

Receptor Binding Kinetics of Human IL-3 Variants with Altered Proliferative Activity

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The binding kinetics of native IL-3 and a set of truncated IL-3 variants to the α subunit of the IL-3 receptor (IL-3R α) were studied using surface plasmon resonance. These variants, with amino acid substitutions at residues, 22, 42, 43, 45, 46, 113, or 116, have previously been identified to have altered capacity to stimulate cell proliferation compared to native IL-3₁₋₁₃₃. In this study, variants E43N and F113Y exhibited >100-fold slower association rates than IL-3₁₅₋₁₂₅ consistent with residues 43 and 113 being essential for the binding of IL-3 to the IL-3R α . Variants G42A, G42D, Q45V, D46S, K116V, and K116W exhibited increased association rates (up to 15-fold relative to IL-3₁₅₋₁₂₅) and decreased dissociation rates (up to 7-fold). The results demonstrate that both the association and dissociation rates for the binding of IL-3 to the IL-3R α are altered by truncation and by amino acid substitution at individual sites. Intracellular signaling studies using K116W and E43N demonstrate that differences in the IL-3 α binding characteristics are reflected in magnitude and kinetics of STAT5 phosphorylation. © 2001

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Human interleukin-3 (IL-3) is a 133 amino acid, multi-lineage hematopoietic growth factor that promotes the growth of many blood cell precursors. The three-dimensional structure of a truncated form of the polypeptide, SC-65369, consists of an up-up-down-down four-helical bundle with an additional short helix that is not present in structurally similar cytokines (1). Studies on the proliferation activity of a large number of variants have defined tolerant and intolerant amino acid positions within the IL-3₁₅₋₁₂₅ polypeptide (2); at

least 69 positions can be substituted without loss of proliferation activity. These are classified as tolerant positions. Sixteen positions cannot be substituted without substantial loss of activity, i.e., <5% the proliferative activity of native IL-3₁₋₁₃₃, and are classified as intolerant positions. Single substitutions at 12 positions were found to result in variants that were >5-fold more active in proliferation than native IL-3₁₋₁₃₃.

The IL-3 cell surface receptor, IL-3R α/β_c , to which the cytokine binds to initiate a series of intracellular events leading to proliferation, is composed of at least two chains, α and β_c . In competitive binding experiments, it has been shown that IL-3₁₋₁₃₃ binds with low affinity ($K_d = 173$ nM) to IL-3R α , and in the absence of β_c receptor; IL-3 has no detectable affinity for β_c when β_c is not associated with the IL-3R α . The affinity of IL-3₁₋₁₃₃ for the IL-3R α/β_c is increased ~50-fold ($K_d = 3.8$ nM) compared to binding to IL-3R α alone (3). Signal transduction and cell proliferation require the expression of both α and β_c chains (4, 5). Binding of IL-3 to the receptor complex results in phosphorylation of the β_c chain and subsequent activation of JAK2 and STAT5 (6). While the complete role of the JAK/STAT pathway in cell proliferation has not been elucidated, several lines of evidence suggest a link between STAT5 activation and cell proliferation. For example, dominant negative STAT5 significantly inhibited IL-3 induced proliferation (7).

In previous studies, equilibrium binding to the IL-3R α has been compared for IL-3₁₋₁₃₃ and several variants with single substitutions (8–11). Binding data and cell proliferation activity measurements, in combination with NMR-derived structural information, have led to a proposed binding site on IL-3 for IL-3R α (8). Residues Asp21, Gly42, Glu43, Asn45, Asp46, Met49, Arg94, Pro96, Phe113 and Lys116 of IL-3 were suggested to lie within or near the binding site for the IL-3R α . Others corroborated the role for residues 21, 113, and 116 in IL-3R α binding (10). Additional studies suggested a position that is involved in the interaction with the β_c subunit, i.e., Glu22 (11). Substitutions at

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position 22 have no apparent effect on direct binding of IL-3 to IL-3R α .

