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A human interleukin 3 analog with increased biological and binding activities

(hemopoiesis/growth factors/function/allergy)

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ABSTRACT **Human interleukin 3 (IL-3) variants generated by site-directed mutagenesis were analyzed in multiple biological and binding assays to identify residues critical for IL-3 activity. Two mutants carrying substitutions in the predicted hydrophilic region within the first α-helix, [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 showed loss of biological activity and high-affinity binding. Mutants in a second predicted hydrophilic region, [Ala^{44},Leu^{45},Ala^{46}]IL-3 and [Ala^{44},Ala^{46}]IL-3, however, showed similar biological and binding activities to wild-type IL-3. Mutations in a C-terminal hydrophilic region that overlaps the fourth predicted α-helix led to either loss or gain of function. IL-3 analogs [Glu^{104},Asp^{105}]-, [Leu^{108}]-, [Asn^{108}]-, [Thr^{108}]-, and [Ala^{101},Leu^{108}]IL-3 were less active than wild-type IL-3, whereas [Ala^{101}]IL-3 and [Val^{116}]IL-3 were 2- to 3-fold more potent. Significantly, the double mutant [Ala^{101},Val^{116}]IL-3 exhibited a 15-fold greater potency than native IL-3. Receptor binding studies showed that [Ala^{101},Val^{116}]IL-3 exhibited increased binding to the high- and low-affinity receptors of monocytes. These results show the generation of an IL-3 analog with increased biological and binding activities and support a model where the C terminus of IL-3 interacts with the α chain of the IL-3 receptor, making this region a useful focus for the development of more potent IL-3 agonists or antagonists.**

Interleukin 3 (IL-3) is a T-cell-derived glycoprotein of 133 amino acids that is central in the control of hemopoiesis and inflammation (1). *In vitro* studies showed that IL-3 stimulates the proliferation and differentiation of normal hemopoietic cells of different lineages directly (2–5) and in combination with lineage-restricted growth factors (6), granulocyte–macrophage colony-stimulating factor (GM-CSF), and mast cell growth factor (7). *In vivo*, IL-3 alone elevates the numbers of several hemopoietic cell lineages in the blood (8–10) and can also act synergistically with GM-CSF (11). Because of its pleiotropic effects, IL-3 appears ideally suited for clinical application in situations where extensive bone marrow reconstitution is required. On the other hand, IL-3 is likely to play a regulatory role in inflammatory conditions through its activation of mature cell function. For example, IL-3 can affect the migration of monocytes to sites of inflammation by modulating their adherence to endothelial cells (12), and through its enhancement of histamine release on basophils, IL-3 can significantly exacerbate allergic reactions (13, 14).

Despite its multiple biological activities, it is not clear how IL-3 exerts its effects on various cell types. Cell surface receptors for IL-3 have been identified on several primary cells (14–17). However, information regarding residues of IL-3 required for binding to these receptors and for eliciting a biological response is scarce. This type of information is essential for understanding the structural basis for the multiple biological activities of IL-3 and for the design of more potent agonists and of antagonists that can block the effect of IL-3 in situations such as allergic reactions. Recent experiments have shown that residues 33 and 111 are important for the proliferative activity of IL-3 on the MO-7 cell line (18, 19), suggesting that different regions of the IL-3 molecule can participate in function. We show here that specific substitutions in the N terminus of IL-3 lead to loss of multiple functions and binding, whereas substitutions in the C terminus lead to a decrease or increase in activities. Significantly, we found that the double mutant [Ala^{101},Val^{116}]IL-3 exhibited 15-fold greater biological and binding activities. This region of IL-3 may constitute a useful focus for the design and construction of further IL-3 analogs with more powerful agonistic or with antagonistic activities.

