Optimizing the Binding Affinity of a Carrier Protein

A CASE STUDY ON THE INTERACTION BETWEEN SOLUBLE ifnar2 AND INTERFERON β

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The occurrence of soluble proteins corresponding to the extracellular domains of transmembrane receptors and adhesion molecules in biological fluids are widely spread. These proteins retain their binding characteristics and typically function as natural antagonists, carrier molecules, or chaperones, protecting their ligands from proteolytic degradation; in some cases, they are biological agonists (1–5). The cellular release of these soluble receptors is tightly regulated. It is typically controlled by one of two mechanisms. First, differential mRNA splicing that leads to the production and secretion of only the extracellular domain (i.e. interleukin 4, epidermal growth factor, and ifnar2 (6–8)). Second, specific proteolysis of the receptor from the cell surface (i.e. interleukin 1, interleukin 2, and tumor necrosis factor-a) (7).

If binding is tight and discharge is slow, binding proteins maintain the cytokines in circulation, but inactive, resulting in an antagonistic behavior. However, the line between antagonist and agonist is blurred, and the same soluble receptor can act as both, depending on the circumstances (7). The factors that most influence the role of soluble receptors as agonists or antagonists are the concentrations of the ligands, binding proteins, and unbound cytokines required for activity. The exact definition of quantities is complicated by the fact that cytokines may assume multiple roles with different partners, and in different cell lines. Thus, a binding protein may inhibit one activity, but promote a second.

Type I interferons (IFNs)1 are proteins initiating an antiviral and an antiproliferative response. The human type I interferons act through a common cell-surface receptor complex composed of two subunits, ifnar1 and ifnar2. Human Ifnar2 binds all type I IFNs at nanomolar affinities, whereas Ifnar1 binding is weak and hardly detectable on its own (9). Ifnar2 is produced in three forms: long, short, and as the extracellular soluble protein only. The three forms are the result of alternative processing of the human ifnar2 gene product (8, 10). The long form was recognized as the functional subunit, and its expression in an ifnar2 mutant strain is sufficient to reconstitute a fully functional receptor (11). Expression of the short form generates a low-affinity binding site but no functional receptor. The biological role of the short and the extracellular forms of ifnar2 (ifnar2-EC) remain unclear. It was lately reported that ifnar2-EC possesses some antiviral activity on its own after prolonged incubation times (12). All forms of ifnar2 bind a and β interferons at approximately nanomolar affinity (9, 13). The structure of ifnar2-EC and of interferon was determined independently, and their complex was modeled (14–17). The binding site of the two proteins was mapped using site-directed mutagenesis, double-mutant cycles, and distance-constrained docking (14, 15, 18–20). A comparison of the ifnar2 binding epitope with IFNα2 versus IFNβ revealed that both proteins competitively bind the same functional epitope on ifnar2, but with distinct binding centers. Residues on ifnar2 that cause the largest decrease in IFNα2 binding include Met-48, Gln-79, and Val-82; mutating these residues had only a moderate effect on IFNβ binding. However, mutating Thr-102 proved to be most destructive for IFNβ binding (18, 19). Interestingly, we also found 2 residues on ifnar2-EC that specifically increase binding toward IFNβ but not IFNα2 (His-78 and Asn-100) (19). Whereas the differences in the mapped binding epitopes of IFNα2 and IFNβ may be because of differences in the angular orientation of the two proteins in the complex (with ifnar2-EC),
they may also be because of differences in the composition of the binding sites of the interferons.

IFNβ is approved for treatment of multiple sclerosis (21–23). It is administered either subcutaneously or intramuscularly, and is rapidly cleared. In this paper, we examine the possibility of using the soluble part of the ifnar2 receptor as a carrier protein to prolong the circulatory half-life of IFNβ. We investigated the relationship between binding affinities, protein concentrations, and the potential of ifnar2-EC to serve as an agonist or antagonist of interferon in vitro. These questions were analyzed using theoretical simulations and experimental data. A range of ifnar2-EC mutants that bind IFNβ, but not IFNα2, at affinities up to 50-fold tighter compared with the wild-type, were prepared and tested in an attempt to probe the optimal binding affinity of a carrier protein.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Mutagenesis**—Ifnar2-EC and IFNα2 were expressed and purified as previously described (13). IFNβ was a generous gift from Prof. Menachem Rubinstein (Weizmann Institute of Science). Site-directed mutagenesis was carried out by PCR amplification of the complete plasmids for expression (pT72Ca2) with 18–21 nucleotide primers containing the mutated codon using high fidelity polymerases pwo (Roche Applied Sciences) and pfu (Stratagene) as previously described (24).

