *et al.*, 1998), which can be ascribed to distinct functional epitopes for the interaction with ifnar1 and ifnar2 (Bandu *et al.*, 1994; Seto *et al.*, 1995; Uze *et al.*, 1995). Much less structural and functional data is available for the receptor proteins, which belong to the class II helical cytokine receptor (hCR) family (Bazan *et al.*, 1990; Uze *et al.*, 1995). Their homology to hCRs with known structure, e.g. TFR (Harlos *et al.*, 1994; Muller *et al.*, 1996), IFN γ R (Walter *et al.*, 1995) and GHR (de Vos *et al.*, 1992), allows for modeling of the 3D structure with reasonable reliability. For GHR and IFN γ R, the crystal structure of the complex of the receptor in its ligand is also available (de Vos *et al.*, 1992; Walter *et al.*, 1995). Based on these structures, models for the receptor and the complex with IFNs have been proposed (Seto *et al.*, 1995; Uze *et al.*, 1995) and several residues contributing to binding were identified (Lewerenz *et al.*, 1998).

Understanding the mechanisms of how distinct activity profiles are exerted by different type I IFN subtypes through the same cell surface receptor proteins ifnar1 and ifnar2 is a prerequisite for designing IFN-like drugs with specific activities. We believe that this requires detailed biophysical characterization of the formation of the complex of IFN with its receptor, which initiates the signaling cascade. We are investigating the interactions of IFNs with its receptors on the level of purified recombinant proteins to elucidate structure, thermodynamics and kinetics of the complex formation. Recently we have succeeded in refolding active extra-cellular part of ifnar2 (ifnar2-EC) expressed in *Escherichia coli* and we have purified it to homogeneity with yields of a few milligrams per liter of cell culture (Piehler & Schreiber, 1999). Moreover, we have established methods for studying thermodynamics and kinetic of the interaction of ifnar2-EC with IFNs. Here, we present a systematic mutational analysis of the binding interface between ifnar2 and IFN α 2 to define the functional epitope on both proteins in quantitative terms. Protein-protein interactions were investigated by heterogeneous phase detection monitoring the binding kinetics in real time. By determining the contributions of a number of residues on both ifnar2-EC and IFN α 2, we defined the binding inter-

face of the ifnar2-IFN α 2 complex on both proteins and we quantitatively mapped differences in binding of IFN α 2 and IFN β to ifnar2.

Results

Theoretical considerations

We have shown before, that the simple relation:

$$K_D = \frac{k_d}{k_a} \quad (1)$$

between rate constants of association k_a and dissociation k_d , and the equilibrium dissociation constant K_D is valid for the formation of the ifnar2-EC-IFN α 2 complex (Piehler & Schreiber, 1999). The interaction between two proteins A and B is typically described by a three-state energy profile including an encounter complex A:B, and a transition state with increased free energy compared to the free and complexed proteins (Figure 1). These early states of interaction A:B and AB ‡ are stabilized by long-range electrostatic forces (Schreiber & Fersht, 1996; Selzer & Schreiber, 1999), while the final complex AB is stabilized mainly by short-range interactions. The dissociation constant K_D describes the interaction energy of the complex as a difference of the free energy of the free proteins and the free energy of the final complex AB according to:

$$\Delta G_{K_D}^0 = RT \ln K_D \quad (2)$$

Thus, changes in $\Delta G_{K_D}^0$ upon mutation:

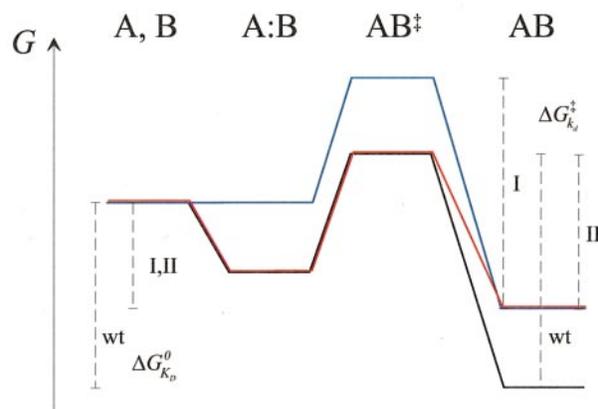


Figure 1. Free energy profiles for protein A and B forming a complex AB *via* an encounter complex A:B and a transition state AB ‡ . The profiles for two different types of mutations of the original proteins (black) are schematically depicted: mutation I (blue) exclusively removes long-range electrostatic forces, while mutation II (red) exclusively removes short-range forces. While these two mutated complexes have the same $\Delta G_{K_D}^0$, the difference in $\Delta G_{K_D}^0$ is considerable.

$$\Delta\Delta G_{K_D}^0 = RT \ln \frac{K_D^{\text{mut}}}{K_D^{\text{wt}}} \quad (3)$$

identifies changes in both the long-range and short-range interactions. However, precisely defining residues in the binding interface requires discrimination of long and short-range interactions. According to transition-state theory, the kinetic rate constants describe the formation of the transition state. The rate of association has been shown to be affected mainly by the magnitude of long-range electrostatic forces between the proteins, which can be altered by mutations outside the binding site (Schreiber & Fersht, 1996; Selzer & Schreiber, 1999). Dissociation rates reflect the free energy $\Delta G_{k_d}^\ddagger$ required for breaking the short-range interactions in the binding site of the complex:

$$\Delta G_{k_d}^\ddagger = -RT \ln \frac{hk_d}{kT} \quad (4)$$

where k_d represents the dissociation rate constant. The change in $\Delta G_{k_d}^\ddagger$ upon mutation:

$$\Delta\Delta G_{k_d}^\ddagger = RT \ln \frac{k_d^{\text{mut}}}{k_d^{\text{wt}}} \quad (5)$$

is a much better measure for determining residues making short-range interactions than $\Delta\Delta G_{K_D}^0$, as it directly maps changes in the stability of the complex (cf. Figure 1). Measuring association and dissociation rate constants therefore allows for discriminating long and short-range forces contributing to affinity. Changes in the dissociation rate constant can also be determined with higher precision than changes in affinity because they are independent of the active protein concentration.

Rate constants of association and dissociation can be measured conveniently by label-free solid phase detection. Even though absolute values obtained by this method are often doubtful, relative parameters, such as $\Delta\Delta G$ values, have been determined to be very reliable (Cunningham & Wells, 1993; Albeck & Schreiber, 1999).

The ifnar2 binding site on IFN α 2

The AB-loop and the D-helix have been identified before as being important for IFN activity (Mitsui *et al.*, 1993) and are assumed to interact with ifnar2 (Seto *et al.* 1995; Uze *et al.*, 1995). We mutated in IFN α 2 most of the exposed residues from S25 to D35 on the AB-loop and from K121 to K134 on the D-helix to alanine, and determined the effect of mutation on the kinetics and thermodynamics of the interaction with ifnar2. All investigations were carried out with wild-type ifnar2-EC immobilized to the sensor surface, since rate constants and affinity determined by this configuration are self-consistent (Piehler & Schreiber, 1999). Typical binding curves for the interaction of wild-type IFN α 2 and the mutant L26A are shown in Figure 2(a). Dissociation rate constants were deter-

mined at high protein concentrations to obtain a maximum amplitude. The dissociation curves for all IFN α 2 mutants were fitted by a single exponential decay without systematic deviations. The dissociation rate constants were measured at least five times for each mutant, with typical standard deviations of less than 10%. Association rate constants were determined under pseudo-first-order conditions at concentrations of 50 nM. Within the first 30 seconds, the association phase of the binding curve followed a single exponential function and was determined by direct fitting of a simple 1:1 kinetic model. Owing to the fast interaction kinetics and the additional parameters (concentration and the dissociation rate constant) the typical standard deviation of the association rate constants was ~20%.