MATERIALS AND METHODS

Site-Directed Mutagenesis of Human IL-3. Human IL-3 mutants were constructed by either site-directed mutagenesis (20) on a synthetic human IL-3 cDNA in an M13 vector (21) or polymerase chain reaction (PCR) mutagenesis (22). A two-part PCR using three primers (22) was used to create mutants in the expression vector pJLA$^+$IL-3 as described (21). IL-3 analogs created by site-directed mutagenesis were subcloned into *Bam*HI–*Eco*RI sites of pJL4 (21). Wild-type IL-3 and the mutant [Ala^{101},Val^{116}]IL-3 were also subcloned, using a PCR to generate *Hin*dIII and *Bam*HI sites, into the *Escherichia coli* expression vector pINIIIOmpH$_3$ (gift of R. Kastelaine and A. Shanafelt, DNAX), a derivative of the vector pINIIIOmpA2 (23). For each IL-3 analog, the complete cDNA was sequenced to confirm the presence of the desired mutation and the absence of other mutations that could have been introduced by PCR.

Expression of IL-3 and Its Analogs. IL-3 analogs in the pJL4 expression vector were transiently transfected in COS cells as described (24). Wild-type IL-3 and the analog [Ala^{101},Val^{116}]IL-3 were expressed in the *E. coli* system after induction by isopropyl β-D-thiogalactoside; protein was recovered from the periplasmic space by osmotic shock (25) and partially purified by gel filtration. IL-3 protein detection was carried out by Western blot analysis using a rabbit anti-human IL-3 (gift from S. Clark, Genetics Institute, Cambridge, MA) and visualized by autoradiography after the addition of ^{125}I-labeled protein A (26).

Quantitation of IL-3 Protein. The amount of IL-3 protein present in COS cell supernatants was quantitated by a competitive radioimmunoassay (RIA) using human IL-3 (a gift from L. Park, Immunex, Seattle) labeled with ^{125}I and an anti-IL-3 serum (gift from S. Clark, Genetics Institute) as

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Abbreviations: IL-3, interleukin 3; CML, chronic myeloid leukemia; GM-CSF, granulocyte–macrophage colony-stimulating factor.

described (27). Wild-type IL-3 and [Ala^{101},Val^{116}]IL-3 produced in *E. coli* were also quantitated directly by scanning densitometry (28). Briefly, wild-type IL-3, [Ala^{101},Val^{116}]IL-3, or RNase standards were electrophoresed over a concentration range of 0.5–5 μg, and the gel then was stained with Coomassie brilliant blue R250 and analyzed using an LKB-Pharmacia Ultrascan XL scanning laser densitometer. Data analysis was performed with GSXL densitometer software. The protein concentrations of the unknown samples were calculated using the area under the peak. In some cases direct protein quantitation was also performed by HPLC peak integration by calculating the area under the IL-3 peak using the extinction coefficient of 0.83 arbitrary units·ml·mg^{-1}. An IL-3 preparation (gift from Genetics Institute) at 0.6 μg/ml (by amino acid analysis) measured 0.59 ± 0.1 μg/ml (mean ± SD) by scanning laser densitometry and 0.6 ± 0.07 μg/ml by RIA. In parallel, an [Ala^{101},Val^{116}]IL-3 concentration of 1.45 ± 0.06 μg/ml by scanning laser densitometry compared with 1.32 ± 0.08 μg/ml by HPLC peak integration and 1.35 ± 0.2 μg/ml by RIA.

Stimulation of Hemopoietic Cell Proliferation. Two types of assay were performed:

(*i*) *Colony assay.* CD34-positive bone marrow cells were purified with the monoclonal antibody MY10 and anti-mouse IgG antibodies coupled to magnetic beads (Dynard, Oslo); 10^3 cells were cultured in the presence of IL-3 analogs.

(*ii*) *Proliferation of chronic myeloid leukemic (CML) cells.* CML cells incorporated [^{3}H]thymidine in response to IL-3 as described (29). Data are expressed in cpm, and each point is the mean of six replicates.