To gain a better understanding of how particular amino acid substitutions influence the activity of IL-3, a kinetic study of binding was undertaken on truncated IL-3₁₅₋₁₂₅ variants that contain single substitutions at intolerant positions (22, 43 and 113) and for variants containing substitutions that increase cell proliferation activity (at positions 42, 45, 46 and 116). This study provides for an increased understanding of how amino acid substitutions influence the biological efficacy of IL-3.

MATERIALS AND METHODS

Expression and purification of IL-3R α /Fc. The IL-3R α /Fc is a fusion protein that contains the extracellular domain of hIL-3R α (4) at the N-terminus and a modified mouse IgG2a Fc (17) at the C-terminus. The protein was expressed in SF-9 insect cells. The SF-9 supernatants were concentrated ~20-fold by ultrafiltration resulting in an IL-3R α /Fc concentration of approximately 2 μ g/ml as determined by ELISA and used for plasmon resonance studies without further purification.

Generation and purification of IL-3 variants. General DNA methods, bacterial hosts and culture conditions have been described previously (2). IL-3 variants were expressed in *E. coli* as inclusion bodies, refolded and purified by reverse phase chromatography to greater than 90% homogeneity as determined by SDS-PAGE electrophoresis using protocols similar to those described previously (2). The truncated IL-3 variants were produced with an additional N-terminal alanine residue (2). Protein concentrations were determined by amino acid composition. Native IL-3₁₋₁₃₃ used in this study is full length recombinant, human interleukin 3 produced in *E. coli*.

Receptor binding studies-surface plasmon resonance. Determination of the binding kinetics was performed on a BIAcore 2000 surface plasmon resonance instrument using the CM5 sensorchip with carboxymethylated dextran (CMD) surface (18). Two flow cells were monitored simultaneously: a blank, containing no rabbit anti-mouse Fc (RAMFc), and an active cell with RAMFc bound to the surface. To covalently immobilize RAMFc to the chip, the CMD surface was activated for seven minutes with a mixture of 0.2 M *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.5 M *N*-hydroxysuccinimide (NHS), followed by a seven minute reaction with Hepes buffered saline (for the negative control cell surface) or with 0.05 mg/ml RAMFc solution (for the active cell surface) at a flow rate of 10 μ l/min. Excess activated groups were deactivated with ethanolamine for seven minutes and the cell was washed with 1.0 N formic acid for 3 min before use in the binding experiments.

During each run, a freshly immobilized or regenerated RAMFc surface was exposed to a six minute injection of the IL-3R α /Fc solution containing 5 mg/ml CMD and 0.05% NaN₃, at a flow rate of 10 μ l/min to yield approximately 400 resonance units (RU) of captured IL-3R α /Fc. The surface then was washed for 25 min with the running buffer until changes in the refractive index were negligible. After which the flow rate was changed to 20 μ l/min and IL-3₁₋₁₃₃ or truncated IL-3 variants were injected for 3 min for the association phase. The dissociation phase was initiated by passing running buffer across the flow cell for 2 min. The flow cell could be regenerated with three minutes injection of 1.0 N formic acid, which removed the receptor while leaving the RAMFc intact. To determine the binding kinetics of each variant, a series of samples varied in concentrations was analyzed using a global fitting program supplied with the BIAcore (Uppsala, Sweden) system (13). This program fit the entire association and dissociation data for all concentrations

simultaneously to yield k_a and k_d values. The K_d was obtained from dividing k_d by k_a .

Signal transduction study. Human leukemic cell line, TF-1 was maintained in RPMI 1640 media with 10% heat inactivated fetal bovine serum (FBS) and IL-3 (2 ng/ml). Cells, starved with 0.5% serum and no IL-3 for overnight, were stimulated with various agonists. Cells, washed twice with PBS, were lysed with cell lysis buffer [150 mM NaCl, 20 mM Tris pH 7.5, 1% Triton X-100, 5 mM EDTA, 50 mM NaF and 10% glycerol with freshly added 1 mM Na₃VO₄ and Protease Inhibitor Cocktail Tablets (Roche Molecular Biochemicals)]. Lysates of cells were clarified by centrifugation and the protein content of the lysate was determined by Coomassie (Pierce). Equal amount of cell lysate protein were immunoprecipitated with anti-STAT5b (C-17) (Santa Cruz Biotechnology) and protein A/G agarose beads for 4 h at 4°C. Immune complexes were collected by centrifugation, washed three times with lysis buffer and resuspended in SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and incubated with anti-Tyr(P) (4G10) (Upstate Biotechnology). The blot was reprobed with anti-STAT5b (C-17). Immunoblots were developed with ECL according to the manufacturer's instructions (Amersham).