All kinetic and thermodynamic constants for the IFN α 2 mutants binding to immobilized wild-type ifnar2-EC are summarized in Table 1. The association and the dissociation rate constants of the IFN α 2 mutants in comparison to wild-type IFN α 2 are shown in Figure 3. The precision of measurement allowed for the detection of down to ~20% change in the dissociation rate constant (2σ criterion, $\alpha = 0.95$). A significant increase of the dissociation rate constant was found for the mutants L26A, F27A, L30A, K31A, D32A, R33A, H34A, D35A, R125A and K133A (Figure 3(b)). For the two mutants, R33A and L30A, the kinetics were too fast to be resolved by this method ($>0.5 \text{ s}^{-1}$). A dissociation constant K_D of approximately 5 μM was found for the mutant L30A by titration on the surface (Figure 2(b) and (c)). Affinities determined by this method were shown to be consistent with the rate constants (Piehler & Schreiber, 1999). For the mutant R33A, no significant interaction with immobilized ifnar2-EC was detectable by this method up to concentrations of 50 μM of this mutant. Significant contributions of the association rate constant were observed for the three positively charged residues K31, K131 and K133 with the strongest contribution by K133. Since kinetic measurement allows for discrimination of long and short-range forces, the energetic contributions of the mutated residues to the stability of the complex were determined both from the dissociation rate constants ($\Delta\Delta G_{k_d}^\ddagger$) and from the affinity constant ($\Delta\Delta G_{K_D}^0$) (Table 1). The residues L30 and R33 are hot-spots on IFN α 2, contributing approximately 15 kJ/mol and >15 kJ/mol, respectively. Except for residue L26, for which the energetic contribution is approximately 4 kJ/mol, all other residues investigated contribute less than 2 kJ/mol.

Anti-viral activity versus affinity of IFN α 2 mutants

Binding of IFN to ifnar2 is the very first step initiating a complex signal transduction cascade which activates the anti-viral state in cells. Thus,

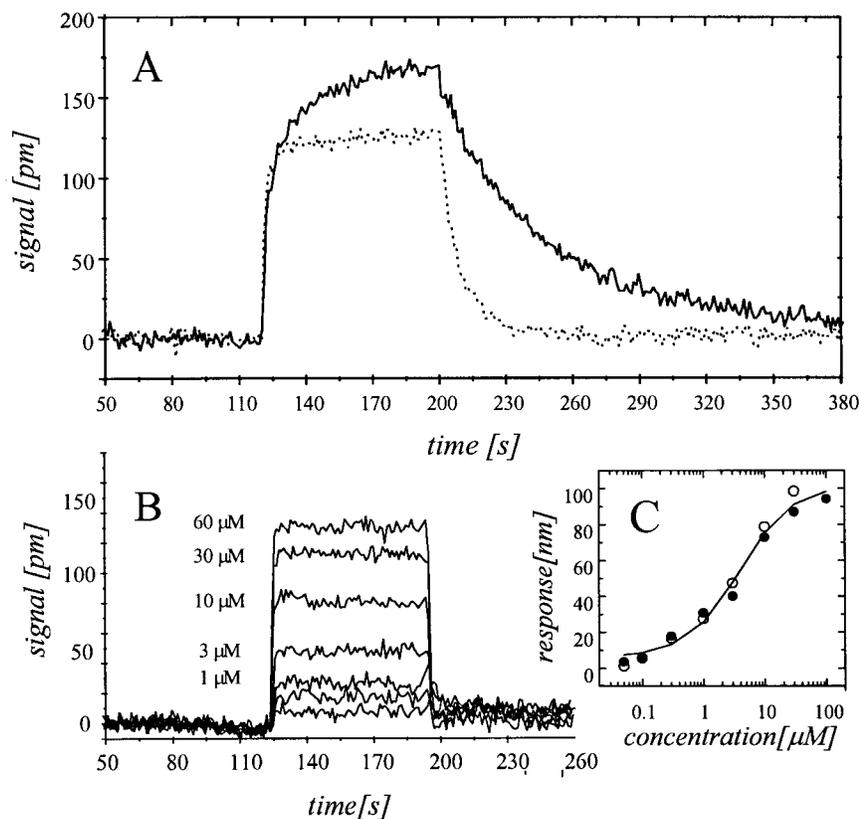


Figure 2. Measurement of kinetics and thermodynamics of the IFN α 2 binding to immobilized wild-type ifnar2-EC by heterogeneous phase detection. (a) Association and dissociation of wild-type IFN α 2 (500 nM, continuous line) and the mutant L26A (1.4 μ M, broken line). (b) Binding curves for IFN α 2 L30A at various concentrations. (c) Determination of K_D from a plot of the equilibrium response determined from B versus the concentration of L30A (two measurements for each concentration) according to Piehler & Schreiber (1999).

correlation between kinetics and thermodynamics of binding of different IFN mutants to ifnar2-EC and the relative potency of their antiviral response could define limiting steps in formation of the ternary complex required for signaling. The anti-viral activity of the IFN α 2 mutants relative to wild-type IFN α 2 were determined from the concentrations required to protect 50% of human WISH cells challenged with vesicular stomatitis virus (VSV). We found that the relative activity of the mutants decreased proportional to the relative decrease in affinity determined by heterogeneous phase detection (Figure 4). Assuming that this correlation is also valid at lower activities, the affinity of the mutant R33A can be estimated from its relative anti-viral activity (of 6×10^5 units/mg) to be approximately 30 μ M.

The binding site for IFN α 2 on ifnar2

Three crystal structures of receptors from the hCR super-family with a similar topology as ifnar2-EC have been solved: TFR, IFN γ R and GHR. While TFR has 21% identity and 33% similarity with ifnar2-EC, and IFN γ R has 17% identity and 33% similarity, the homology of GHR to ifnar2-EC is significantly lower. Therefore, we used only the

structures of TFR and IFN γ R for homology modeling. The final alignment chosen for modeling is shown in Figure 5. Because of the rather low homology, the sequence alignment is ambiguous and different models are possible. The positioning of the gaps within the sequence of ifnar2 compared to the TFR sequence proved to be particularly critical. The sequence alignment of ifnar2 to IFN γ R appeared more reliable: in the N-terminal domain where the binding site was mapped, only a single gap of two residues occurs, which location is defined by structural conserved regions. We therefore took the model based on IFN γ R for all further structural considerations. A model of ifnar2-EC based on TFR as template with the alignment shown in Figure 5, however, gave a RMS deviation of all backbone atoms of 1.35 Å compared to the IFN γ R based structure.

The region presumably involved in the interaction with IFN can be identified by comparison with the homologous systems GH/GHR and IFN γ /IFN γ R, for which the structures of the complex have been solved (Seto *et al.*, 1995; Lewerenz *et al.*, 1998). Functional contributions of some residues in this region have already been confirmed by a mutational study on ifnar2 (Lewerenz *et al.*, 1998). To confine this binding site on ifnar2, we

Table 1. Thermodynamic and kinetic constants for the interactions of wild-type IFN α 2 and several mutants with wild-type ifnar2-EC

IFN α 2 mutant	k_a^a ($10^6 \text{ M}^{-1}\text{s}^{-1}$)	k_d^a (s^{-1})	K_D^b (nM)	$\Delta\Delta G_{k_d}^{\ddagger d}$ (kJ/mol)	$\Delta\Delta G_{K_D}^0 e$ (kJ/mol)	Activity ^f (10^8 units/mg)
wt	2.1	0.020	10	-	-	4.0
S25A	2.1	0.015	7	-0.8 (0.2)	-0.8 (0.4)	n.d.
L26A	2.1	0.114	55	4.2 (0.2)	4.1 (0.4)	0.9
F27A	1.9	0.046	24	1.9 (0.1)	2.1 (0.3)	2.4
S28A	2.3	0.022	10	0.2 (0.2)	-0.0 (0.4)	n.d.
L30A	-	-	$\sim 5000^c$	-	~ 15	0.04
K31A	1.3	0.030	23	1.0 (0.2)	2.0 (0.3)	2.8
D32A	2.1	0.041	19	1.7 (0.2)	1.6 (0.4)	2.6
R33A	-	-	$>5000^c$	-	>15	0.006
H34A	1.8	0.037	20	1.4 (0.2)	1.7 (0.4)	2.8
D35A	2.1	0.031	15	1.0 (0.1)	0.9 (0.4)	3.0
E41A	2.1	0.021	10	0.1 (0.1)	0.0 (0.5)	4.4
K121A	1.9	0.024	12	0.4 (0.2)	0.0 (0.4)	4.0
R125A	1.9	0.034	18	1.2 (0.2)	1.4 (0.4)	3.4
L128A	2.1	0.021	9	0.1 (0.3)	-0.2 (0.4)	5.1
K131A	1.4	0.023	16	0.3 (0.1)	1.2 (0.3)	3.0
E132A	2.4	0.019	8	-0.2 (0.1)	-0.5 (0.4)	3.8
K133A	0.9	0.033	37	1.1 (0.1)	3.2 (0.4)	1.3
K134A	1.8	0.021	11	0.1 (0.3)	0.3 (0.5)	n.d.