Stimulation of Human Monocyte and Basophil Function. Monocytes were purified from the peripheral blood of normal donors as described (16) and IL-3-mediated adhesion was measured by an isotopic method as described (12). Basophil histamine release was measured as described (14).

Radioreceptor Assay. Human IL-3 (gift from L. Park, Immunex) was radioiodinated by the ICl method (30) and used in competitive binding experiments as described (16, 31). The results are expressed as percent competition, where 100% is the competition observed in the presence of a 100-fold excess of wild-type IL-3.

Statistical Analysis. Each mutant was tested over a range of concentrations using three to six replicates per point, and the concentration of IL-3 analog giving 50% maximal response was determined. Percent potency was calculated by dividing the concentration of wild-type IL-3 giving 50% maximal response by the concentration of IL-3 mutant giving 50% maximal response × 100%. Significance was established by one-way analysis of variance using the 50% values of at least three experiments for each assay.

RESULTS

Predictive studies of human IL-3 structure (32, 33) indicated that this molecule contains four α-helices and several hydrophilic regions (Fig. 1). Since these regions have been implicated in function and receptor binding of related growth factors such as GM-CSF (24, 34–36) and growth hormone (37), they were targeted for mutagenesis. IL-3 cDNA mutants were expressed transiently in COS cells, and Western blot analysis revealed that IL-3 mutants and wild-type IL-3 exhibited similar size and levels of glycosylation (data not shown).

Biological Analysis of N-Terminal Mutations. Mutation of residues Asp^{21}, Glu^{22}, and Thr^{25} located in a hydrophilic region of the predicted first α-helix of IL-3 to Ala, Leu, and Ala, respectively, was predicted to reverse the hydropathy of this region (Fig. 1). The two IL-3 mutants tested, [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 were unable to stimulate the proliferation of CML cells, monocyte adherence, and histamine release from basophils (Table 1) or to bind to the high-affinity IL-3 receptor of monocytes (Table 2).

Mutations in a different hydrophilic region comprising residues Asp^{44}, Gln^{45}, and Asp^{46} also led to a predicted inversion of hydropathy. In this case, the IL-3 mutants [Ala^{44},Ala^{46}]- and [Ala^{44},Leu^{45},Ala^{46}]IL-3 exhibited properties similar to wild-type IL-3 in stimulating CML cell proliferation, monocyte adherence, and basophil histamine release (Table 1).

Biological Analysis of C-Terminal Mutations. Initial deletion mutagenesis revealed that the 13 most C-terminal residues of IL-3 were not essential for activity (Table 1). However, deletions into the predicted fourth α-helix illustrated by analogs IL-3-(1–118) and -(1–116) caused a dramatic decrease in activity (data not shown), emphasizing the essential nature of this helix for IL-3 function. Substitution mutagenesis within and adjacent to the fourth α-helix resulted in IL-3 analogs with modified activities. Replacement of Asp^{101} with Ala caused a decrease in the predicted hydrophilic nature of