RESULTS

Using surface plasmon resonance (SPR), we have measured the binding kinetics of native, IL-3₁₋₁₃₃, truncated IL-3₁₅₋₁₂₅ and truncated singly-substituted IL-3₁₅₋₁₂₅ variants to a recombinant IL-3R α fusion protein, IL-3R α /Fc (see Materials and Methods). The IL-3R α /Fc was captured to a rabbit anti-mouse Fc antibody covalently linked to the sensorchip. This method resulted in a stable receptor surface with a dissociation rate of less than 10⁻⁵/s for the IL-3R α /Fc prior to the binding study (data not shown). A modest density (0.3–0.5 ng of immobilized IL-3R α /Fc per mm²) on the sensorchip was used to minimize rebinding of IL-3 and variants during the dissociation phase (12).

Figure 1 illustrates the experimental and fitted SPR sensorgrams of variant (G42D) for binding kinetics determination. Association rate (k_a) and dissociation rate (k_d) were calculated from the entire set of concentration-dependent curves for this variant and the others (13). The k_a and k_d values for each of the variants are compiled in Table 1. As shown, the binding of IL-3₁₋₁₃₃ to IL-3R α /Fc is low affinity, i.e., K_d = 910 nM, as calculated by $K_d = k_d/k_a$. This K_d is ~5-fold weaker than the value reported for IL-3₁₋₁₃₃ binding to the α subunit of the receptor using competitive binding (3). The low affinity of IL-3₁₋₁₃₃ is a consequence of slow k_a (1.1×10^5 /sM) and fast k_d (96×10^{-3} /s). In contrast, the dissociation rate of IL-3₁₅₋₁₂₅ was faster than that of native IL-3₁₋₁₃₃ ($k_d = 170 \times 10^{-3}$ /s versus 96×10^{-3} /s). However the binding constant, K_d , for IL-3₁₅₋₁₂₅ was better ($K_d = 510$ nM versus 910 nM). This is due to a 3-fold faster k_a for IL-3₁₅₋₁₂₅ compared to IL-3₁₋₁₃₃ (3.3×10^5 /sM versus 1.1×10^5 /sM).

To evaluate the effect of amino acid substitutions on the binding kinetics, a set of truncated single amino acid variants were compared to IL-3₁₅₋₁₂₅. In comparison with IL-3₁₅₋₁₂₅, truncated variants with substitu-