^a All rate constants were measured by heterogeneous phase detection with immobilized wild-type ifnar2-EC. The standard deviations from at least five independent measurements were $\sigma < 10\%$ for all k_d and $\sigma < 20\%$ for all k_a .

^b Determined from the rate constants $K_D = k_d/k_a$ if not noted otherwise; $\sigma < 20\%$.

^c Determined from equilibrium binding at the surface as shown in Figure 2(b) and (c); the standard error is $\sim 25\%$.

^d Determined from k_d according to equation (3) (25°C); σ is in parentheses.

^e Determined from K_D according to equation (5) (25°C); $\sigma < 20\%$.

^f Anti-viral activity on human WISH cells with VSV as challenging virus relative to NIH IFN β standard. The standard error from a fit of 16 data points was $\sim 10\%$.

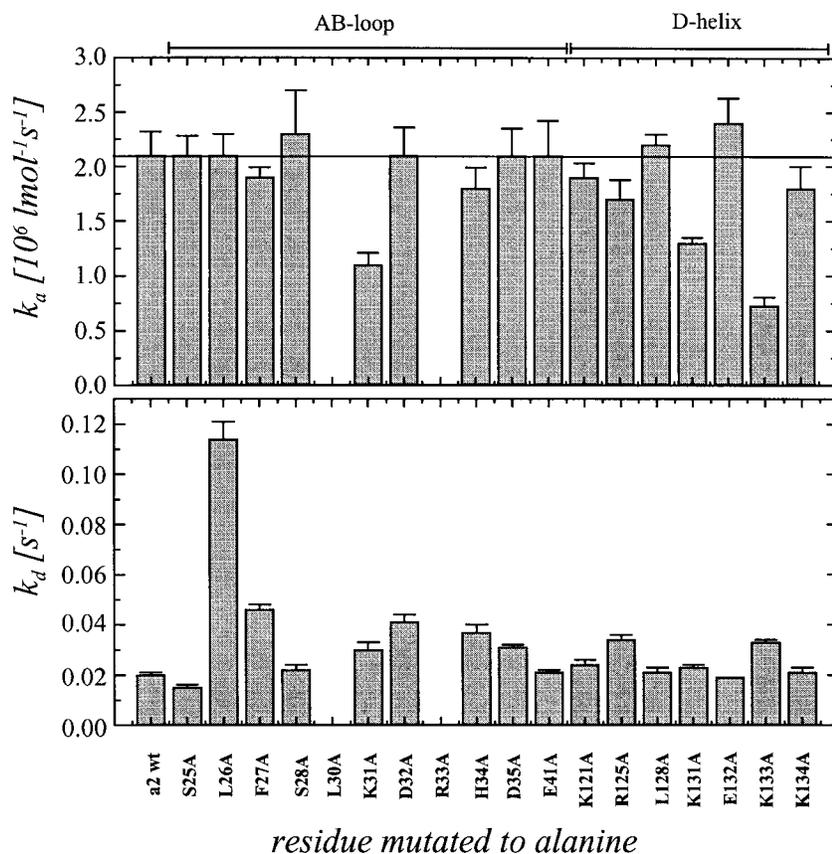


Figure 3. Comparison between the kinetic rate constants determined for the interaction of wild-type and mutant IFN α 2 with immobilized wild-type ifnar2-EC (data from Table 1). Rates of L30A and R33A were too fast to be determined. Error bars indicate the standard deviations from at least five independent measurements.

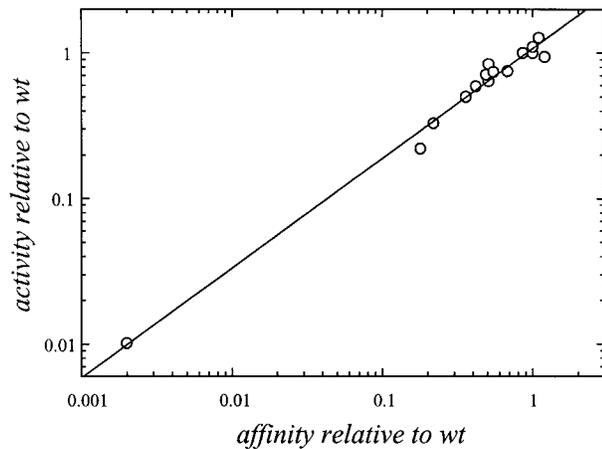


Figure 4. Correlation between the relative antiviral activity of different IFN α 2 mutants with the relative affinity of the mutants determined by heterogeneous phase detection (data from Table 1).

probed long-range electrostatic interactions by a set of single charge reverse mutants: D8R, E30R, E52R, D72R, E86R, E134R, D140R and D191R. The effects on the dissociation kinetics was investigated by heterogeneous phase detection with IFN α 2 wild-type being immobilized (Table 2). Based on these data, a number of residues in the binding site were individually mutated into alanine: T46, I47, M48, S49, K50, E52, D53, W74, S76, T77, H78, E79, N100, W102, I105, H189, D191 and E192. The dissociation kinetics of these mutants was compared to wild-type ifnar2-EC with immobilized IFN α 2 (Figure 6(a)). In order to characterize the energetic contribution of residues towards binding more precisely, changes in kinetics and thermodynamics of the interaction with IFN α 2 were investigated with the ifnar2-EC mutants being immobilized. Immo-

bilization of ifnar2-EC mutants gave active surfaces for most mutants with typical R_{\max} values between 100 and 300 pm (60-200 pg/mm²) and a similar stability as the wild-type. For some mutants, however, no stable and active surfaces were obtained: the mutants E134R and W74A quickly lost their activity when immobilized on a surface, whereas W74F remained stable on the surface. For immobilized E79A, no binding activity could be measured for the immobilized protein indicating complete denaturation of this mutant by the immobilization procedure. The affinity of this mutant was estimated by a binding inhibition assay in solution. All the thermodynamic and kinetic data measured for the interaction of these mutants with wild-type IFN α 2 are given in Table 2. Though the absolute dissociation rate constants are different for the ifnar2-EC mutants with either IFN α 2 or ifnar2-EC immobilized, the relative change in the dissociation rate constant, and thus the $\Delta\Delta G_{\text{kd}}^{\ddagger}$, determined are in good agreement (Figure 6(b)). This result proves that these relative parameters are widely independent from experimental artifacts. A total of 14 out of the 24 mutated residues investigated significantly affected the interaction with IFN α 2. The residue M48 was identified as a hot-spot for the binding of IFN α 2, contributing more than 15 kJ/mol of binding energy. Mutations of T46 and I47 to Ala decrease binding by more than tenfold. E79 to Ala (but not to Gln) as well as I105 to Ala decreased the affinity to IFN α 2 by four- to fivefold. The very low stability of the mutant E79A suggests that the residue E79 stabilizes not only the integrity of the binding site, but also seems to be important for the stability of the protein. A more precise characterization of the contribution of this residue towards binding of IFN α 2 might be achieved by a more stable homologue mutant or by a different immobilization procedure. The mutations S49A, K50A, W74F, H78A and

