We documented that α-helices A, C, and D in human interleukin-13 (IL13) participate in interaction with its respective receptors. We hypothesized that α-helix D is the site II of the cytokine that binds IL13Rα1, a component of the normal tissue heterodimeric signaling IL13/4 receptor (IL13/4R), and that α-helix D independently binds a monomeric IL13Rα2 receptor, which is a non-signaling glioma-restricted receptor for IL13. Therefore, we alanine-scanned mutagenized helix D of IL13 to identify the residues involved in the respective receptors interaction. Recombinant mutants of IL13 were produced in Escherichia coli, and their structural integrity and identity were verified. The alanine mutants were tested in functional cellular assays, in which IL13 interaction with IL13Rα2 (glioma cells) or an ability to functionally stimulate IL13/4R (TF-1 cells) were examined, and also in binding assays. We found that residues 105, 106, and 109 of the α-helix of IL13 are responsible for interacting with the glioma-associated receptor. Moreover, glutamic acids at positions 92 and 110, and leucine at position 104 was found to be important for IL13/4R stimulation. Thus, α-helix D of IL13 is the primary site responsible for interaction with the IL13 binding proteins. We propose a model that illustrates the binding mode of IL13 with cancer-related IL13Rα2 and physiological IL13/4R.

**Materials**

A fast protein liquid chromatographic system, columns, and media and a unique site elimination mutagenesis kit were obtained from stomach, colon, and skin cancers (9). Furthermore, Caput et al. (10) has cloned another IL13 binding protein, IL13Rα2 that exhibits high affinity toward IL13, and unlike the IL13/4 receptor, it binds to IL13 even in the absence of IL4Rα. We had found that malignant glioma cells express restricted, IL4-independent, intermediate to high affinity binding sites for IL13, the feature resembled by IL13Rα2 receptor (11, 12). In fact, we have recently identified IL13Rα2 as this glioma-restricted receptor (13). Thus, there are two types of IL13 receptors: (a) one present on normal cells and shared with IL4, IL13/4R and (b) one that is more restricted, in that it does not bind IL4 and is expressed primarily in malignancy, IL13Rα2 (13). Consequently, our research effort has been to design a form of IL13, which would have preserved more affinity toward glioma-associated receptor, IL13Rα2, than toward IL13/4 receptor to specifically and effectively target brain tumors. Our earlier rational mutational studies of IL13 revealed several mutants in A, C, and D helices with altered reactivity toward the IL13/4 receptor (14–16). For example, we documented the importance of glutamic acid in positions 13 and 16 in helix A in cellular activation through IL13/4R. Moreover, our results initially suggested potential importance of arginine at position 109 in α-helix D, because its mutation to aspartic acid impaired an ability of IL13 to bind to the IL13/4R as well as to glioma-associated receptor (16). We have also demonstrated that arginine and serine at positions 66 and 69 in helix C, respectively, were important for inducing biological signaling, because their mutation to aspartic acid resulted in reduced signaling through IL13/4R. In other words, our studies revealed site I composed of regions within α-helix A and C, which is most likely responsible for the binding of IL13 to IL4Rα of the IL13/4 receptor (17). Our earlier observations led us to hypothesize that the site II in IL13, which is responsible for the binding to IL13Rα2, is located at the C-terminal region of IL13 molecule in the α-helix D (17). The recently documented solution structure of IL13 is very much in line with this hypothesis (5, 18). These facts prompted us to examine in more detail α-helix D of IL13, which we believed interacts non-concomitantly with both glioma-associated receptor IL13Rα2 and/or a segment of IL13/4R, IL13Rα1 (5, 14, 18).

**Experimental Procedures**

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Amersham Biosciences (Piscataway, NJ). Oligonucleotide primers were synthesized in house at the Macromolecular Core Laboratory, Pennsylvania State University College of Medicine, Hershey, PA. The GMB cell line, U-251 MG, was from American Type Culture Collection (ATCC) (Rockville, MD). G-26 mouse glioma cells were transfected with IL13Re1cDNA and used to obtain G-26-IL13(26R) + (15). TF-1 cell line was obtained from the ATCC. Tissue culture equipment was from Corning Glass (Corning, NY). MTS/MTS-PMS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt/phenazine methosulfate was purchased from Promega (Madison, WI). SDS-PAGE and Western blot transferring equipment were from Bio-Rad. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal Substrate for chemiluminescent detection was from Pierce.