**Receptor Binding Affinities**—Binding affinities were evaluated using RiS or BIAcore measurements (25). Chip immobilization by ifnar2-EC and the preparation of the surface of RiSs were carried out according to Piehler et al. (26). Briefly, Ifnar2-EC was immobilized to the surface using the non-neutralizing anti-ifnar2-EC mAb 46.10 followed by cross-linking with a second mAb (117.7) (gift from Daniela Novick, Weizmann Institute of Science). The binding curves were evaluated with the BI-Aevaluation software (Biacore AB, Sweden) using a simple one-to-one kinetic model.

**Stopped-flow**—Experimental values of $k_{on}$ were measured using stopped-flow fluorescence in either second-order conditions with the concentrations of interferon and ifnar2-EC being equal, or using pseudo-first order conditions with IFN being in at least a 5-fold excess over ifnar2-EC (13). Both conditions produced the same rate constants. Measurements were done in phosphate-buffered saline.

**In Vivo Experiments**—These experiments were performed using male Wistar rats (150–170 g). Rats were injected either subcutaneously or intravenously (0.2 ml/rat).

**Antiviral Activity of IFNβ**—Antiviral activity was determined by the capacity of the cytokine to protect human amnion WISH cells against vesicular stomatitis virus-induced cytopathic effects (27). In this assay, native IFNo2 and IFNβ show 50% protection of vesicular stomatitis virus-induced WISH cells (ED50) at concentrations of 0.3 (1.7 × 10^5 units/mg) and 0.15 pm (3.4 × 10^-11 units/mg), respectively.

**ELISA for Binding of IFNβ**—Two residues on ifnar2 were identified to specifically promote tighter binding toward IFNβ, but not toward IFNo2, His-78 and Asn-100 (19). Both residues are located on the edge (but on opposite sides) of the binding site of interferon on ifnar2 (Fig. 1) (15). The two single mutants (H78A and N100A) were found to increase the binding affinity toward IFNβ by 3–5-fold each (Table I). To increase the affinity even further, three double mutant ifnar2-EC proteins were produced, and their binding toward IFNβ and IFNo2 was evaluated: H78A/N100A, H78A/N100D, and H78A/N100H. Binding was measured using RiSs, with ifnar2-EC immobilized to the surface via two mAb as previously described (26). Typical binding curves of IFNo2 or IFNβ interacting with surface bound ifnar2-EC (wild-type and mutants) are shown in Fig. 2.

The rate constants are given in Table I. Only $k_{on}$ values were determined from these data, since $k_{on}$ measurements for IFNβ binding ifnar2-EC are not reliable using this method (19). IFNo2 binds all ifnar2-EC variants with comparable affinities.
Kinetic and thermodynamic properties of IFN-ifnar2 complex

Values of \( k_{\text{on}} \) were determined using RIfS. Values of \( k_{\text{off}} \) were determined using a stopped-flow, \( K_D = k_{\text{off}} / k_{\text{on}} \). Relative to wt affinities were calculated from either \( K_D \) or (for H78A/N100D and H) \( k_{\text{off}} \). All measurements were carried out in phosphate-buffered saline. The standard error ± mean for \( k_{\text{off}} \) and \( k_{\text{on}} \) is 20% and accordingly for \( K_D \) is 37%.