ifnar2-EC	4	DSPDYTDES	CTFKISLRNF	RSILSWELKN	HSIVPTHYTL	LYTIMSKPED
TFR	1	SGTTNTVAA	YNLTWKSTNF	KTILEWEPKP	V----NQVYT	VQISTK---S
IFN γ R	9	DLGPSSVPTP	TNVTIESYNY	NPIVYWEYQI	MPQVEVFTVE	VKNYGVKNSE
ifnar2-EC	54	LKVVKNCANT	TRSFCDLTDE	WR-STHEAYV	TVLEGF----	-SG-NTTLFS
TFR	43	GDWKSCKFYT	TDTECDLTDE	IVKDVKQTYL	ARVFSYPAGN	VES-TEPLYE
IFN γ R	59	--WIDACINI	SHHYCNISDH	VG-DPSNSLW	VRVKAR----	-VGQKESAYA
ifnar2-EC	97	CSHNFWLAI	MSFEPPEFEI	VGFT-NHINV	MVKFP-----	-----S
TFR	96	NSPEFTPYLE	TNLGQPTIQS	FEQVGTKVNV	TVEDERTLVR	RNNTPLSLRD
IFN γ R	101	KSEEFVAVCRD	GKIGPPKLDI	RKEEKQIMID	IFHPSVFNNG	DEQEVVDYDPE
ifnar2-EC	132	IVVEELQFDL	SLVIEEQSEG	IVKHKHPEIK	GNMSGNFTYI	IDKLIPTNTNY
TFR	146	VFGKDLIYTL	YYWKSS----	-GKKTAKTNT	-----NEFL	IDVDK-GENY
IFN γ R	151	TTCYIRVYNV	YVRMNG-SEI	QYKILTQKED	DCDEIQCQLA	IPVSSLNSQY
ifnar2-EC	182	CVSVYLEHS-	--DEQAVIKS	PLKCTLLPPG	QSEFS	
TFR	186	CFSVQAVIPS	RTVNRKSTDS	FVECMG----	-----	
IFN γ R	200	CVSAEGVLH-	-VWGVTTEKS	KEVCITIFNS	SI----	

Figure 5. Sequence alignment of ifnar2-EC to IFN γ R and TFR used for homology modeling. Note that alignment of ifnar2 to IFN γ R along the first fibronectin like domain (residues 1-105) is almost perfect, with only one short (two residue) gap.

Table 2. Thermodynamic and kinetic constants for the interaction of wild-type IFN α 2 with wild-type and mutant ifnar2-EC

ifnar2-EC mutant	IFN α 2								IFN β		
	k_a^a ($10^6 \text{ M}^{-1}\text{s}^{-1}$)	k_d^a (s^{-1})	k_d^b (s^{-1})	K_D^c (nM)	$\Delta\Delta G_{k_d}^{\ddagger f}$ (kJ/mol)	$\Delta\Delta G_{k_d}^{\ddagger g}$ (kJ/mol)	$\Delta\Delta G_{K_D}^0^h$ (kJ/mol)	k_d (s^{-1})	K_D (nM)	$\Delta\Delta G_{k_d}^{\ddagger f}$ (kJ/mol)	$\Delta\Delta G_{K_D}^0^h$ (kJ/mol)
wt	2.1	0.020	0.038	10	-	-	-	0.017	5 ^e	-	-
D8R	-	-	0.034	-	-	-0.2 (0.2)	-	-	-	-	-
E30R	-	-	0.036	-	-	-0.1 (0.2)	-	-	-	-	-
T46A	-	0.189	-	130 ^d	5.5 (0.3)	-	6.5 (0.5)	0.081	-	3.8 (0.4)	-
I47A	-	0.289	-	-	6.5 (0.3)	-	-	0.289	-	7.0 (0.3)	-
M48A	-	> 0.4	-	~5000 ^d	-	-	15.4 (0.6)	0.148	-	5.3 (0.3)	-
S49A	2.1	0.047	0.086	22	2.1 (0.2)	2.0 (0.2)	2.1 (0.4)	0.071	-	3.5 (0.3)	-
K50A	2.6	0.053	0.125	17	2.3 (0.2)	3.0 (0.3)	1.9 (0.5)	0.019	-	0.3 (0.3)	-
E52R	1.3	0.040	0.053	30	1.6 (0.2)	0.9 (0.2)	2.8 (0.5)	0.026	-	1.0 (0.3)	-
E52A	2.2	0.032	0.046	14	1.1 (0.3)	0.5 (0.2)	1.0 (0.5)	-	-	-	-
D53A	-	-	0.033	-	-	-0.3 (0.3)	-	-	-	-	-
K55A	1.8	0.034	0.098	19	1.3 (0.2)	2.2 (0.4)	1.7 (0.4)	0.025	-	0.9 (0.2)	-
D72R	-	-	0.035	-	-	-0.2 (0.3)	-	-	-	-	-
W74A	-	-	0.116	-	-	2.8 (0.3)	-	-	-	-	-
W74F	1.6	0.055	0.113	34	2.5 (0.2)	2.7 (0.4)	3.2 (0.6)	0.022	-	0.7 (0.5)	-
S76A	2.0	0.033	0.081	17	1.2 (0.2)	1.9 (0.2)	1.4 (0.3)	0.017	-	-0.1 (0.6)	-
T77A	-	0.021	0.035	-	0.1 (0.1)	-0.2 (0.3)	-	0.021	-	0.5 (0.3)	-
H78A	1.9	0.055	0.120	29	2.5 (0.1)	2.9 (0.2)	2.7 (0.4)	-	-	-1.9 (0.6)	-
E79A	-	-	-	~50 ^e	-	-	~4	-	~10 ^e	-	1.7
E86R	-	-	0.042	-	-	0.3 (0.2)	-	-	-	-	-
N100A	2.2	0.016	0.033	7	-0.6 (0.2)	-0.3 (0.2)	-0.7 (0.4)	0.005	-	-3.1 (0.5)	-
W102A	1.8	0.063	0.111	35	2.7 (0.1)	2.7 (0.3)	2.9 (0.5)	-	~500 ^d	-	11.0
I105A	-	0.107	-	-	4.1 (0.3)	-	-	0.093	-	4.2 (0.3)	-
E134R	-	-	0.033	-	0.0 (0.1)	-0.3 (0.3)	-	-	-	-	-
D140R	1.8	0.017	0.026	9	-0.5 (0.1)	-0.9 (0.3)	0.1 (0.4)	0.020	-	0.5 (0.2)	-
H189A	-	0.024	0.054	-	0.4 (0.2)	0.9 (0.3)	-	-	-	-	-
D191R	1.5	0.034	0.061	23	1.3 (0.2)	1.2 (0.2)	2.1 (0.5)	-	-	-	-
D191A	2.2	0.017	0.040	8	-0.4 (0.3)	0.2 (0.2)	-0.4 (0.4)	0.022	-	0.7 (0.4)	-
E192A	2.1	0.030	0.051	13	0.7 (0.2)	0.8 (0.2)	0.7 (0.3)	0.014	-	-0.5 (0.5)	-

^a Measured by heterogeneous phase detection with wild-type and mutant ifnar2-EC being immobilized. The standard deviations (σ) from at least five independent measurements were <10% for all k_d with IFN α 2, <25% for all k_d with IFN β and <20% for all k_a .

^b Measured by heterogeneous phase detection with wild-type IFN α 2 immobilized, σ < 10%.

^c Determined from the rate constants $K_D = k_d/k_a$, σ < 20%.

^d Determined from equilibrium binding at the surface, σ ~ 20%.

^e Estimated from binding inhibition assay in solution as described before (Piehler & Schreiber, 1999), σ ~ 25%.

^f $\Delta\Delta G_{k_d}^{\ddagger}$ determined from k_a (ifnar2-EC immobilized) according to equation (5) (25 °C), with σ in parentheses.

^g $\Delta\Delta G_{k_d}^{\ddagger}$ determined from k_d (IFN α 2 immobilized) according to equation (5) (25 °C), with σ in parentheses.

^h $\Delta\Delta G_{K_D}^0$ determined from K_D according to equation (3) (25 °C), with σ in parentheses.

W102A each increased the dissociation rate constant between two- and threefold compared to wild-type ifnar2. The mutants E52A, K55A and S76A increased the dissociation rate constant by less than twofold, suggesting that these residues are positioned in the periphery of the binding site. For all other alanine mutants, dissociation rate constants similar to wild-type ifnar2-EC were observed. Significant changes in the association rate constant were observed for the charge reverse mutants E52R and D191R (decreased k_a), as well as for K50A (increased k_a), indicating that a negative field on ifnar2 is required for electrostatic steering.