Methods

Mutations in the gene encoding for wild type IL13 were introduced by a unique site-elimination method (21) in which site-specific mutations are introduced in the plasmid using a target mutagenic primer (Table I) and a selection primer as suggested by the manufacturer. Primers were designed using Vector NTI Suite software (Bethesda, MD). A selection primer 5'-TGAGGATCCGAGCTCTAATCTAGAGGCTGCTAAC-3' was used to convert EcoRI enzyme site in the plasmid to XhoI site, to achieve efficient mutagenesis through restriction selection digestion and transformation. All the mutated plasmids were isolated, purified, and subsequently sequenced in house.

Expression and Purification of Recombinant Protein Expression in E. coli—All the plasmids encoding the protein of interest were constructed with T7 promoters and an ampicillin-resistant gene as described previously (16). Escherichia coli BL21 (DE3) cells, carrying an IPTG-inducible T7 RNA polymerase gene, and an IPTG-inducible form was used for host for the expression of alanine mutant proteins. BL21 E. coli cells were transformed with appropriate plasmid and cultured in 1.0 liter of LB broth. When Amax reached a value of 2.0, IPTG was added to a final concentration of 250 μM and incubated for 2 h. The proteins localized in the inclusion body pellets were isolated after tissue-suspending, washing, and denaturing it in 7 M guanidine HCl and renaturing it in presence of L-arginine and oxidized glutathione as described previously (22). The renatured proteins were then dialyzed against 10 mM sodium acetate buffer, pH 5.2, and purified on two ion exchange columns, SP-Sepharose and Mono S using a fast protein liquid chromatography system (fast protein liquid chromatography, Amersham Biosciences).

SDS-PAGE and Western Blot Analyses—The molecular weight and purity of alanine mutants of α-helix D of IL13 were verified by SDS-PAGE, under non-reducing conditions. The mutants were electrophoresed in 15% SDS-PAGE gel. The separated proteins in the gel were stained in Coomassie Blue for visual inspection. For the Western blot, the proteins from the gel were transferred to polyvinylidene fluoride membrane in a transfer buffer at 4 °C. Then, the membrane was blocked with 5% nonfat milk in phosphate-buffered saline for about 2 h at room temperature and incubated for 45 min in 5% milk containing the primary antibody, polyclonal goat anti-human IL13 antibody (1: 1000 dilution). The membrane was washed three times for 5 min each in 0.05% Tween 20/PBS and was incubated for 45 min in 5% milk containing anti-goat IgG conjugated to horseradish peroxidase (1:15,000 dilution), a secondary antibody, which was raised against the peptide derived from C terminus of IL13. Subsequently, the membrane was washed three times with 0.05% Tween 20/PBS. The proteins were detected on the film by enhanced chemiluminescent substrate (ECL) detection system (Pierce).

For UV Circular Dichroism Spectroscopy—For CD spectral analysis, each mutant protein was suspended in phosphate-buffered saline (0.1 mg/ml). All the mutants were analyzed under similar condition at 37 °C, and the spectra were recorded using a demountable “strain-free” quartz cuvette with path length of 2 mm, using a Jasco J-710 spectropolarimeter calibrated with (+)-camphor-10-sulfonic acid. The spectra were obtained in the wavelength range of 195–280 nm and subtracted with that of blank PBS spectrum. Reported spectra were the average of three scans for each sample.

Cell Proliferation Assay—TF-1 cells, the pre-leukemic human B cells, which express the shared IL13/4 receptor, but not IL13Rα2 (23), were grown in the presence of different concentrations of wild type IL13 and their mutants in 96-well culture plates. After 72 h of incubation at 37 °C, the rate of proliferation of TF-1 cells was determined by a colorimetric MTS/PMS cell proliferation assay. The cell samples were incubated with the dye for 4 h, and then their absorbance at 490 nm was recorded for each well using a microplate reader. The cells with wells treated with high concentrations of cycloheximide served as background for the assay. The experiment was performed in duplicate. The relative potency of IL13 mutants versus wild type cytokine was estimated as follows.

\[
\text{Proliferation (wild type IL13)} = \frac{\text{Proliferation (IL13 mutant)}}{\text{Proliferation (wild type IL13)}} \times 100 \quad (\text{Eq. 1})
\]

Cytotoxicity Blocking Assay—The blocking or neutralizing efficiency of the alanine mutants of human IL13 against IL13-based cytotoxin, IL13-PE38QQR, were tested in U-251 MG and G-26-hIL13Rα2(+) cell lines. IL13-PE38QQR is a cytotoxin obtained by fusing a derivative of Pseudomonas exotoxin, PE38KDEL, to IL13 (9, 14, 15, 24). In our studies, 1 × 10^5 cells/well of U-251 MG cells and 2.5 × 10^5 cells/well of G-26 cells were plated in 96-well culture plates and incubated at 37 °C, 5% CO_2, and 90% humidity for 24 h. Then, IL13 or its mutants were added to cells for 1 h at a final concentration of 1 μg/ml. In the positive control wells, 0.1% PBS/bovine serum albumin of volume equal to IL13 or its mutants were added. After 1 h of incubation, increasing concentrations of the IL13-based cytotoxin (0.01–100 ng/ml, final concentration) were added, and the cells were incubated for 2 days. The number of proliferating cells in each well was determined by colorimetric MTS/PMS method described above. The experiment was performed in duplicate. The neutralization efficiency of the alanine mutants with respect to the wild type IL13 at a cytotoxin concentration of 100 ng/ml were calculated as follows.