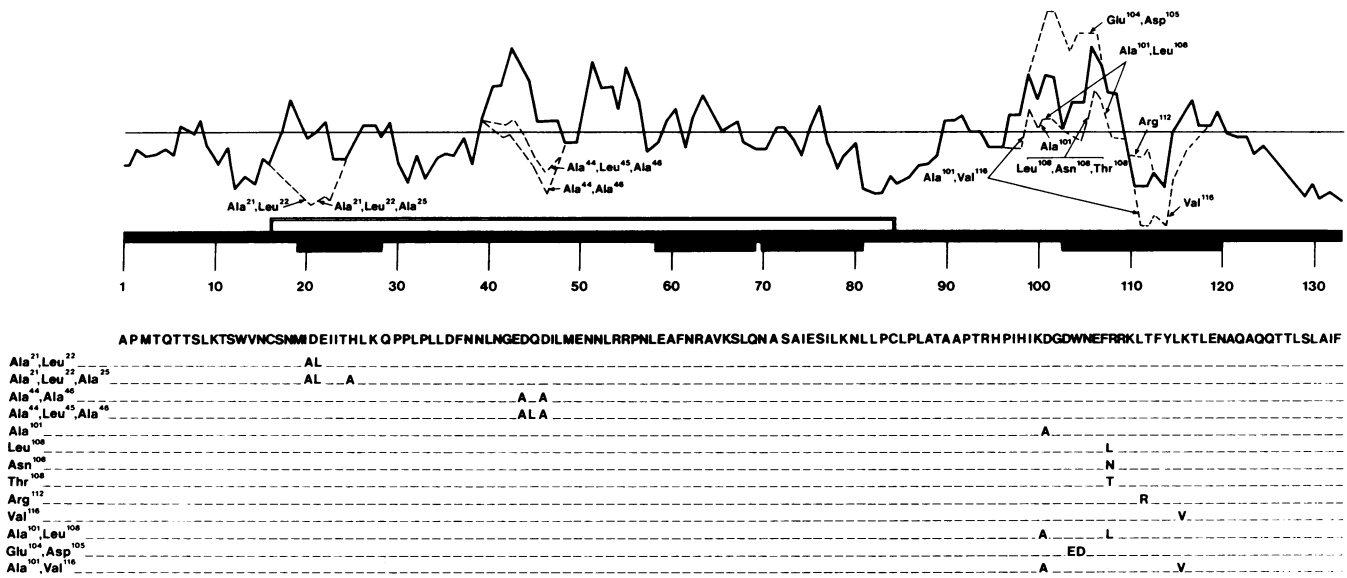

FIG. 1. Hydropathy profile and α-helix prediction of human IL-3. Hydropathy profile was determined using the method of Hopp and Woods (32), and the α-helical boundaries were based on heptad symmetry predictions (33). The four α-helices are represented by the solid rectangular boxes. The open double line joining Cys^{16} to Cys^{84} indicates the disulfide bridge. The IL-3 analogs generated by site-directed mutagenesis and the predicted changes to the hydropathy profile are indicated.

Table 1. Relative potencies of human IL-3 analogs

Analog	Proliferation of CML cells		Monocyte adherence		Basophil histamine release	
	% potency	P value	% potency	P value	% potency	P value
N-terminal mutations						
[Ala^{21},Leu^{22}]IL-3	<10	<0.01	<10	<0.01	<10	<0.01
[Ala^{21},Leu^{22},Ala^{25}]IL-3	<10	<0.01	<10	<0.01	<10	<0.01
[Ala^{44},Ala^{46}]IL-3	103 (1.4)	NS	77 (2.0)	NS	116 (1.6)	NS
[Ala^{44},Leu^{45},Ala^{46}]IL-3	72 (1.6)	NS	81 (1.9)	NS	136 (1.8)	NS
C-terminal mutation(s)						
IL-3-(1–120)	148 (2.1)	NS	ND		181 (1.5)	0.04
[Ala^{101}]IL-3	186 (1.4)	0.05	305 (1.7)	<0.02	259 (2.3)	0.02
[Leu^{108}]IL-3	40 (1.8)	<0.01	49 (1.8)	<0.01	49 (1.2)	<0.05
[Asn^{108}]IL-3	21 (2.2)	0.05	ND		ND	
[Thr^{108}]IL-3	15 (3.2)	<0.01	ND		ND	
[Arg^{112}]IL-3	70 (1.4)	NS	225 (n = 1)		110 (2.2)	NS
[Val^{116}]IL-3	235 (1.4)	0.01	590 (1.9)	<0.01	290 (2.6)	<0.01
[Glu^{104},Asp^{105}]IL-3	24 (1.6)	<0.01	45 (1.2)	<0.01	5 (2.2)	<0.01
[Ala^{101},Leu^{108}]IL-3	<10	<0.01	15 (n = 1)		14 (2.6)	<0.05
[Ala^{101},Val^{116}]IL-3	1153 (1.1)	<0.01	1480 (2.0)	<0.01	1437 (1.5)	<0.01