Comparison of IFN α 2 and IFN β binding to ifnar2-EC

Accurate measurement of the interaction kinetics of single alanine mutations in the binding site of ifnar2 allowed mapping of differences in binding of IFN α 2 and IFN β . Strong non-specific binding interfered with studying the interaction of immobilized ifnar2-EC with IFN β under physiological

ionic strength. Increasing the salt concentration up to 500 mM eliminated the non-specific binding to the transducer surface. Therefore, all investigations involving IFN β were carried out under these conditions. The dissociation rate constant of IFN α 2 decreased by 10% at this salt concentration compared to 150 mM, but still single-phase kinetics were observed. The maximum response by IFN β was approximately 10-20% higher than that of IFN α 2, in agreement with the higher molecular mass of the glycosylated, CHO-produced IFN β . The first 70% of the dissociation amplitude were fitted by a single exponential decay, with a rate constant of 0.017 s^{-1} for immobilized wild-type ifnar2-EC. Binding curves for IFN α 2 and IFN β are shown in Figure 7 in comparison for two different ifnar2-EC mutants. The association kinetics of IFN β significantly deviated from a simple single exponential model and was not further evaluated.

The changes in interaction energy of mutants of ifnar2-EC ($\Delta\Delta G_{k_d}^{\ddagger}$ if available, otherwise $\Delta\Delta G_{K_D}^0$, cf. Table 2) for wild-type IFN α 2 and IFN β are compared in Figure 8. Most residues of

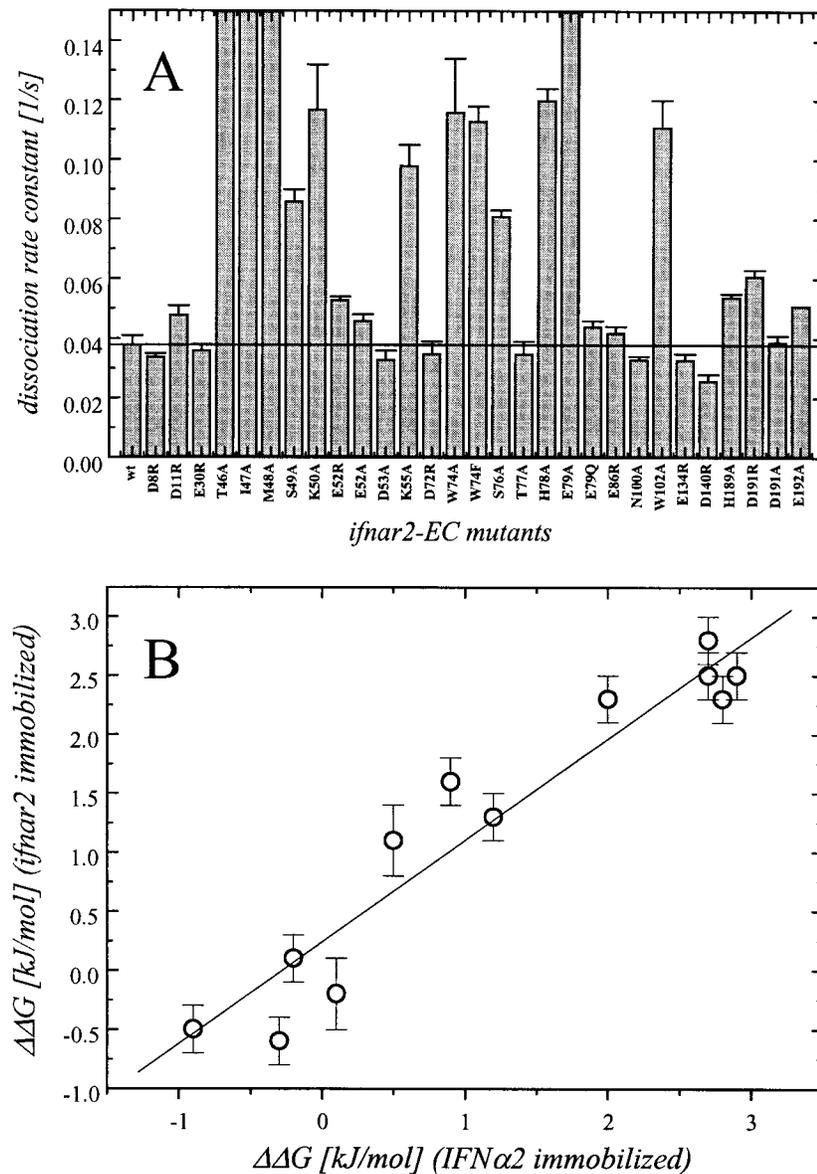


Figure 6. Characterization of ifnar2-EC mutants. (a) Dissociation rate constants measured with wild-type IFN α 2 immobilized to the surface. (b) Comparison of $\Delta\Delta G_{kd}^{\ddagger}$ obtained from measurements with immobilized IFN α 2 and ifnar2-EC, respectively. Data are from Table 2.

ifnar2-EC that are destabilizing the complex with IFN α 2, also affect the binding of IFN β . However, the relative energetic contributions to binding of IFN α 2 and IFN β are very different for most residues. The mutation W102A results in a tenfold stronger decrease in affinity to IFN β compared to IFN α 2. The mutation M48A, which destabilizes the complex with IFN α 2 by about 500-fold, decreased the affinity towards IFN β only eightfold. Furthermore, the mutant H78A stabilizes the complex with IFN β nearly twofold, while destabilizing the complex with IFN α 2 more than twofold. The mutation N100A hardly affects the rates for binding IFN α 2, whereas its decreasing the dissociation rate constant for IFN β by almost fourfold (cf. the binding curves in Figure 7). For the residues K50, E52 and W74F, no significant changes in affinity to IFN β were found upon mutation, while they

significantly contributed to binding of IFN α 2 with ifnar2-EC.

Discussion

Understanding the activity profiles exerted by different type I IFNs requires a thermodynamic, kinetic and structural understanding of the recognition by the cell surface receptor. As a first step towards this goal, we have carried out a rigorous mutational analysis of the binding interface of the complex formed between ifnar2 and type I IFNs. The energetic contributions of amino acid residues were mapped in a quantitative manner using purified proteins for measuring affinity and kinetics of the complex formation. Heterogeneous phase detection was used for precise comparison of the rate constants and the affinity of different IFNs and its mutants. A principal problem of mutational

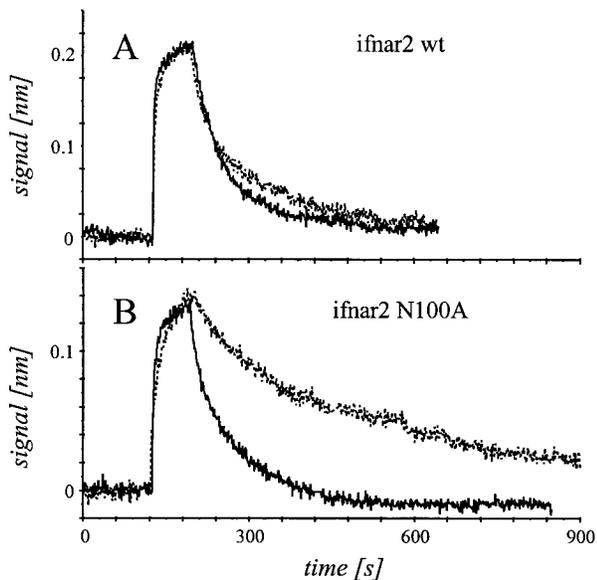


Figure 7. Comparison of the binding curves for wild-type IFN α 2 (—) and IFN β (·····) with immobilized (a) wild-type ifnar2-EC and (b) the mutant N100A.

analysis of a binding site is to discriminate between mutations which affect its structural integrity, from mutations which specifically remove interactions in the interface. In case of IFN α 2, the importance of residues for the structural integrity of the binding site can be estimated from the structure, and only exposed residues in the vicinity of the hot-spot of binding were analyzed. The case is more difficult for ifnar2, because small shifts in sequence alignment can exchange exposed and buried residues. Some mutations on ifnar2-EC affected the stability of the protein (e.g. Y81A, W74A), indicating an effect on the structure of ifnar2. According to our model structure, these two residues are buried within the core of ifnar2. Thus, the stability of the ifnar2 mutants gave at least an estimate of whether residues are exposed or buried. All charge-reverse mutations which are located outside the binding site (D8R, E30R, D72R, E86R, E134R and D140R) did not significantly affect the interaction, suggesting that in general mutations on the surface of ifnar2 affect binding only when they are located at the vicinity of the binding site. This is true even for the extreme case of charge-reverse mutants. Furthermore, very different effects upon mutation on the interaction with IFN α 2 and IFN β were observed. This was most pronounced for the mutants M48A and W102A which are hot-spots of binding for IFN α 2 and IFN β , respectively. These mutants apparently affect the binding very locally, excluding considerable effects on the structural integrity of the entire binding site.