\[
\% \text{Neutralization efficiency} = \frac{A_{\text{wild type}} - A_{\text{mutant}}}{A_{\text{wild type}}} \times 100 \quad (\text{Eq. 2})
\]

\[
\% \text{Loss in neutralization efficiency} = \frac{A_{\text{wild type}} - A_{\text{mutant}}}{A_{\text{wild type}}} \times 100 \quad (\text{Eq. 3})
\]

Autoradiography—IL13 was labeled with 121I using IODO-GEN agent (Pierce) according to the manufacturer’s instructions (25). On each slide, 2.5 × 10^4 U-251 MG cells were spotted in duplicate and ethanol-fixed and dried. The specific activity of 125I-IL13 was 120 μCi/μg of protein. To determine the competition in the binding efficiency of 121I-IL13 in the presence of alanine mutants on U-251 cells, the slides were incubated with the IL13 alanine mutants of varied concentrations (0.1–200 nm) for 1 h. All the dilutions were made in binding buffer composed of 200 mM sucrose, 50 mM HEPES, 1% bovine serum albumin, and 10 mM EDTA. Subsequently, the cell spots were incubated with 1.0 nm 125I-IL13 in binding buffer. The nonspecifically bound radioligand were dissociated by rinsing the spots in three consecutive changes (5 min each) of ice-cold 0.1 mM PBS. After drying, the radiolabeled sections were exposed to Phosphor screen cassettes for 12 h. Autoradiographic images were scanned by Laser Scanning Module PhosphorImager (Molecular Dynamics, Sunnyvale, CA), with integral ImageQuant data system. The area integration was carried out on the radioactive spots using ImageQuant software, and the percent relative intensities were plotted against the concentration of blocker.

Radio-receptor Binding Assay for IL13 Proteins Affinity Determination Toward the IL13/4R (TF-1 Cells)—TF-1 cells growing in a log phase were harvested and sedimented by low speed centrifugation, then washed twice and resuspended in ice cold PBS. For saturation curve binding analysis, 1 × 10^7 cells were incubated in 250 μl of PBS in duplicates containing 0.1–1000 pm concentrations of 125I-IL13 (specific activity, 147 μCi/μg of protein) for 3 h at 4 °C. Cell-bound radioactivity was separated by using a cell harvester (Brandel, Gaithersburg, MD), and the harvested cells were counted in a γ-counter. Non-specific binding was determined at each concentration of labeled IL13 by adding 500-fold molar excess of the non-labeled IL13. Non-specific binding was subtracted from all the data points to produce specific binding values. The results represent an average of duplicate determinations in one to three independent assays with variations being less than 20% from the mean. In competition studies, 1 × 10^6 TF-1 cells were incubated with serial dilutions of wild type and mutant IL13 proteins at 4 °C for 1 h. Next, labeled IL13 was added at a final concentration of 40 pM and incubated with cells for additional 2 h. Then the cell-bound radioactivity was determined as above.
RESULTS AND DISCUSSION

Alanine Scanning Mutagenesis—We carried out alanine scanning mutagenesis of α-helix D of IL13 using IL13-CLS as the template plasmid. pIL13-CLS contains a gene in which several codons were changed without affecting the corresponding amino acid in IL13 and has been optimized in our laboratory to express higher level of proteins. An alanine substitution is preferred as a first step in a mutational analysis of proteins, because it eliminates potential polar interactions and most hydrophobic interactions with minimum effects on the secondary structure of the α-helical ligand domains (26). Moreover, changing a residue capable of non-covalent interaction with the receptors to alanine usually gives an initial indication of whether a particular side chain is required for ligand binding. 23 single-site alanine mutants from position 90–113 encompassing the entire length of α-helix were constructed (Table I). In one instance, we have substituted alanine at position 94, to another hydrophobic neutral amino acid, valine.