Percent potency was calculated by dividing the concentration of wild-type IL-3 giving 50% maximal response by the concentration of IL-3 mutant giving 50% maximal response × 100. The numbers in parentheses indicate the SD of the mean after logarithmic transformation. The P value represents the level of significance between IL-3 analogs and wild-type IL-3 obtained by performing one-way analysis of variance. ND, not determined; NS, not significant.

this region as did the replacement of Arg^{108} with Leu, Asn, or Thr (Fig. 1). However, whereas the mutant [Ala^{101}]IL-3 showed a 2- to 3-fold enhancement in IL-3 function, the mutants [Leu^{108}]IL-3, [Asn^{108}]IL-3, and [Thr^{108}]IL-3 exhibited a 2- to 5-fold decrease in biological activity (Table 1). The double mutant [Ala^{101},Leu^{108}]IL-3 exhibited low activity, indicating the dominant effect of position 108. This may reflect the fact that position 108 but not position 101 is predicted to be part of the fourth α-helix and thus is less tolerant to substitution mutagenesis. Replacement of residues Trp^{104} and Asn^{105} with Glu and Asp, respectively, led to an IL-3 analog with predicted higher hydrophilicity in this region (Fig. 1) but with significantly decreased biological activity (Table 1). Substitutions in a predicted hydrophobic region caused either no change in function as illustrated by [Arg^{112}]IL-3 or a 3- to 6-fold increase in biological activity as shown by [Val^{116}]IL-3.

Binding Properties of IL-3 Analogs. The results showed a correlation between biological activity and high-affinity binding (Table 2). The inactive analogs [Ala^{21},Leu^{22}]IL-3 and [Ala^{21},Leu^{22},Ala^{25}]IL-3 did not show detectable binding; the mutants with decreased biological activity [Glu^{104},Asp^{105}]IL-3 and [Leu^{108}]IL-3 showed decreased binding; the mutants with unaltered activity IL-3-(1–120) and [Arg^{112}]IL-3 showed similar K_d values; and the two analogs [Ala^{101}]IL-3 and [Val^{116}]IL-3 that were more active than wild-type IL-3 also exhibited increased binding affinity (Table 2). These two mutations were then incorporated into a single molecule and tested in biological and binding assays.

Table 2. Binding properties of human IL-3 analogs tested on the high-affinity IL-3 receptors of human monocytes

Analog	n	K_d	P value
Wild type	8	1.15×10^{-11} M	
[Ala^{21},Leu^{22}]IL-3	4	—	
[Ala^{21},Leu^{22},Ala^{25}]IL-3	3	—	
[Ala^{44},Ala^{46}]IL-3	1	5×10^{-11} M	
IL-3-(1–120)	3	1.8×10^{-11} M	NS
[Ala^{101}]IL-3	5	7.5×10^{-12} M	<0.05
[Leu^{108}]IL-3	1	3×10^{-11} M	
[Arg^{112}]IL-3	1	1.3×10^{-11} M	
[Val^{116}]IL-3	5	5.4×10^{-12} M	<0.05
[Glu^{104},Asp^{105}]IL-3	1	1.04×10^{-10} M	
[Ala^{101},Val^{116}]IL-3	5	8.2×10^{-13} M	<0.01

n, Number of experiments; NS, not significant. The K_d values were calculated from a titration curve using increasing concentrations of IL-3 analogs to compete for the binding of 100 pM ^{125}I-labeled IL-3 by using the LIGAND program. Each concentration of competitor was tested in duplicate; 2–4 × 10^6 monocytes per point were used. P values were calculated as described in Table 1. —, No competition was observed up to a 100-fold excess concentration of IL-3 mutant.