Until now, all mutational studies of the binding site on IFN α 2 have been based on the assessment of pleiotropic activities of chimeric or point-

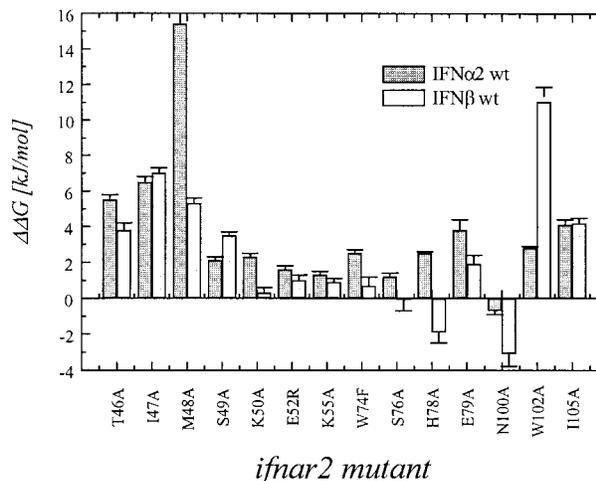


Figure 8. Comparison of the energetic contributions of residues in the binding site of ifnar2 to binding of IFN α 2 and IFN β (data from Table 2).

mutated IFNs (reviewed by Mitsui *et al.*, 1993). We found a good correlation of the anti-viral activity of IFN α 2 mutants with their affinity toward ifnar2-EC, which confirms the principal validity of conclusion drawn from activity assays. The residues on IFN α 2 which contribute to the interaction with ifnar2 are in good agreement with residues identified previously by measuring changes in antiviral activity (Camble *et al.*, 1986; Tymms *et al.*, 1989; Waite *et al.*, 1992; Wang *et al.*, 1994). Assessment of the kinetics and thermodynamics of IFN binding to ifnar2 enables us to characterize its role in IFN signaling. The correlation between affinity and activity of IFN is striking. This correlation holds true not only for the IFN α 2 mutants we investigated here. Our thermodynamic and kinetic data for ifnar2-EC mutants also correlate with anti-viral activities of IFNs measured on cells with mutated ifnar2 (Lewerenz *et al.*, 1998). In particular, differences in anti-viral activity of IFN α 2 and IFN β observed with cells with the ifnar2 mutants M48A and W102A correlate with the differences we found in the affinity. The apparently linear downstream propagation (tyrosine phosphorylation and formation of the transcription factor ISGF3) of these effects shown in the same study corroborates the central role of the affinity of IFNs to ifnar2 for function. Such correlation is not trivial, since the formation of a ternary complex by at least two consecutive binding events is required for signal transduction. In the case of the structural homologous and functionally similar GH-GHR system, the affinity of GH to its receptor surpasses the requirements for activity by about 30-fold, and any further increase in affinity by decreasing k_d did not decrease the EC₅₀ dose (Pearce *et al.*, 1999). The limiting step in formation of the ternary GH complex is probably the association of GH to the first receptor (binding to site 1), while the long half-life

of this complex (\sim one hour) is longer than is required to ensure the formation of a ternary complex within a single binding event. For GH mutants with dissociation rate constants higher than $\sim 0.016 \text{ s}^{-1}$, a linear correlation between affinity and activity has been observed (Pearce *et al.*, 1999). The half-life of the binary complex of $<$ one minute is probably too short to guarantee the formation of a productive ternary complex within a single binding event. In this case, equilibrium occupancy of the receptor (k_a versus k_d) determines the probability of the formation of the ternary complex. This situation corresponds to the IFN α 2-receptor system with a k_d of 0.02 s^{-1} , which is strikingly close to the critical k_d in the GH system. Though the critical k_d , below which no further increase in activity can be achieved, has yet to be verified for the binding of IFNs, increasing the activity of IFNs should be possible by increasing k_a .

The total free energy of the IFN α 2-ifnar2-EC complex ΔG^0 is approximately 54 kJ/mol at room temperature assuming the unitary standard state of 55 M given by the concentration of water molecules. Interaction energies $\Delta\Delta G_{K_D}^0$ determined from single point mutations are not strictly additive, because cooperative effects are not taken into account. Furthermore, interactions with the backbone and C $^\beta$ atoms are not mapped by alanine scanning analysis. However, the sum of $\Delta\Delta G_{K_D}^0$ for individual mutations gives an estimation as to what extent the binding site is mapped by the residues investigated (Cunningham & Wells, 1993). For IFN α 2, the sum of the interaction energies $\Delta\Delta G_{K_D}^0$ of all residues investigated is approximately 53 kJ/mol, which is close to the free energy of the complex formation. This agreement suggests that the functional epitope is essentially built from

the residues we have identified. Further contributions to the total complex free energy have to be ascribed to long-range electrostatic interactions, which considerably increase the basal association rate constant ($\sim 5 \text{ kJ/mol}$ at physiological ionic strength; Piehler & Schreiber, 1999). The two residues L30 and R33 on IFN α 2 account for nearly two-thirds of ΔG^0 , together with L26 and F27 for more than 75 % of ΔG^0 . The functional epitope on IFN α 2 for binding to ifnar2 according to these considerations is depicted by the solution structure of IFN α 2 (Klaus *et al.*, 1997) shown in Figure 9. The key residues L30 and R33 (colored in red in Figure 9) are close to each other and form together with L26 and F27 a hydrophobic patch on IFN α 2 (and probably other IFN α , which have a highly conserved sequence in this region). This region contributes the dominant part of the interaction energy and can be assumed as the very center of the binding site. Six residues adjacent to this patch also show significant interaction with ifnar2; however, none of them decreases the complex stability (as measured by the change in k_d) by more than twofold (orange colored residues in Figure 9). These six residues are all charged, suggesting that they predominantly interact by electrostatic forces. Except for R125 and K133, which are both in the periphery of the binding site, all residues contributing to binding of ifnar2 are localized on the AB-loop (Figure 9(b)). Thus, peptides analogous to the AB-loop might mimic the binding site of IFN α 2, providing a potential lead structure for designing low molecular mass antagonists.

A quantitative determination of the rate constant of the complex formation allows for discriminating of long and short-range forces contributed by specific residues. We have shown before that long-range electrostatic forces considerably enhance the

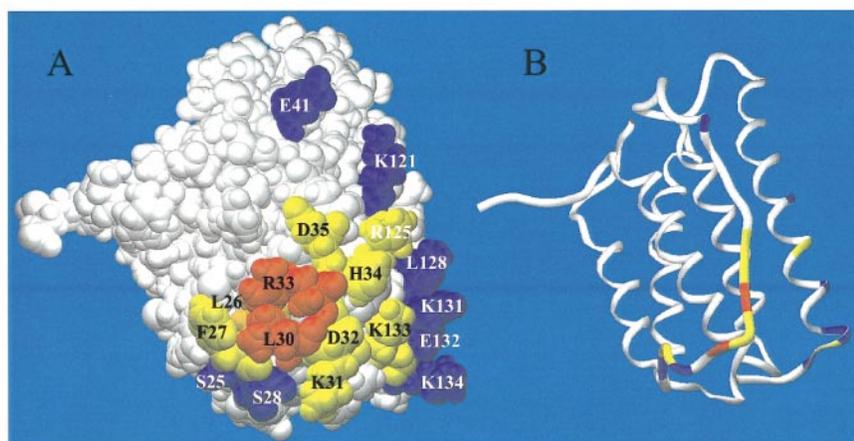


Figure 9. The functional epitope for binding ifnar2 on IFN α 2 (solution structure of IFN α 2 according to Klaus *et al.* (1997)). (a) Space-filling representation; (b) ribbon model including the side-chains of the mutated residues. The hot-spot residues R33 and L30 are colored red, L26 is colored orange and the other residues with significant effect on complex stability (F27, K31, D32, H34, D35, R125, K133) are colored yellow. Indifferent residues (S25, S28, K121, L128, E132 and K134) and residue K131, which contributes exclusively by long-range forces, are depicted in blue. This picture was composed with the Swiss-PDB Viewer (Guex & Peitsch, 1997).

association of the IFN α 2-ifnar2-EC complex (Piehler & Schreiber, 1999). In agreement with the theory of electrostatic rate enhancement, only mutations of charged residues (K31, K131 and K133) on IFN α 2 affected the association rate constant. Mutation of these positive residues into neutral residues decreased k_a , indicating that the positive electric field in the periphery of the functional epitope is important for steering the association with ifnar2. This observation supports our notion that a binding site is preferably mapped using $\Delta\Delta G_{kd}^\ddagger$ as a criterion for allocating a residue in the binding site.