Expression and Purification of IL13 and its α-Helix Mutants—Plasmids encoding each of the mutant proteins were used to transform BL21 E. coli (DE3) bacteria, and all the expressed proteins were localized to the inclusion bodies. The final yield of the protein after purification ranged from 0.5 to 1.5 mg/liter culture, except for mutant proteins like IL13.L100A and IL13.H103A, the expressions of which were below 0.5 mg/liter culture. Each mutant protein was purified from the inclusion body pellets to greater than 90% homogeneity and of proper size of ~13 kDa, as observed from SDS-PAGE analysis (Fig. 1A). Furthermore, all the 24 alanine mutant proteins were immunoreactive toward anti-human IL13 antibody (Fig. 1B) and IL13.K90A was the mutant that showed substantially diminished immunoreactivity in the Western blot. IL13.K90A mutant is of lesser immunoreactivity either because this amino acid represents an important antigenic epitope in IL13 or structurally important β-sheet involving this residue affected the proper folding of the protein resulting in its poor immunoreactivity.

Circular Dichroism Analysis of IL13 Alanine Mutants—The CD spectrum was measured to confirm qualitatively the secondary structural elements in the IL13 mutant proteins. It is important to determine the secondary structural elements, because they are involved in the process of folding of a protein at early stage (27, 28). CD spectra of α-helical-enriched protein should have two spectral minima at 208 and 222 nm. The CD spectral data were obtained for all the 24-alanine mutants, and all the mutant proteins had those spectral minima and confirmed the fact that, by substituting the residues in IL13 with alanine, the helical coiling behavior remained largely unaffected. We have shown in Fig. 2 only the CD spectral data for functionally important mutants, which displayed altered binding to IL13/4R and/or glioma-restricted receptor to confirm the folded nature of those mutants. By comparing the intensity of peaks with wild type IL13, we observed a decrease in intensity with certain mutants like H103A and L100A. It is possible that replacement of bulky groups might have created a kink in the helix resulting in lower intensity bands. Interestingly, these are the mutants of lowest yield of recombinant proteins.

Oligonucleotide mutagenic primers used for alanine scanning mutagenesis of α-helix D segment of IL13

Position Mutagenic primer 5′-3′

| Position | Mutagenic primer 5′-3′ |
|----------|-----------------------|
| K90A     | GTCCGAGAGACCCGCAATCAGGAGT |
| I91A     | CGAGACACCAAAAGCGAGGTGCCCAG |
| E92A     | GACACAAATACTGAGTGCCCAGATT |
| V93A     | ACCAAAATCGAGGGCCAGCTTTTGA |
| A94V     | AAAATCGAGGGCACTTTTGAAGT |
| Q95A     | TCGAGGGAGCAGTTTGAAGATCTG |
| F96A     | ATCGAGGTGGCCAGGATGTTAAGATCTG |
| V97A     | GTGCCGGATTTTGAAGATGTTAAGATCTG |
| K98A     | GCCAGATTTGGTACCAAGATGTTAAGATCTG |
| L99A     | GCCGATTTGGTACCAAGATGTTAAGATCTG |
| K100A    | GCCAGATTTGGTACCAAGATGTTAAGATCTG |
| H101A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| L102A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| L103A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| L104A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| K105A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| K106A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| L107A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| H108A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| L109A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| K110A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| E111A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| G112A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| R113A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |
| F114A    | GCCGAGATTGGTACCAAGATGTTAAGATCTG |

TABLE I

# Alanine in the 94th position is substituted by valine.
Charged Residues in α-Helix D of IL13 Participate in the Interaction with the Glioma-restricted Receptor—IL13-PE38QQR is a recombinant chimera cytotoxic fusion protein that effectively kills glioma cells through IL13Rα2 binding and internalization (13). IL13-based cytotoxin cell killing neutralization by the mutants of IL13 demonstrates, how efficient a mutant competes for the receptor site in cancerous cells that mediates the killing action of a cytotoxin (29). This phenomenon is of importance to us to design cancer-specific cytotoxins and showed its usefulness and predicted structural features of IL13 confirmed in its solution structure (5, 18). Cytotoxicity studies were conducted using two cell lines, U-251 MG human cells and G-26 mouse glioma cells, the latter were transfected to express IL13Rα2 (13). Only G-26 cells transfected with IL13Rα2 and not parental G-26 cells were susceptible to killing by IL13-based cytotoxins.

Most alanine mutants neutralized the cytotoxicity of IL13-PE38QQR cytotoxin to a similar extent when compared with wild type IL13 (Fig. 3). The first set of analysis (Fig. 3A) demonstrates the blocking efficiency of IL13 alanine mutants in the region encompassing amino acids from positions 90–99 in α-helix D, which forms the beginning of helix D and includes the short β-strand preceding this helix. We found that mutation of lysine at position 90 and isoleucine at position 91 to alanine dramatically impaired the neutralization efficiency of the mutants when compared with wild type IL13 (Fig. 3A).