Biological and Binding Properties of [Ala^{101},Val^{116}]IL-3. The mutant [Ala^{101},Val^{116}]IL-3 was 10- to 20-fold more potent than wild-type IL-3 at stimulating CML cell proliferation (Fig. 2A and Table 1) and bone marrow colonies (Fig. 2B). Greater potency by [Ala^{101},Val^{116}]IL-3 was also observed in the stimulation of monocyte adherence (Fig. 3A and Table 1) and basophil histamine release (Fig. 3B and Table 1). This increased potency was reflected in the increased ability of [Ala^{101},Val^{116}]IL-3 to recognize the high-affinity IL-3 receptor of monocytes (Table 2).

Because high-affinity IL-3 receptors contain a low-affinity receptor binding chain and a convertor β chain (38), it was important to establish whether [Ala^{101},Val^{116}]IL-3 was exhibiting increased affinity as a result of increased binding to the low-affinity IL-3 component. To determine this, we used ^{125}I-labeled IL-3 at 2 nM, a concentration at which the majority of occupied receptors in human monocytes are of the low-affinity class (16, 31). For these experiments, [Ala^{101},Val^{116}]IL-3 and wild-type IL-3 were expressed in *E. coli*, partially purified by gel filtration, and tested in conjunction with purified *E. coli*-derived IL-3 (gift from S. Clark, Genetics Institute) for their ability to inhibit the binding of 2 nM ^{125}I-labeled IL-3 to human monocytes. A quantitative inhibition binding experiment showed that [Ala^{101},Val^{116}]IL-3 has a 15-fold greater affinity than wild-type IL-3 in binding to human monocytes under low-affinity conditions (Fig. 4).

DISCUSSION

We show here the construction of a human IL-3 analog with increased biological and binding properties. [Ala^{101},Val^{116}]IL-3 exhibited an ≈15-fold greater potency than native IL-3 at stimulating bone marrow cells to form day-14 colonies, leukemic cell proliferation, and monocyte adhesion and at enhancing the release of histamine from basophils. Paralleling

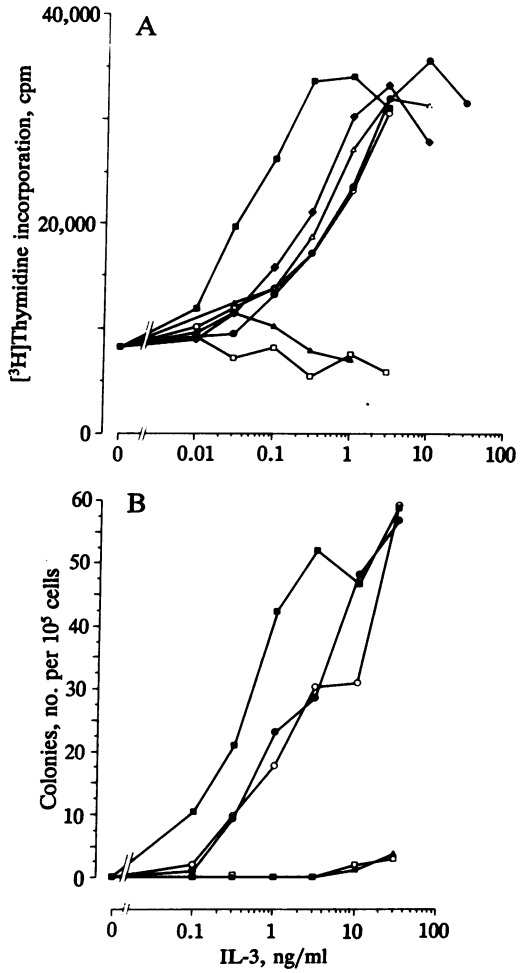

FIG. 2. Titration of [Ala^{101},Val^{116}]IL-3 and other IL-3 analogs for their ability to stimulate cell proliferation. (*A*) [^{3}H]Thymidine incorporation in CML cells. Each value is the mean of six replicates. (*B*) Bone marrow colony formation after 14 days in culture. Each value represents the mean of three replicates. ●, Wild-type IL-3; ◆, [Ala^{101}]IL-3; △, [Val^{116}]IL-3; ■, [Ala^{101},Val^{116}]IL-3; ○, [Arg^{112}]IL-3; □, [Ala^{21},Leu^{22},Ala^{25}]IL-3; ▲, [Ala^{21},Leu^{22}]IL-3.