In comparison to the functional epitope of GH for the interaction with GHR (de Vos *et al.*, 1992; Cunningham & Wells, 1993), the number of contact residues on IFN α 2 is much smaller, suggesting a significant smaller buried surface of the complex with ifnar2. This observation matches the differences observed in binding kinetics of the two complexes. Both association and dissociation rate constants of binding of GH to its receptor ($3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $2.7 \times 10^{-4} \text{ s}^{-1}$, respectively, for binding of GH site 1; Cunningham & Wells, 1993) are much smaller than for IFN α 2-ifnar2. The lower association rate constants indicates less long-range electrostatic forces involved in the GH-GHR interaction, while the lower dissociation rate con-

stant indicates more stabilizing contacts in the interface.

We have characterized the functional epitope on ifnar2 for the binding of IFN α 2 and IFN β . A number of residues on ifnar2 were identified which contributed to binding both IFN α 2 and IFN β . The binding sites resulting for IFN α 2 and IFN β from these investigations are depicted on our homology model of ifnar2-EC (Figure 10). The key residue on ifnar2 for binding of IFN α 2 is M48. Mutating this residue to Ala reduces binding by 500-fold (15 kJ/mol). Two additional residues located nearby (T46 and I47) reduce binding by over tenfold (5.5 and 6.5 kJ/mol, respectively). These three relative hydrophobic hot-spot residues are surrounded by six, mostly polar residues (S49, K50, H78, E79, W102 and I105) which contribute between 2 and 4 kJ/mol binding energy. Residue W74 in our model is buried beneath this binding site. This is in agreement with the low stability of ifnar2-EC observed for the alanine mutant of W74 (which was not observed for the phenylalanine mutant) and the considerable decrease of complex stability upon mutation. In agreement with previous investigations (Lewerenz *et al.*, 1998), our results indicate hardly any contact of IFN α 2/IFN β with the C-terminal domain of ifnar2, which is considerably involved in the homologous complex of GH and IFN γ with their receptors (de Vos *et al.*, 1992;

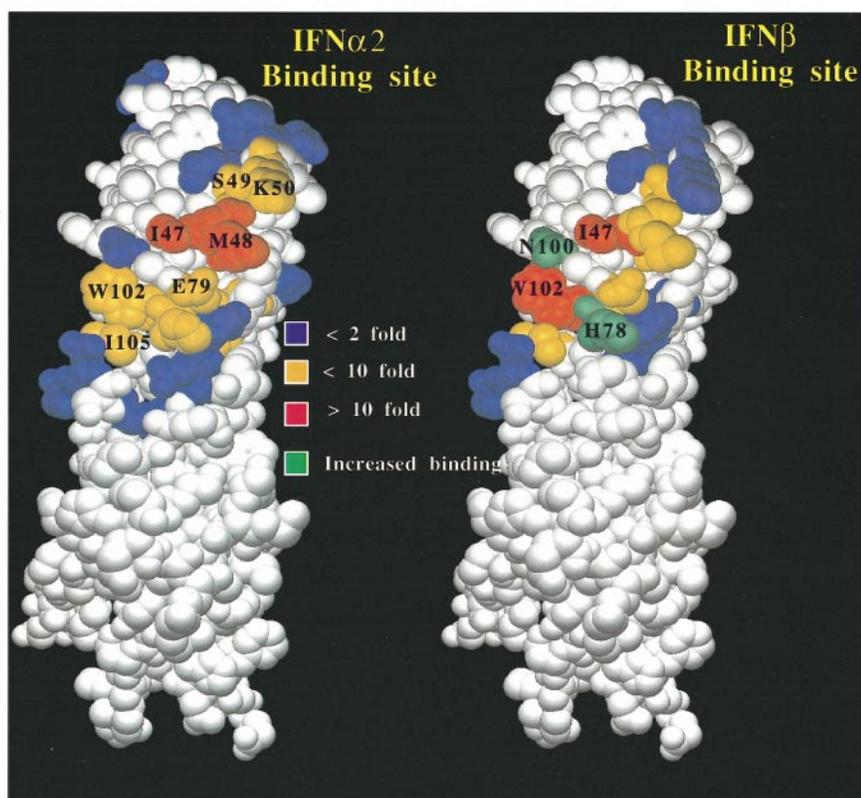


Figure 10. Comparison of the functional epitope of ifnar2 for IFN α 2 and IFN β (homology model of ifnar2-EC with IFN γ R as template). The residues are color-coded according to their contribution to binding IFN α 2 or IFN β as indicated in the Figure. This picture was composed with the Swiss-PDB Viewer (Guex & Peitsch, 1997).

Clackson *et al.*, 1998; Walter *et al.*, 1995). Some contribution of residue E192 was found and none for the residues E134, D140, H189 and D191. However, this perfectly agrees with the small functional epitope identified on IFN α 2 and the binding site on ifnar2 fits very well to its binding site on IFN α 2. The key residues M48 and I47 form a hydrophobic patch which could interact with the hydrophobic patch on IFN α 2 (F27 and L30). For ifnar2-EC, patches of negatively charged residues at the periphery of the binding site (E52/D53, E134-E137 and D191/E192) contribute to steering of association through long-range attraction of the positive residues in the periphery of the functional epitope of IFN α 2, driving the fast rate of association of this complex. It is interesting to note that all the residues on ifnar2 identified by us to be important in binding are located on loops and not beta-sheets. A similar distribution of binding residues has been observed for the interaction of GH with GH-R, and on antibodies. The sum of the interaction energies for all residues involved in binding IFN α 2 identified so far accounts for 52 kJ/mol, which is close to the total interaction energy of the complex.

Dissecting the binding epitope of IFN β on ifnar2 shows a similar location of the binding site, but with a very different distribution of binding energies of specific residues. The most important residues on ifnar2 binding IFN β are W102 (11 kJ/mol) and I47 (7 kJ/mol). Other residues important for binding include T46, M48, S49 and I105 (3-5 kJ/mol). Two mutations on ifnar2 (H78 and N100) result in an decreased rate of dissociation (and thus a higher affinity) for IFN β but not for IFN α 2. It would be interesting to explore the phenotype of a H78, N100 double mutation in ifnar2, which should have about 20-fold tighter binding for IFN β compared to IFN α 2. Overall, much lower effect of the mutations investigated on the binding of IFN β was observed. The sum of the interaction energies is 39 kJ/mol for IFN β , which is approximately two-thirds of the total interaction energy (~57 kJ/mol). Further residues of the functional epitope on ifnar2 interacting with IFN β have yet to be identified.

Structural differences have been proposed to be responsible for different activity profiles of different IFNs, especially IFN α 2 and IFN β (Domanski *et al.*, 1998; Lewerenz *et al.*, 1998; Russell-Harde *et al.*, 1999). Comparing the contributions of different residues located on ifnar2 to the binding of IFN α 2 and IFN β , allows mapping such differences in a quantitative manner. Our results suggest that IFN α 2 and IFN β essentially bind to the same binding site on ifnar2 with very similar kinetic and thermodynamic parameters. Albeit the contributions of specific residues to binding are significantly different. Comparing the functional epitope of IFN α 2 and IFN β on ifnar2 (Figure 10) suggests that despite the overlapping binding site, the angular orientation of both ligands binding ifnar2 may differ. This could lead to a different orientation of the intra-cellular domains of ifnar2 and ifnar1 in

the ternary complex, which could be critical in the pattern of activation of the cellular response to IFN α 2 *versus* IFN β . This fact is in good agreement with mutational studies of the binding site on IFN β (Runkel *et al.*, 1998). Mutation of the residue R35 of IFN β resulted in a much less dramatic loss in activity than the homologous mutation of R33 on IFN α 2. Despite the considerable sequence homology between IFN α 2 and IFN β in the AB-loop, the spatial arrangement of the residues most important for binding is quite different (Figure 11). The overall structural deviation in the homologue sequence C29-R33 (C31-R35 on IFN β) is 1.75 Å, while for the residues L26 and F27, which play a considerable role in forming the functional epitope on IFN α 2, no homology on IFN β is observed. The fact that a loop structured so differently binds to essentially the same epitope on ifnar2 might indicate structural changes upon binding.