Lys-90 and Ile-91 both lie in the β-strand preceding α-helix D. Cytotoxic neutralization study indicated the total impairment of neutralization efficiency in the case of IL13.K90A and 64% loss in neutralization for IL13.K91A when compared with wild type IL13 (Fig. 3A). Substitution of alanine for valine at position 92 in the α-helix D partially impaired its neutralization efficiency against cytotoxin, with a progressive decrease with respect to cytotoxin concentration, reaching a value of 49% at 100 ng/ml cytotoxin. Similar partial impairment in neutralization efficiency was observed for another mutant, IL13.F96A. Lesser loss in neutralization efficiency was observed for mutants like IL13.E92A and IL13.D99A, although they were not significant (Fig. 3A). However, the cytotoxicity neutralization pattern for IL13.Q95A, IL13.V93A, and IL13.V97A appeared to be similar to that for wild type IL13.

The neutralization of cytotoxicity by the alanine mutants corresponding to the middle portion of the α-helix D, from positions 100 to 109, is shown in Fig. 3B. We found that the cytotoxic neutralization effect was almost nullified for the mutant IL13.K105A. Similar loss in blocking effect was observed for IL13.K106A, although less pronounced (Fig. 3B). There was also 52% loss in blocking efficiency for IL13.R109A at a higher concentration of cytotoxin (100 ng/ml). Although other mutants like IL13.L101A and IL13.L107A exhibited an apparent decrease in blocking activity, they were less prominent than that for IL13.K105A and IL13.K106A.

Fig. 3C includes the cytotoxic neutralization pattern of the alanine mutants located toward the end of helix D at the C-terminal end of the protein (positions 110–113). The respective alanine mutants did not show dramatic alterations toward binding to glioma-restricted receptor, because there was no change in their blocking efficiency of cytotoxin killing (Fig. 3C) when compared with wild type IL13, with the exception of IL13.E110A and IL13.R112A where we observed a partial decrease in blocking efficiency at 100 ng/ml cytotoxin concentration.

Neutralization of Cytotoxicity Pattern for Alanine Mutants in G-26-hIL-13Rα2(+) Cells Correlates with That Observed in U-251 MG Cells—U-251 MG cells not only overexpress IL13Rα2, but also express IL13/4R (11). Therefore, we re-analyzed the blocking efficiency of α-helix mutants of IL13 in a cell system, in which IL13/4R does not contribute to IL13-based cytotoxin cell killing as evidenced by the fact that G-26 mouse glioma parental cells are not killed by IL13-PE38QQR toxin (13). Fig. 4 demonstrates the cytotoxicity neutralization pattern for all the alanine mutants in G-26-IL13Rα2(+) cells. In general, the cytotoxicity-blocking pattern of the α-helix D alanine mutants was similar to that observed in U-251 MG cell line (Fig. 3). Thus, mutations introduced in positions 90 and 91 were associated with loss in neutralization efficiency of more than 50% even at 10 ng/ml concentration of cytotoxin in G-26-IL13Rα2(+) cells (Fig. 4A). Similarly, the cytotoxicity neutralization ability of the mutants corresponding to the middle portion of helix D (positions 100–109) showed similar trend as observed in U-251 MG cells, and, in fact, it was even more pronounced in the G-26 cell line (Fig. 4B). For example, mutant IL13.K105A lost 50% of neutralization potency in G-26-IL13Rα2(+) cells, when compared with wild type IL13, even at low cytotoxin concentration of 1 ng/ml, whereas IL13.K106A lost 40% activity at the concentration of 10 ng/ml cytotoxin. Alanine mutant corresponding to the position 109 retained about 30% of neutralization potency at 10 ng/ml cytotoxin concentration and at the concentration of 100 ng/ml, it lost 78% with respect to wild type IL13. The remaining mutants of the middle region (positions 100–109) such as IL13.L100A, IL13.L102A, and IL13.L104A retained 70% or more wild type IL13 neutralization potency. Toward the tailing region of the helix D, in G-26-IL13Rα2(+) cell lines, we observed a partial loss in neutralization efficiency of the cytotoxicity by the mutants IL13.E110A and IL13.R112A similar to that we observed in U-251 MG cell line (data not shown).