| ifnar2          | IFNα2 | IFNβ | IFNγ |
|----------------|-------|------|------|
|                | \( k_{\text{off}} \) | \( k_{\text{on}} \times 10^6 \) | \( K_D \) | Relative to wt | \( k_{\text{off}} \) | \( k_{\text{on}} \times 10^6 \) | \( K_D \) | Relative to wt | ELISA, 50% binding | Relative to wt |
| wt             | 0.01  | 10   | 1    | 1.0  | 0.005 | 50 | 0.1 | 1.0 | 16 | 1.0 |
| H78A           | 0.022 | 13   | 1.7  | 0.6  | 0.0012 | 50 | 0.024 | 4.5 | 8 | 2 |
| N100A          | 0.013 | 22   | 0.6  | 1.6  | 0.0009 | 100 | 0.009 | 11 | 1.6 | 10 |
| H78A/N100A     | 0.014 | 18   | 0.8  | 1.2  | 0.00013 | 67 | 0.002 | 50 | 0.35 | 45 |
| H78A/N100D     | 0.006 | 1.6  | 0.00023 | 22 |
| H78A/N100H     | 0.006 | 1.6  | 0.0037 | 14 |

![Graph](image_url)

Fig. 2. Binding of IFNα2 and IFNβ to wt and mutant H78A/N100A ifnar2-EC measured by heterogeneous phase detection in real time. Binding was followed using RIfS, with ifnar2-EC being immobilized to the surface using the non-neutralizing anti-ifnar2-EC mAb 46.10 followed by cross-linking with a second mAb (117.7).

with a 3-fold difference between the weakest (H78A) and the tightest (H78A/N100D and H78A/N100H) ifnar2-EC mutants. The picture for IFNβ binding ifnar2-EC is fundamentally different. The dissociation rate constants measured between the ifnar2-EC mutants and IFNβ are significantly slower compared with the wild-type protein (Table I). The largest decrease in \( k_{\text{off}} \) was measured for the three ifnar2-EC double mutants, which were 14–40-fold slower compared with the wild-type protein. The H78A/N100A double mutant seems to be the tightest binder. For this mutant, dissociation is so slow that it cannot be measured in real-time using RIfS/BIAcore.

The Fast Rate of Association of IFNβ toward ifnar2-EC Is because of Long Range Electrostatic Steering—Binding affinities can be determined from kinetic data according to \( K_D = k_{\text{off}} / k_{\text{on}} \). To determine the \( k_{\text{on}} \) values of IFNα2 or IFNβ binding ifnar2-EC reliably, we measured those reactions in solution under second-order conditions using a stopped-flow. At low ionic strength (\( I = 25 \) mM), IFNβ binds ifnar2-EC about 20-fold faster compared with IFNα2 (Table II). Even at physiological ionic strength (using phosphate-buffered saline), IFNβ binds ifnar2-EC 5-fold faster than IFNα2, with a rate constant of \( 5 \times 10^7 \) M\(^{-1}\) s\(^{-1}\). This very fast rate of association is the result of the strong electrostatic steering of IFNβ, but not of IFNα2, toward ifnar2-EC. IFNβ displays a strong positive electrostatic potential at its ifnar2 binding site, which is complementary to the negatively charged interferon-binding site on ifnar2 (Fig. 3). Much of the electrostatic attraction between IFNβ and ifnar2 stems from residues located close to the C terminus of IFNβ.

PARE (28) is a computer program developed by us to calculate the rate of association of protein complexes using a known structure of the complex. Calculating the expected relative rate increase of IFNβ versus IFNα2 binding ifnar2 accurately simulated the observed rates (Table II). As the calculations are based on the atomic details of the complex (charge distribution and protein structure), the success of PARE in predicting the \( k_{\text{on}} \) of IFNβ suggests that our structural model is accurate (29). Moreover, the accurate simulation suggests that the difference in \( k_{\text{on}} \) between IFNα2 and IFNβ in binding ifnar2-EC is because of electrostatic steering. The strong positive charge of the ifnar2 binding site on IFNβ may also explain the problems in measuring the association of IFNβ and ifnar2-EC (but not of IFNα2) using BIAcore or RIfS. This strong positive electrostatic potential may promote nonspecific interactions of IFNβ with the negatively charged carboxymethylated dextran layer on the surface of the sensor chips used in both BIAcore and RIfS. This would also explain our observation that binding of IFNβ, but not IFNα2, using RIfS has to be done in 500 mM salt, to reduce nonspecific binding to the surface.