With the quantitative, high-resolution description of the functional epitopes on IFN α 2 and ifnar2, and the correlation of thermodynamics with activity, we have made an important step towards an understanding of IFN recognition by its receptor on a molecular level. We were able to prove the importance of the affinity of IFN α 2 toward ifnar2 for their activity, which may account for the varying potency of different IFNs. Although IFN α 2 and IFN β bind competitive to the same functional epitope, mutational analysis revealed distinct centers of binding for these IFNs on ifnar2. In the ternary complex IFN-ifnar1-ifnar2, small shifts of the binding site may result in different angular orientation, which can be critically coupled to cytoplasmic signaling as been shown for another hCR (Livnah *et al.*, 1998).

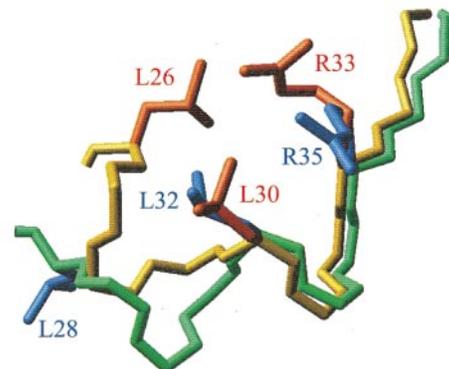


Figure 11. The backbone of the AB-loop from L26 to D35 for IFN α 2 (orange) and L28 to D37 for IFN β (green), with the most important residues on IFN α 2, L26, L30 and R33 in red and the corresponding residues on IFN β (L28, L32 and R35) in blue. This picture was composed with the Swiss-PDB Viewer (Guex & Peitsch, 1997).

Materials and Methods

Protein expression and purification

IFN α 2 and ifnar2-EC were expressed in *E. coli* and were purified by ion-exchange and size-exclusion chromatography as described (Piehler & Schreiber, 1999). The levels of expression of IFN α 2 and ifnar2-EC mutants were as high as the wild-type. The fraction of successfully refolded IFN α 2 mutants varied, with typical yields between 10 and 50 mg protein per liter of cell culture, purified to over 95% homogeneity. The IFN α 2 mutants Y129A and R120A gave very low yields of active protein (<1 mg/l cell culture). These mutants were not further investigated. Significantly lower yields of properly refolded ifnar2-EC (approximately 20% compared to the wild-type) were obtained for the mutants W74A, E79A and E134R. However, the mutant W74F again gave yields similar to the wild-type. Y81A did not give active protein. Wild-type, glycosylated IFN β produced in CHO cells was a gift from Professor M. Rubinstein. Protein concentrations were determined from the absorbance at 280 nm (Piehler & Schreiber, 1999) with $\epsilon_{280} = 18,070 \text{ M}^{-1}$ for IFN α 2, $\epsilon_{280} = 30,050 \text{ M}^{-1}$ for IFN β and $\epsilon_{280} = 26,500 \text{ M}^{-1}$ for ifnar2-EC (corrected to $\epsilon_{280} = 21,100 \text{ M}^{-1}$ for the tryptophan mutants of ifnar2-EC W102A and W74F). Protein purity was analyzed by SDS-PAGE under non-reducing conditions and concentrations were verified by quantitative evaluation of the protein bands compared to wild-type standard. Concentrations of active IFN α 2 protein were determined by analytical gel filtration with ifnar2-EC as described (Piehler & Schreiber, 1999).

Site-directed mutagenesis

Site-directed mutagenesis was carried out by PCR of the plasmids for expression (pT72C α 2 and pT72CR2) with 18-21 nucleotide primers containing the mutated codon using high fidelity polymerases *pwo* (Boehringer Mannheim) and *Pfu* (Stratagene) as described in detail (Albeck & Schreiber, 1999). After phosphorylation and ligation, the mutated plasmids were transformed into TG1 cells. The sequence of the whole expressed gene containing the mutation was verified by DNA sequencing.

Thermodynamic and kinetic analysis

All thermodynamic and kinetic data were obtained from label-free heterogeneous phase detection. The interaction between IFN α 2 and ifnar2-EC was monitored by reflectometric interference spectroscopy (RIfS) under flow-through conditions as described (Piehler & Schreiber, 1999). Either ifnar2-EC (wild-type or mutant) or wild-type IFN α 2 was immobilized by standard amide coupling chemistry. All measurements with IFN α 2 and ifnar2-EC were carried out in 50 mM Hepes with 150 mM NaCl and 0.01% Triton X100 at pH 7.4. The interaction with IFN β was measured at 500 mM NaCl in order to eliminate non-specific interactions with the surface, which was observed at 150 mM NaCl.

Association and dissociation kinetics were measured by standard injection protocols and corrected by blank runs. Dissociation rate constants were measured at protein concentration of 0.5-2 μM in order to saturate the surface. The total range of dissociation was used for fitting a 1:1 kinetic model. Association rate constants were determined by incubating IFN α 2 (wild-type and

mutants) at a concentration c of 50 nM under pseudo-first-order conditions. Consistency of the observed rate constants at different concentrations with a single association rate constant under these conditions has been shown before (Piehler & Schreiber, 1999). The first 30 seconds of the binding curve $R(t)$ was fitted by a 1:1 kinetic model:

$$R(t) = R_{\text{eq}}(1 - e^{-(k_{\text{a}}c + k_{\text{d}})t}) \quad (6)$$

(R_{eq} , equilibrium response) using the mean dissociation rate constant determined in previous experiments. All rate constants were determined as mean values from at least five independent measurements and the errors are given by their standard deviations σ . Differences between two mean values of more than 2σ were considered significant ($\alpha = 0.95$). For fast dissociating mutants, dissociation constants K_{D} were determined from the equilibrium binding on the surface at different concentrations as described before (Piehler & Schreiber, 1999). For signal correction, the immobilized ifnar2-EC was denatured and the background response was measured at the same concentrations. Equilibrium dissociation constants in solution were determined by a binding inhibition assay with immobilized ifnar2-EC as described (Piehler & Schreiber, 1999).

Anti-viral protection activity assay

Anti-viral activity of wild-type and mutant IFN α 2 was assayed as the inhibition of the cytopathic effect of VSV on human WISH cells (Rubinstein *et al.*, 1981). The activity of wild-type and mutant IFN α 2 was determined from the relative concentration needed for 50% protection of the cells compared to NIH IFN β standard. The dissociation constant K_{D} of IFN β binding to wild-type ifnar2-EC was determined by a binding inhibition assay as described (Piehler & Schreiber, 1999).

Homology modeling of ifnar2-EC

A structural model of the extracellular part of the ifnar2 (212 amino acid residues) from SYDSP (N-ter) to ESEFS (C-ter) (Novick *et al.*, 1994) was built using homology modeling. Sequence alignment was carried out using Ψ -BLAST and clustalX (with ten different homologous protein sequences), taking into account the secondary structure motifs in TFR and IFN γ R. The alignment was corrected manually by moving gaps from β -sheets into loops. Homology modeling was done using Swiss-PDBViewer 3.0 and the automated comparative protein modeling server SWISS-MODEL in optimized mode (Guex & Peitsch, 1997). As primary template for modeling we used the structure of IFN γ R.

Acknowledgments

We thank M. Rubinstein and D. Novick for supplying IFN β and for many helpful discussions, S. Barak for performing numerous IFN cytopathic activity assays, and S. Albeck for the critical reading of the manuscript. This work was supported by grant no. 96-00439/1 from the US-Israel Binational Science Foundation (BSF). J.P. is an EMBO postdoctoral fellow, 1998-1999.

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Edited by J. A. Wells

(Received 9 July 1999; received in revised form 28 September 1999; accepted 28 September 1999)