The results obtained in both U-251 MG and G-26-IL13Rα2(+) cells demonstrate that the competition for the glioma-restricted IL13Rα2 receptor is altered to a large extent in mutants IL13.K105A, IL13.K106A, and IL13.R109A. This is indicative that the three positions in α-helix D of IL13, 105, 106, and 109, take part in binding toward glioma-associated IL13Rα2. Previously, we identified Lys-105 position to be the “hot spot” in the α-helix A, which most likely interacts with IL4Rαε of the signaling IL13/4R (16). Here, we provide evidence that Lys-105 in the α-helix D represents a hot spot for the interaction with IL13Rα2. It is noteworthy that Lys-105, as Glu-13, is highly conserved between both human and murine IL13 and IL4 (23). Moreover, the arginine at position 109 is conserved between human and murine IL13 and human IL4.
FIG. 3. Cytotoxicity neutralization behavior of IL13 alanine mutants in the presence of IL13-PE38QQR toxin in G26-hIL13-Res2(+) cell lines. A, alanine mutants corresponding to positions 90–99 in IL13. B, alanine mutants corresponding to the positions 100–109 in IL13. C, alanine mutants corresponding to the positions 110–113 in IL13.
Our study also suggests that mutation of Lys-90 and Ile-91 at the structurally important α-strand impairs the ability of the mutants to bind to glioma-restricted receptor.

**Competitive Radio-receptor Binding Study of Alanine Mutants**—Autoradiographic receptor binding studies were performed to demonstrate how effectively certain mutants that showed impaired cytotoxicity neutralization, competes for the IL13Ra2 receptor site on U-251 MG cell line in the presence of 125I-IL13 at 4°C. However, IL13.K105A, IL13.K106A, and IL13.R109A mutants did not compete with 125I-IL13 for IL13 receptors on U-251 MG cells, even at high concentrations. This finding indicates that those alanine mutants at positions 105, 106, and 109 in the α-helix D segment of IL13 lost their ability to bind the glioma-associated receptor for IL13 indeed. Thus, as we expected it to be the case, the reason for the impaired cytotoxicity neutralization potency in mutants, such as IL13.K105A, IL13.K106A, and IL13.K109A (Fig.
is their lack of binding affinities toward the glioma-restricted receptor.

The Cytotoxicity Neutralization Pattern of the Alanine Mutants and the Orientation of Amino Acids on α-Helix D—Taking into consideration the results obtained from neutralization of the cytotoxicity and binding assays, using alanine mutants, the orientation of amino acids in α-helix D of IL13 on a helical wheel and helical net models was analyzed (Fig. 6, A and B, respectively). In these models, residues Asp-99, Glu-110, Lys-106, Glu-95, Leu-102, Arg-109, Lys-98, Lys-105, and Ala-94 of IL13 all lie on the exposed face of the helix (Face-1, Fig. 6A). The helical wheel projection indicates that almost all the hydrophobic residues, such as His-103, Phe-96, Leu-107, Leu-100, Val-93, Leu-104, Val-97, and Phe-108 with the exception of Leu-102 are in the non-exposed interior face of the helical wheel (Face-2, Fig. 6A). These hydrophobic residues most likely do not interact directly with the receptor. Current results are in line with our hypothetical consideration of the importance of α-helix D in IL13 for the binding to glioma-restricted receptor moiety. All the three positively charged residues, showing impairment in ability to block IL13-based cytotoxins in both U-251 and G-26-IL13Ra2(+) cells, namely Lys-105, Lys-106, and Arg-109, are located within a hydrophilic-exposed region (Face-1, shaded spheres in the helical wheel model in Fig. 6A), which is an anticipated IL13Ra2 binding site. Somewhat surprisingly, we could not observe a similar trend for the mutant IL13.K98A, although the lysine residue at position 98 is expected to lie in the same exposed Face-1 of D helix (Fig. 6A). However, the lysine residue at position 98 is present in the other (“upper”) portion of the helix than the one containing residues at positions 105, 106, and 109, as depicted in the helical net model of α-helix D (Fig. 6B). Thus, according to this helical net model, the interacting site of IL13Ra2 receptor may span rather in the “lower” portion of the helix. As suggested by the helical wheel model (Fig. 6A), Ala-94 is on the same face, Face-1 as Lys-105, and the corresponding mutant A94V demonstrated partial impairment in the cytotoxicity-blocking pattern. Although the overall positive charge of Face-1, the exposed region of α-helix D in IL13, may be important for binding to the IL13Ra2 receptor, substitution of larger hydrophobic residues with a smaller hydrophobic residue (I91A) and substitution of smaller hydrophobic residue with a larger one (A94V) also resulted in a partial loss of cytotoxicity blocking. However, phenylalanine at position 113, which is at the C-terminal end beyond α-helix D boundaries has no role in the interaction with the receptor.

It is also notable that the middle region of the α-helix D, comprising motif LLLHLKKLFR, which corresponds to amino acids from positions 100 to 109, has the maximum number of IL13Ra2-interacting ligands. The region is composed primarily of hydrophobic residues, such as leucine, hydroxyproline, and phenylalanine. Residues Lys-105, Lys-106, and Arg-109 are the
only three residues that are hydrophilic in this middle region, and we found that all these hydrophilic residues in the amphipathic middle region of α-helix D contribute toward the binding to the IL13Rα2 receptor.