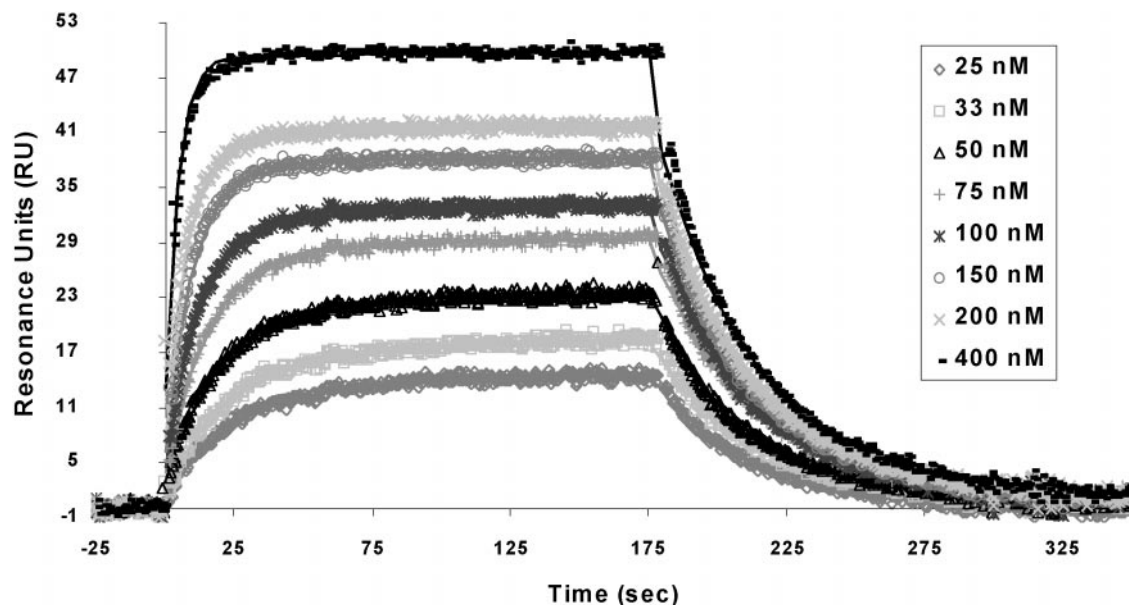


FIG. 1. Sensograms showing the binding of the truncated IL-3 variant G42D to immobilized IL-3R α . The concentration-dependent interaction of the IL-3 variant with IL-3R α /Fc is demonstrated using a series of G42D concentrations ranging from 25 to 400 nM. The response is measured in resonance units (RU). The simulated curves used to obtain the binding properties (k_a , k_d , and K_d) are indicated.

tions at position 22 (E22S, E22A, E22R) exhibited similar k_a (3.3×10^5 /sM versus 4.3, 4.1 and 4.7×10^5 /sM respectively) and 2-fold or less change in the k_d (Table 1), resulting in minimal differences in the binding constant, K_d . This finding is consistent with the noninvolvement of residue 22 in the interaction of IL-3 with IL-3R α .

The K116W variant exhibited the most marked improvement in K_d compared to IL-3₁₅₋₁₂₅ (5.3 nM versus 510 nM). The K116W variant also exhibited the fastest

association rate, 47×10^5 /sM. The related variant, K116V, also exhibited an accelerated association rate (15.2×10^5 /sM) suggesting that residue 116 is an important position involved in the k_a . The Q45V variant exhibited a 3-fold increased association rate over that of IL-3₁₅₋₁₂₅ (9.1×10^5 /sM versus 3.3×10^5 /sM). Variants with substitutions at intolerant positions 43 and 113, E43N and F113Y, exhibited significantly slower association rates than IL-3₁₅₋₁₂₅. The association rates of these variants were too slow to be measured in this study (Table 1).

Variants with substitutions at positions 42, 45, 46 and K116W exhibited 3- to 7-fold slower dissociation rates compared with IL-3₁₅₋₁₂₅ (Table 1). The k_d for K116V is similar to that for IL-3₁₅₋₁₂₅.

The K_d values derived from SPR measurements were found to correlate with previously published competitive equilibrium receptor binding values (IC_{50} values) for IL-3 and variants (8). IC_{50} values were obtained in competition studies wherein unlabeled IL-3 and variants were co-incubated with a radiolabeled IL-3 derivative and the binding of the radiolabeled derivative to BHK cells transfected with the human IL-3R α was quantified. The correlation between SPR-derived K_d and the IC_{50} values is $R^2 = 0.99$, (data not shown). The SPR-derived K_d values also correlated with the enhanced cell proliferation activities of the variants (Fig. 2). Enhancements in cell proliferation activity may be related to contributions by both the association and dissociation rates (Fig. 3). A stronger correlation was observed between the k_a and AML193.1.3 cell proliferative activity ($R^2 = 0.81$), compared to the k_d and