Combining the kinetic data of \( k_{\text{off}} \) (obtained from RIfS/BIAcore) and \( k_{\text{on}} \) (from stopped-flow) enabled us to calculate the binding affinities. The binding data and affinities are summarized in Table I. The affinity of IFNβ toward wild-type ifnar2-EC is in the range of 0.1 nM, which is about 10-fold tighter than the affinity of ifnar2 toward IFNα2. This difference in affinity is mainly because of a significantly faster rate of association. The tightest binding ifnar2-EC mutant binds IFNβ extremely tightly with an affinity of ~2 pM at physiological ionic strength, which is about 50-fold tighter than the wild-type protein.

Direct Determination of Binding Affinities Using ELISA—Because it was difficult to precisely evaluate the \( k_{\text{off}} \) of the very tight binding ifnar2-EC mutants, a second independent method was applied to measure binding, based on sandwich ELISA. Varying concentrations of IFNβ were added to Exiqon™ ELISA plates that were precoated with ifnar2-EC (wt and mutants). Binding of IFNβ to ifnar2-EC was evaluated using the non-neutralizing Ab 117.1 (gift of Daniela Novick). The IFNβ concentration yielding 50% saturation was determined using Equation 1 (“Experimental Procedures”) and summarized in Table I. Whereas the 50% saturation concentration determined from ELISA does not directly correspond to the binding affinity measured in real-time using RIfS or BIAcore, the relative differences between the mutants were expected to be maintained. Indeed, a very good correlation between the
differences in binding affinities of the mutant proteins measured using different methods was observed. The concentration of IFNβ yielding 50% saturation on an H78A/N100A ifnar2-EC-coated plate was 45-fold lower than that determined for the wt. The ELISA served as an independent confirmation of the data obtained from the kinetic experiments.

Occlusion of Interferon by wt and Mutant ifnar2-EC—Soluble receptors have been shown to occlude free ligand from solution, when administered in large excess. Here, we measured the potential of ifnar2-EC to occlude IFNβ as reflected through the inhibition of the anti-viral activity of IFNβ in tissue culture. Briefly, WISH cells were incubated with a mixture of 5 pM IFNβ and increasing concentrations of ifnar2-EC (between 20 and 2500 pM) for 4 h prior to being challenged with the vesicular stomatitis virus. The concentration of free IFNβ was determined relative to a standard curve of the antiviral response of IFNβ (Fig. 4, inset).

About 2 nM wt-ifnar2-EC were required to reduce the antiviral response of 5 pM IFNβ by half. This can be interpreted as being the 50% occlusion point (Fig. 4). When the H78A/N100A

| IFN   | ifnar2-EC       | NaCl | $k_{on}$ (experimental) | $\Delta G_{elect}$ | $k_{on}^b$ (calculated) |
|-------|-----------------|------|------------------------|--------------------|-------------------------|
|       |                 |      | [M] s$^{-1}$ | kcal/mol | [M$^{-1}$ s$^{-1}$] |
| a2    | wt              | 50   | $1.1 \times 10^7$ | 1.26 x $10^7$ |
| β     | wt              | 50   | $2 \times 10^8$   | 1.14 x $10^8$ |
| a2    | H78A/N100A      | 50   | $3 \times 10^7$   |                     |
| β     | H78A/N100A      | 50   | $5 \times 10^8$   |                     |
| a2    | wt              | 150  | $1 \times 10^7$   | $-3.74$            | 9 x $10^6$      |
| β     | wt              | 150  | $5 \times 10^7$   | $-5.9$             | 5.7 x $10^7$      |
| a2    | H78A/N100A      | 150  | $1.8 \times 10^7$ |                     |
| β     | H78A/N100A      | 150  | $6.7 \times 10^7$ |                     |

* Values of $k_{on}$ were measured using a stopped-flow under second-order conditions.

* Values of $k_{on}$ were calculated using the program PARE.

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**Fig. 3.** Electrostatic surface representation of IFNo2, IFNβ, and ifnar2-EC (created using Swiss-PDB viewer). Residues on the ribbon are color coded according to: red, negatively charged, blue, positively charged. The electrostatic potentials on the binding surfaces were calculated using formal charges in 0.15 M salt (blue for positive and red for negative charge).
ifnar2-EC double mutant was added, only 0.05 nM ifnar2-EC were required: 40-fold less compared with the wt. The 50% occlusion concentrations of the other ifnar2-EC mutants were between these values, approximately according to their relative binding affinities. Whereas these concentrations cannot be taken as representing the IFNβ-ifnar2-EC binding affinity (biological activity is activated by the continuous presence of interferon for at least 4 h), the relative difference between the concentrations of the ifnar2-EC mutants measured to occlude 50% of the antiviral activity of IFNβ was in good agreement with the relative binding affinities of these mutants (Table I).