Charged Residues in α-Helix D Also Play a Significant Role in the Activation of IL13/4R—The functional interaction of IL13 with the shared IL13/4R complex can be measured by TF-1 cells responses. TF-1 cells proliferate in a dose-dependent
manner in the presence of IL13 or hIL4 as they express the functional IL13/4R (23, 30). At a concentration of 100 ng/ml, wild type IL13 produced a maximal proliferative response in TF-1 cells throughout the concentration range we measured (0.01–100 ng/ml), and a further increase in the IL13 concentration brought about only a minimal increment in TF-1 cell proliferation.\(^2\) Fig. 7 demonstrates the varying abilities of IL13 alanine mutants to proliferate the TF-1 cells. Mutations at residues Lys-90 and Ile-91, which lie in the structurally important /H9251 SHEET preceding the /H9251 HELIX D, had detrimental effect on TF-1 cell-proliferative capacity, displaying abrogated activity when compared with wild type IL13 (Fig. 7A). Mutation of glutamic acid at position 92 resulted in a similar dramatic loss in proliferation of TF-1 cells (Fig. 7A). Furthermore, mutants IL13.K105A and IL13.K106A were poorer stimulants of TF-1 cell proliferation than IL13 (Fig. 7B). Similar to IL13.K105A and IL13.K106A is the mutants IL13.H103A and IL13.R109A (Fig. 7B). Interestingly, IL13.E110A and IL13.R112A induced a prominently lower proliferation of TF1 cells with respect to wild type IL13 (Fig. 7C).

The alanine substitution of most of the hydrophobic residues in α-helix D did not significantly impair the proliferation potency of the mutants on TF-1 cells (Fig. 7). On the contrary, one of these mutants, IL13.L104A, behaved like a superagonist inducing proliferation of TF-1 cells to 122% with respect to wild type IL13 (Fig. 7B). We made this mutant in three separate batches, and, in all three, the mutant showed the same superagonistic trend. Thus, a pattern emerged in which the substitution of hydrophobic residues Val-93, Gln-95, Phe-96, Val-97, Leu-100, Leu-102, Leu-104, and Phe-108 retained more than 70% of wild type IL13 proliferation ability. In contrast, IL13 alanine mutants corresponding to the hydrophilic residues (Glu-92, Lys-90, Lys-105, Lys-106, Arg-109, Arg-112, and Glu-110) exhibited an impaired proliferation of TF-1 cells. His-103

\(^2\) A. B. Madhankumar, A. Mintz, and W. Debinski, unpublished.
is the only hydrophobic residue, which on mutation to alanine displayed a loss of TF-1 cell proliferation ability when compared with IL13 (Fig. 7B).

**IL13 Mutants That Do Not Proliferate TF-1 Cells Lack Affinity Toward IL13/4R**—To ascertain the reason for the inability of several of the IL13 α-helix D mutants to stimulate TF-1 cell proliferation, we performed an IL13/4 receptor binding assay on TF-1 cells. Fig. 8 (A and B) represents the saturation binding curve and Scatchard plot analysis of 125I-IL13 binding to TF-1 cells, respectively. The equilibrium dissociation constant ($K_d$) for the binding of 125I-IL13 on TF-1 cells was determined by the GraphPad Prism program to be 40.3 ± 10 pM, and the number of binding sites was found on average to be 106 per cell.

Next, we used several mutants of IL13, which, contrary to

| Residue in α-helix D | Interaction with respective receptors$^a$ | Corresponding residue in IL4 from sequence alignment (Ref. 23) |
|----------------------|------------------------------------------|-----------------------------------------------------------------|
|                      | IL13/4R (physiological)  | IL13R(a2) (glioma-associated) | |
| Lys-90               | ++                        | ++                                | Lys-102              |
| Ile-91               | ++                        | ++                                | Glu-103               |
| Glu-92               | ++                        | --                                | Ala-104              |
| Val-93               | --                        | --                                | Asn-105              |
| Ala-94               | --                        | +                                 | Gin-106              |
| Gin-95               | --                        | --                                | Ser-107              |
| Phe-96               | --                        | --                                | Thr-108              |
| Val-97               | --                        | --                                | Leu-109              |
| Lys-98               | --                        | --                                | Glu-110              |
| Asp-99               | --                        | --                                | Asn-111              |
| Leu-100              | +                         | --                                | Phe-112              |
| Leu-101              | --                        | --                                | Leu-113              |
| Leu-102              | --                        | --                                | Glu-114              |
| His-103              | ++                        | --                                | Arg-115              |
| Leu-104              | ++                        | --                                | Leu-116              |
| Lys-105              | ++                        | ++                                | Lys-117              |
| Lys-106              | ++                        | +                                 | Thr-118              |
| Leu-107              | --                        | --                                | Ile-119              |
| Phe-108              | --                        | --                                | Met-120              |
| Arg-109              | +                         | ++                                | Arg-121              |
| Glu-110              | ++                        | --                                | Glu-122              |
| Gly-111              | --                        | --                                | Leu-123              |
| Arg-112              | ++                        | --                                | Tyr-124              |
| Phe-113              | --                        | --                                | | |