TABLE 1

Binding Kinetics to the IL-3R α as Determined by SPR

	k_a (1/sM $\times 10^5$)	k_d (1/s $\times 10^{-3}$)	K_d (k_d/k_a) (nM)
IL-3 ₁₋₁₃₃	1.1 \pm 0.3	96 \pm 8	910 \pm 250
IL-3 ₁₅₋₁₂₅	3.3 \pm 0.4	170 \pm 20	510 \pm 72
E22S	4.3 \pm 0.2	200 \pm 27	460 \pm 75
E22A	4.1 \pm 0.7	120 \pm 16	295 \pm 37
E22R	4.7 \pm 0.5	160 \pm 19	330 \pm 22
G42A	5.5 \pm 0.6	44 \pm 6	80 \pm 4
G42D	4.6 \pm 0.2	29 \pm 1	60 \pm 4
Q45V	9.1 \pm 0.4	40 \pm 2	45 \pm 4
D46S	5.6 \pm 1.0	59 \pm 8	110 \pm 24
K116V	15.2 \pm 1.5	230 \pm 20	153 \pm 20
K116W	47 \pm 4	25 \pm 1	5.3 \pm 0.4
E43N	<0.02		>5000
F113Y	<0.03 ($n = 1$)		>5000 ($n = 1$)

Note. Values are the average of at least 3 independent experiments, \pm SD, except for F113Y ($n = 1$). k_a , association rate; k_d , dissociation rate; K_d binding constant.

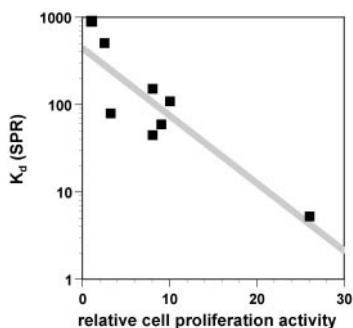


FIG. 2. Plot of cell proliferation activity of individual variants relative to native IL-3 (8) versus K_d values determined by SPR. Data for native IL-3, IL-3₁₅₋₁₂₅ and variants, G42A, G42D, Q45V, D46S, K116V, and K116W, are included.

proliferative activity ($R^2 = 0.14$), suggesting that association of IL-3 with the IL-3R α may have a greater influence in increasing cell proliferation activity.

To further investigate the biological properties of the IL-3 variant proteins, the extent of STAT5 phosphorylation was determined in the IL-3 dependent human cell line, TF-1. STAT5 is tyrosine phosphorylated in response to IL-3 stimulation and is believed to be a key mediator of IL-3 receptor signal pathway. Treatment of TF-1 cells with IL-3₁₅₋₁₂₅, K116W and E43N at 1 nM, increased STAT5 tyrosine phosphorylation (Fig. 4).

IL-3₁₅₋₁₂₅ and K116W were found to have similar effects on the kinetics of STAT5 tyrosine phosphorylation. For these proteins, STAT5 tyrosine phosphorylation reached its peak around 5 min, and gradually decreased after 15 min. While the intensity of the STAT5 tyrosine phosphorylation signal was very similar in response to IL-3₁₅₋₁₂₅ and K116W agonists, the magnitude and kinetics of E43N-induced STAT5 ty-

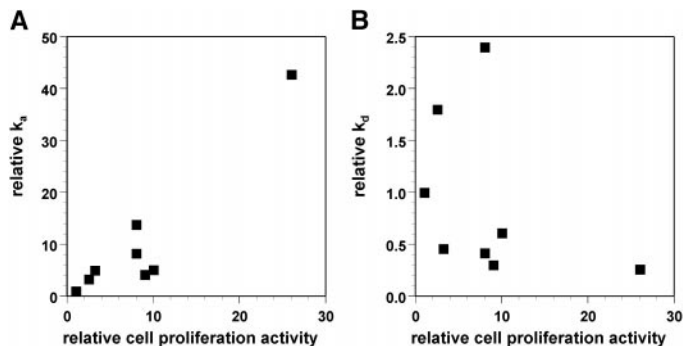


FIG. 3. Plot of cell proliferation activity of individual variants relative to native IL-3 versus association (k_a) and dissociation (k_d) rates determined by SPR. (A) The association rates (k_a) of variants relative to that of IL-3 are plotted versus the AML 193.1.3 cell proliferation activity of variants relative to IL-3 (8). (B) The dissociation rates (k_d) of variants relative to that of IL-3 are plotted versus the AML 193.1.3 cell proliferation activity relative to IL-3 (8). Data for native IL-3, IL-3₁₅₋₁₂₅, and variants, G42A, G42D, Q45V, D46S, K116V, and K116W, are included in both panels.