Co-administration of Ifnar2-EC Prolongs Circulation of IFNβ in Rats—Small and medium sized proteins (up to 40 kDa) are rapidly removed from circulation by kidney filtration. Therefore, it is not surprising that the half-life of interferons in circulation is only 1 h (Fig. 5 and Ref. 30). We wished to determine whether the circulatory half-life of interferon could be prolonged by binding to ifnar2-EC, as an inactive pro-drug (the combined size of the complex is ~45 kDa). As the molecular mass of ifnar2-EC produced heterologously in Escherichia coli is only 25 kDa and that of interferon is ~20 kDa, we assumed that either unbound protein is rapidly removed from circulation, but not the complex. Therefore, IFNβ and ifnar2-EC were administered at equal concentrations, keeping most of the proteins in their bound form. To determine the residual concentration of IFNβ in circulation, the antiviral potency of rat serum was measured (relative to an IFNβ standard) at different time points following administration. Two methods of injection were used, subcutaneous and intravenous. Following subcutaneous administration of IFNβ alone, initial serum concentrations were about 5-fold lower compared with those determined after intravenous administration: when 3.5 g/rat were injected, initial plasma levels of 0.5 and 2.5 nM were detected, respectively (extrapolated to time 0, Fig. 5, A and C). Assuming that the circulation volume of a rat weighing ~180 g is ~20 ml, the yield of active IFNβ in plasma is 5 and 25% (subcutaneous and intravenous, respectively), in line with values obtained previously. Native IFNβ (3.5 μg/rat) was removed from circulation within 10 h of injection. The short half-life of IFNβ (~1 h) is independent on whether the protein was administered subcutaneously or intravenously (Fig. 5, A and C), suggesting a rapid transfer of IFNβ from the subcutaneous volume to the circulation. The measured plasma concentration of IFNβ scaled with the concentration injected. Subcutaneous administration of 20 μg/rat of IFNβ resulted in an initial plasma concentration of ~3 nM (Fig. 5B): 6-fold higher compared with that measured for low concentrations injected.

Next, we administered a 1:1 molar mixture of IFNβ and ifnar2-EC (wt or H78A/N100A) at two concentrations, 3.5 μg of IFNβ + 4 μg of ifnar2-EC, and 20 μg of IFNβ + 25 μg of ifnar2-EC. Monitoring the concentration of IFNβ in circulation displayed a rapid decline for the lower dosage, with a half-life...
of ~1 h for IFNβ co-injected with wt ifnar2-EC. This suggests that at this concentration, wt ifnar2-EC had no effect on prolonging the half-life of IFNβ in circulation. However, co-injecting H78A/N100A ifnar2-EC with IFNβ showed a marked increase in the concentration of IFNβ in the serum; even at these low concentrations, IFNβ was still detected up to 24 h post-administration (Fig. 5C). When the same experiment was repeated intravenously, a marked increase in IFNβ concentrations was observed when the H78A/N100A ifnar2-EC double mutant served as the carrier protein (Fig. 5A). Administration of a higher concentration of proteins (20 μg of IFNβ + 25 μg ifnar2-EC) resulted in a marked increase in the half-life of IFNβ in circulation for either wt or H78A/N100A ifnar2-EC as the carrier protein. However, the measured concentration of IFNβ co-injected with H78A/N100A ifnar2-EC was about 10-fold higher compared with that found using wt ifnar2-EC. Moreover, IFNβ co-injected with the double mutant was detected in circulation up to 50 h post-administration, compared with 28 h for wt ifnar2-EC. Thus, administration of a mixture of IFNβ with H78A/N100A ifnar2-EC had three main effects: 1) the concentration of IFNβ in circulation was dramatically increased; 2) levels of IFNβ in the serum declined at a much slower rate; and 3) IFNβ remained in the plasma after extended time periods.