$^a$**“++” indicates a loss of functional interaction with the receptor; “–” indicates no measurable loss of functional interaction with the receptor.**

**Fig. 9. Solution structure of IL13 indicating the proximity of α-helix A and α-helix D.** The alignments of residues in (i) Plane-P for binding to glioma-restricted, IL13R(a2), and (ii) Plane-Q for activation of physiological IL13/4R are illustrated.
wild type IL13, were incapable of stimulating TF-1 cells, in competition displacement studies on TF-1 cells (Fig. 8C). None of those mutants studied, namely IL13.K90A, IL13.E92A, IL13.K105A, and IL13.E110A, could compete for $^{125}$I-IL13 binding sites, in sharp contrast to what was observed with wild type IL13 (Fig. 8C). This result supports the notion that these mutants are unable to proliferate TF-1 cells due to their lack of affinity for the IL13/4R rather than being bindable but non-signaling muteins.

Based on all of the above-described experiments on TF-1 cells, we suggest that charged residues like Glu-92, Glu-110, and Arg-112 are involved in activating physiological IL13/4R. Among these three amino acids, glutamic acid at position 110 is conserved among human IL4 and human IL13 (position 122 of IL4, see Table II). The structural homology of IL4 to IL13, our previous and current data, and the solution structure of IL13 all document that the residues in IL13, crucial for signaling through IL13/4R, are present in the C-terminal helix of IL13 (31). In our current study, we observed the helix D mutants of IL13, which have altered binding to glioma-restricted IL13R2, namely K105A, K106A, and R109A, do not proliferate TF-1 cells efficiently either despite their preserved structural integrity and proper folding. This suggests that the binding mechanism of α-helix D to glioma-restricted IL13R2 may involve the same residues as for the binding of IL13 to IL13R1 and thus would support our notion that IL13 cannot bind to IL13/4R and IL13R2 concomitantly (17).

Alanine-scanning Mutagenesis Shows α-Helix D Correlates with IL13 Solution Structure—In general, mutational analysis of a helical protein reveals several features of the static structure of the protein, including the lateral proximity of helices and the lateral orientation of each helix (32, 33). In the case of IL13, the results obtained from the alanine-scanning mutagenesis of the helix D segment, and our earlier mutational studies (14–16), revealed the structural significance of the α-helix D, α-helix A, and β-strand preceding the α-helix D toward spatial orientation and binding appropriate receptors. Fig. 9 depicts the three-dimensional structure of IL13 (only α-helix A and α-helix D are shown for simplicity) as viewed using RASMOL based on the co-ordinates obtained from the recently reported solution structure for IL13 (5, 18). The model reveals in particular the spatial closeness of α-helix A and α-helix D. All the residues that are functionally important for interaction with the glioma-restricted receptor, IL13R2, namely Lys-105, Lys-106, and Arg-109, lie in the hydrophilic exposed face of the amphipathic α-helix D of the human IL13 protein molecule in one plane (Fig. 9, Plane-Q). Lys-105, Lys-106, and Arg-109 are also spatially close to each other, which is not surprising, when assigning their roles in binding to IL13R2. It is noteworthy that the mutants we found crucial for activation of the IL13/4R, namely Glu-92, Arg-112 and Glu-110, align with each other and lie in the other plane, which we termed Plane-P, as well. This is compatible with making more favorable contact sites with the receptor. We propose that the regions in IL13 responsible for interacting with the physiological IL13/4R and glioma-restricted IL13R2 receptors are present in the interface of helix A and helix D. Indeed, helices A and D are inclined with an inter-helical angle of 157° (18) and are not separated far apart, which facilitates the formation of a binding interface (7–10 Å).

As described above, mutants IL13.K90A and IL13.I91A lost cytotoxicity blocking and TF-1 cell proliferation potential. We suspected residues Lys-90 and Ile-91 to be important structurally, because they lie in the rigid β-strand preceding α-helix D. In other words, the Lys-Ile motif in that region appears to primarily play a structural role. In this arrangement, isoleu-
which are close to each other. We also propose that the receptor interacting sites span in the “lower” portion of the α-helix D in IL13.

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