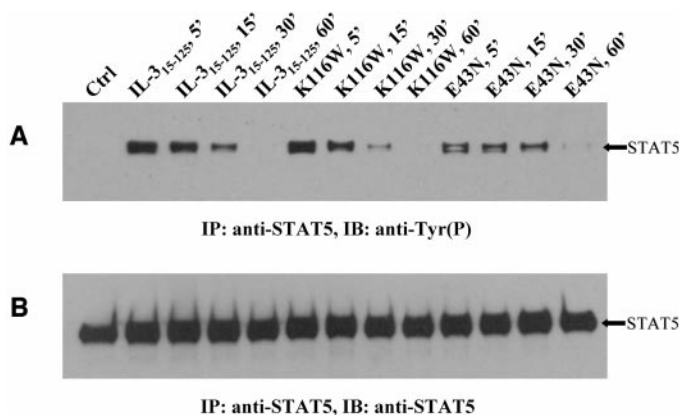


FIG. 4. IL-3 stimulated phosphorylation of STAT5. TF-1 cells were stimulated with 1 nM of IL-3₁₅₋₁₂₅, K116W, E43N for the indicated times, respectively. Cell lysates were immunoprecipitated with anti-STAT5b antibody (which recognized both STAT5a and STAT5b). (A) Samples were subjected to SDS-PAGE and immunoblotted with anti-Tyr(P) antibody. (B) The immunoblot probed with anti-STAT5b antibody.

rosine phosphorylation differed. The phosphorylation intensity was weaker, and did not change substantially between 5 and 30 min. These results demonstrate different signal transduction kinetics for the E43N variant protein compared to either IL-3₁₅₋₁₂₅ or K116W and may be consistent with the altered IL-3R α binding properties of E43N.

DISCUSSION

The binding constants (K_d values), derived by SPR measurement of the k_a and the k_d for binding to immobilized IL-3R α /Fc, have been found to correlate with measurements of competitive equilibrium binding (IC_{50} values) using cell surface IL-3R α . There was approximately a 5-fold difference in the K_d values derived by SPR compared to IC_{50} values. The difference may be inherent in the indirect nature of the competitive binding studies versus SPR measurements that monitor a direct binding to the receptor. Another potential factor for the observed differences may be related to temperature effects. SPR studies were performed at ambient temperature whereas the competitive cell binding studies were done at 4°C. The correlation between the K_d and IC_{50} values suggests that the methodology used herein is suitable for measuring K_d values.

The association rate of IL-3₁₅₋₁₂₅ was 3-fold faster than IL-3₁₋₁₃₃ and the K_d is tighter (510 nM versus 910 nM). These findings are consistent with earlier observations regarding IL-3₁₅₋₁₂₅; (1) the binding at equilibrium of IL-3₁₅₋₁₂₅ to cells which express IL-3R α is 1.3 to 1.7 times greater than the binding of IL-3₁₋₁₃₃ and (2) the proliferation activity of IL-3₁₅₋₁₂₅, using a human hematopoietic cell line AML193.1.3 which expresses IL-3 α/β_c , is 2.5 fold better than IL-3₁₋₁₃₃ (8).

To focus on the effects of single amino acid substitutions, subsequent comparisons were made between the single substituted IL-3₁₅₋₁₂₅ variants and unsubstituted IL-3₁₅₋₁₂₅. The k_a of IL-3₁₅₋₁₂₅ was improved (3- to 5-fold) by Q45V and K116V substitutions and improved greatly (15-fold) by the K116W substitution. Corresponding with increased k_a , each of these later variants has been shown to exhibit enhanced proliferation activity. This result suggests that the capture of IL-3 to the cell receptor is a key driver for proliferation whereas the k_d is not equally significant for cell proliferation. The k_d values for these variants were found not to correlate as well with proliferation. This observation is consistent with studies on human growth hormone (hGH) where the k_d was found to be unrelated to proliferation activity for variants whose k_d were within 30-fold of native hGH (14).