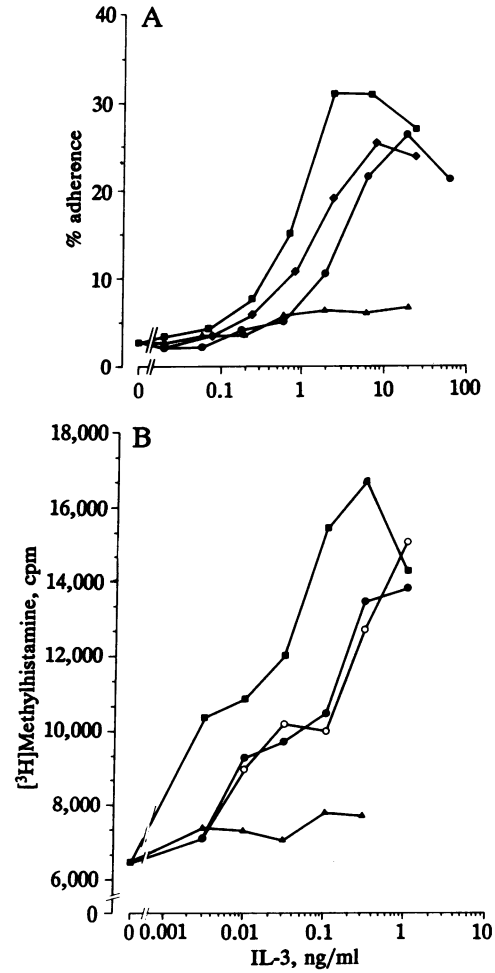

FIG. 3. Titration of [Ala^{101},Val^{116}]IL-3 and other IL-3 analogs for their ability to stimulate monocyte adherence (*A*) and basophil histamine release (*B*). The values represent the mean of five replicates. ●, Wild-type IL-3; ◆, [Ala^{101}]IL-3; ■, [Ala^{101},Val^{116}]IL-3; ▲, [Ala^{21},Leu^{22}]IL-3; ○, [Arg^{112}]IL-3.

this increased function, [Ala^{101},Val^{116}]IL-3 also bound with 15-fold greater affinity to the high-affinity and low-affinity IL-3 receptors of monocytes.

These results show that the potential to generate IL-3 analogs with increased potency appears to be restricted so far to substitution of residues at positions 101 and 116, inasmuch as mutations at positions 104, 105, and 108 (Tables 1 and 2) and replacement of residues Lys^{110} or Leu^{111} (19) and Glu^{106} (39) greatly reduce the potency of IL-3. These results are consistent with other structure–function studies on IL-3 (40) and on GM-CSF and IL-5 where the C terminus has been shown to be required for functional integrity (34–36, 41) and raise the possibility that analogous mutations to [Ala^{101},Val^{116}]IL-3 in GM-CSF and IL-5 may also lead to more potent agonists.