**DISCUSSION**

The half-life of interferon in circulation is between 1 h in rats and 4 h in larger primates and humans (30, 31). This short circulatory half-life suggests that an optimal activation of interferon-induced responses requires either a constant supply of newly produced interferon or a reduction in its rate of elimination (20). The rapid clearance of interferon may be advantageous in tightly regulating the initiation of interferon-induced responses, but is problematic in achieving an optimal response following a single administration. A number of technologies have been successfully tested and implemented for prolonging the half-life of interferons, foremost using PEGylation. Indeed, a PEGylation formulation of IFNa2 was recently approved for the treatment of hepatitis C (32). A different approach, commonly utilized by nature, is the use of carrier proteins, which slowly release the active component into the plasma (7). This may explain the abundance of soluble cytokine receptors in circulation, among them ifnar2 (33, 34). Mimicking this strategy would require the co-administration of interferon with its soluble receptor (ifnar2-EC). Whereas the interferon-ifnar2-EC complex is neither active nor subjected to rapid clearance, discharged interferon is. The equilibrium between the complex and the free components is a function of their affinity and the concentrations of the protein. Therefore, the balance between the soluble receptor as an efficient carrier protein, and a sufficiently high concentration of unbound active interferon is quantitative and not qualitative. Knowing the optimal concentrations and affinity ensures a constant supply of free interferon in circulation. The pool of inactive protein (i.e. interferon) bound to the carrier protein (i.e. ifnar2-EC) refurbishes the supply of free protein in circulation. To assess the optimal protein concentrations and the desired binding affinity between the carrier protein and its partner, the following questions should be addressed. What is the concentration range of active protein needed in circulation? How long is it desired to maintain the protein in circulation? What is the rate of passage from the subcutaneous volume into the circulation? What are the rates of depletion from the circulation of the discharged active protein, the carrier protein, and of the complex? These questions can be answered using a numerical simulation that estimates the optimal binding affinity and initial concentrations of the components.

Scheme 1 is a description of the kinetic barriers of an injected protein-complex (I and R), from injection to clearance.

**Scheme 1. A general description of the kinetic pathway of a protein (I) co-injected with a carrier protein (R), from injection to clearance.**

![Scheme 1](https://example.com/scheme1.png)
concentrations of all species. The following rate constants were used as input for the simulations: $k_1$ and $k_4$ were estimated from Fig. 5 to be in the order of 1 h$^{-1}$, $k_2$ and $k_{-2}$ were taken from Table I. Two separate simulations were performed for $k_5$. In the first, R was removed from circulation at a similar rate as I, whereas in the second, the concentration of R in circulation was constant ($k_5 = 0$). The first case may provide a better description for the E. coli produced ifnar2-EC protein used throughout this work (molecular mass = 24.5 kDa). The second case holds for high molecular mass carrier proteins, as may be the case for the native, glycosylated form of ifnar2-EC (molecular mass = 40 kDa), which can be produced in large quantities in Chinese hamster ovary cells. In both cases, the complex, I-R, was assumed to be stable in circulation ($k_3 = 0$). Fig. 6 is the result of the simulation where $k_3 = 1$ h$^{-1}$, and the proteins are administrated subcutaneously. The concentrations used throughout the simulation are the highest concentrations measured in rat plasma (Fig. 5B, 20 $\mu$g injected, in vivo concentration, 3 nM). At this concentration, about 75% of the interferon is in complex with wt ifnar2-EC. As a result, the rate of depletion is relatively fast (Fig. 6, A and B).

When $k_5 = 0$, assuming that ifnar2-EC is not removed from circulation, the residence time of interferon increases from 12 to 35 h (Fig. 6C). The effect of co-injecting IFN$\beta$ with the ifnar2-EC H78A/N100A double mutant is much more dramatic. Here, most of the IFN$\beta$ is in complex, and the concentration of unbound IFN$\beta$ is maintained at $\sim$100 pm ($\sim$800 units/ml) for a prolonged period of time (Fig. 6, A and B). The simulation shows that even at a 10-fold lower concentration (0.4 nM), the mutant receptor maintains an IFN$\beta$ concentration of 40 pm over a prolonged period of time (data not shown). Again, assuming that ifnar2-EC is not removed from circulation ($k_5 = 0$), the residence time of interferon bound to the mutant ifnar2-EC substantially increases (Fig. 6C).