As found for other cytokines (15, 16), the magnitude of the change in K_d (e.g., ~100-fold for the K116W variant over IL-3₁₅₋₁₂₅) does not translate into as large an increase in proliferation activity (e.g., 10-fold for K116W versus IL-3₁₅₋₁₂₅) (2) suggesting that there are additional events subsequent to binding to the IL-3R α which modulate the magnitude of cell proliferation activity. Among these might be the formation of the high affinity

IL-3/IL-3R α / β_c complex or signal transduction. For example, an hGH variant with 400-fold enhanced binding affinity (primarily as a result of a slower k_d) demonstrated virtually no improvement in EC₅₀ for proliferation (14).

The association rates for intolerant variants E43N and F113Y were too slow to be measured by SPR in this study (Table 1). These kinetic findings provide insight into the decreased proliferation properties (~20-fold less potent than IL-3₁₅₋₁₂₅) and the diminished competitive binding abilities (<0.01 the value of IL-3₁₅₋₁₂₅) of these variants (8). Thus, the SPR-derived data support the conclusion that Glu at position 43 and Phe at position 113 are essential for the interaction of IL-3 with the IL-3R α and the subsequent initiation of cell proliferation.

Signal transduction studies are one approach to bridge receptor binding studies with the cell proliferation results. IL-3 variants that associate rapidly with IL-3R α (IL-3₁₅₋₁₂₅ and K116W) demonstrate strong phosphorylation of STAT5 in as little as 5 min. In contrast, E43N binds slowly to IL-3R α and demonstrates lower levels of STAT5 phosphorylation that are steady and maintained for at least 30 min.

Comparison of the k_a and K_d values for K116V and K116W variants is interesting. These differences point out the merits of evaluating a variety of amino acids to determine the optimal substitutions at each position, as was done in the initial publication of this series (2). During the initial screening, K116W was chosen for further analysis because it exhibited greatly enhanced

activity. Interestingly, Trp is not an amino acid commonly used for substitution in structure-activity studies. The K116W variant would have been missed using conventional mutagenesis strategies that substitute with a single amino acid (e.g., alanine scanning).

Generally, charged side chains are involved in the initial contact and thus effect association, while side chains which form hydrophobic contacts stabilize the interaction and thus effect dissociation. For the variants investigated in this study, there is not a consistent effect of charge changes on the magnitude of increase in association rate. G42D and D46S, which add and subtract a negative charge respectively, exhibited no change in association rate. Substitutions, such as K116W, may stabilize a local conformation within human IL-3 and enable the cytokine to have a more favorable conformation for receptor interaction, such as presentation of key residues to the IL-3 binding pocket of the receptor.

The dissociation rate of variants with substitutions at sites 42, 45, 46 and K116W slowed by up to 7-fold compared to native IL-3₁₅₋₁₂₅. This slower k_d may be the result of stabilizing interactions with IL-3R α as suggested previously (8) for reasons such as: improved hydrophobic interactions (G42A, Q45V and K116W), increased charge interaction (G42D) and optimized hydrogen bonds (D46S). In contrast to K116W, substitution of Val at position 116 results in a k_d which is similar to IL-3₁₅₋₁₂₅ suggesting that a small hydrophobic side chain of valine is not sufficient for additional stabilization. These results reinforce the unique role of residue 116 in the interaction of IL-3 with the IL-3R α .

It is noteworthy that the interaction of IL-3 (like other members of the hematopoietic cytokine superfamily) with its receptor can be improved. The effect of association rate on proliferation activity suggests that improved variants such as K116W can be identified using quantitative binding assays based upon the kinetics of receptor interaction. A kinetic-based selection procedure could yield more potent IL-3 receptor agonists. Study of variants, such as those within this report should lead to an increased understanding of the interaction between IL-3 and the IL-3R.

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