The N terminus of IL-3 is also likely to contribute to function and binding. Replacements and deletions in the N terminus of IL-3 result in decreased activity (18, 39) except for one IL-3 analog, [Gly^{33}]IL-3, that exhibited a 14-fold enhanced biological activity, albeit without increased binding capacity. The results shown here with the analogs [Ala^{21},Leu^{22},Ala^{25}]IL-3 and [Ala^{21},Leu^{22}]IL-3 showing greatly reduced IL-3 binding and activity also illustrate the involvement of the N terminus. On the other hand, the analogs [Ala^{44},Leu^{45},Ala^{46}]IL-3 and [Ala^{44},Ala^{46}]IL-3 showed similar potency to wild-type IL-3 (Table 1). This difference may reflect different structural constraints in that positions 21 and 22 are predicted to be part of an α-helix and positions 44–46 are not (Fig. 1) or that residues 21 and 22 are directly involved in binding and function. In support of the latter, it is worth noting that both IL-3 and GM-CSF interact with their respective specific α chains (38, 42) and with a β chain that is common to both ligands (38, 43). Glu^{22} is in a predicted exposed face of the first α-helix of IL-3 and is in an analogous position to Glu^{21} in GM-CSF (33). Since the mutation of Glu^{21} in GM-CSF impaired binding to the β chain while fully retaining binding to the α chain (24), it is likely that similar substitutions at position 22 in IL-3 will also selectively affect binding to the β chain.

The mechanism by which [Ala^{101},Val^{116}]IL-3 stimulates function with greater potency is not known but is probably related to its increased binding to high-affinity IL-3 receptors (Table 2). Since these are composed of at least two chains, a low-affinity α chain and a convertor β chain (38), it was important to determine to which component of [Ala^{101},Val^{116}]-IL-3 was preferentially binding. The finding that in monocytes, and under low-affinity conditions, [Ala^{101},Val^{116}]IL-3 exhibited a 15-fold higher affinity than wild-type IL-3 suggests that the mutated residues are involved in binding to the IL-3 receptor α chain.

The construction of the more potent analog [Ala^{101},-Val^{116}]IL-3 has important implications. (*i*) It argues that the

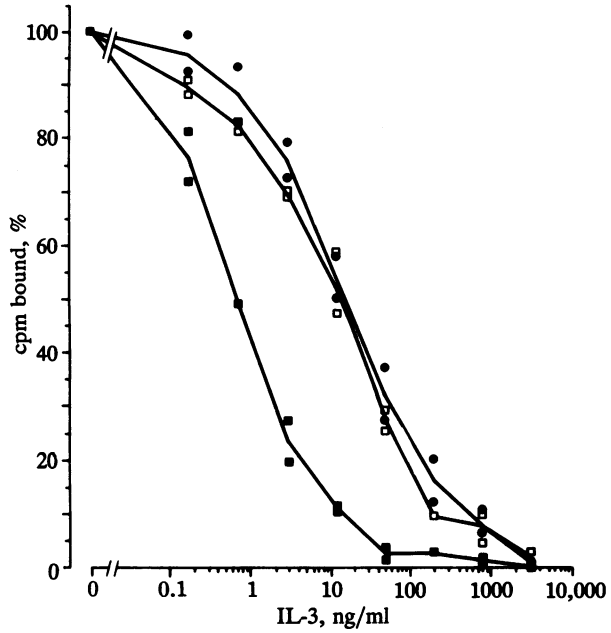

FIG. 4. Competition for binding to low-affinity IL-3 receptors of monocytes by partially purified *E. coli*-derived wild-type IL-3 (● and □) and [Ala^{101},Val^{116}]IL-3 (■). The purified wild-type IL-3 (□) was a gift from S. Clark. ^{125}I-labeled IL-3 was used at 2 nM. Duplicate determinations were performed with 4×10^6 monocytes per point.

C-terminal region of IL-3 and probably of related cytokines such as GM-CSF and IL-5 are involved in binding to low-affinity (α chain) receptors. (*ii*) This type of mutant with increased potency may be useful in situations such as hemopoietic reconstitution where clinical trials are already showing a beneficial effect of IL-3 (44). (*iii*) The identification of residues involved in IL-3 binding to the receptor α chain may facilitate the construction of IL-3 antagonists for use in allergic reactions where IL-3 can exacerbate these conditions (45) and in follicular cell tumors where IL-3 binds and promotes their growth (46).

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