The simulation provides an explanation for an interesting phenomenon observed in the experimental measurements. Initially, the concentration of IFN$\beta$ is kept relatively constant, and is then suddenly dropped (Figs. 5B and 6). The reason for this is the depletion of both IFN$\beta$ and ifnar2-EC from circulation, but not of the complex. As long as the concentration of both proteins is significantly higher than the $K_D$ of the complex, the rate of clearance is relatively slow. However, once the concentrations decline, the rate of clearance is accelerated, and the concentration of IFN$\beta$ drops rapidly. If ifnar2-EC would not be removed from circulation (as may occur for the glycosylated form), the time-dependent depletion of IFN$\beta$ is expected to be similar to that simulated in Fig. 6C.

Comparing the free IFN$\beta$ concentrations simulated in Fig. 6 clearly shows that using a high molecular mass carrier protein is advantageous in keeping high concentrations of the cytokine
in circulation over a prolonged period of time. The glycosylated form of ifnar2-EC may just be suitable for this task. The wild-type glycosylated ifnar2-EC has been used as a carrier protein for IFNβ in mice, showing a clear increase in the half-life of IFNβ in circulation, as well as improved antiproliferative activity in BALB/cByJSm-nsc/scid mice infected with Daudi cells (35).

An important question is whether increasing the half-life of interferon in vivo would result in an improved therapeutic potency (36). Dupont and co-workers (36) have demonstrated that cells grown in tissue culture lose their responsiveness toward IFNβ after a first round of administration, rendering a second dose 24 h later ineffective. Therefore, it has been argued that it is sufficient to keep high levels of interferon for 8–24 h to achieve maximum efficiency. This conclusion is contradicted by the successful clinical trials of PEG-IFNα2b, which was shown to be more potent against hepatitis C compared with native IFNα2 (32). Moreover, as mentioned above, the in vivo antiproliferative response of IFNβ co-administered with wild-type glycosylated ifnar2-EC was significantly better compared with IFNβ alone (35).

In this paper we have shown that increasing the affinity between a carrier protein and its ligand improves the pharmacokinetic profile of the ligand. Obviously, this trend is limited. This strategy works so well for IFNβ because of the low concentrations required for its optimal activity. Standard plasma concentrations after treatment are in the order of 100 units/ml IFNβ; this is equivalent to a concentration of 10 μM, with the 50% antiviral activity in a WISH cell assay being 0.3 μM. This is in contrast to other cytokines like interleukin 18, which require plasma concentrations in the millimolar range for optimal activity. Fig. 7 shows the results of simulating the concentrations of unbound IFNβ using the same conditions as in Figs. 6, B and C (for Fig. 7, A and B, respectively). Here the initial concentrations of ifnar2-EC and IFNβ are 5 nM, whereas the affinity of the complex was probed to be 0.3–1000 pM. If the carrier protein is depleted from circulation (Fig. 7A), an affinity of 1 μM would assure a prolonged supply of >10 μM of free protein in circulation. However, if the carrier protein is not depleted, a binding affinity of 10–30 pM would be optimal. As a general rule, the optimal affinity of a carrier protein should not exceed the desired concentration of the unbound, active species in circulation. Thus, in our case, the optimal affinity of a carrier protein would be in the low picomolar range, which is the activity measured for ifnar2-EC H78A/N100A binding IFNβ.

Increasing the binding affinity further would result in the binding protein becoming an antagonist, instead of a tool to prolong the half-life of the active protein.

We have demonstrated the relation between binding affinity, concentration, and functionality of a binding protein acting as an agonist for a specific ligand. The experimental work, using a range of mutant binding proteins with enhanced binding affinities to prolong the half-life of IFNβ in rats was accompanied by numerical simulations, providing a theoretical basis for the experimental observations. These tools are generally applicable for the design of optimized binding proteins for a specific task. In the case presented here, the ifnar2-EC double mutant H78A/N100A is practically the ideal carrier protein for IFNβ.

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