Identification of a CD4 Domain Required for Interleukin-16 Binding and Lymphocyte Activation*

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Interleukin-16 (IL-16) activates CD4+ cells, possibly by direct interaction with CD4. IL-16 structure and function are highly conserved across species, suggesting similar conservation of a putative IL-16 binding site on CD4. Comparison of the human CD4 amino acid sequence with that of several different species revealed that immunoglobulin-like domain 4 is the most conserved extracellular region. Potential interaction of this domain with IL-16 was studied by testing murine D4 sequence-based oligopeptides for inhibition of IL-16 chemoattractant activity and inhibition of IL-16 binding to CD4 in vitro. Three contiguous 12-residue D4 region peptides (designated A, B, and C) blocked IL-16 chemoattractant activity, with peptide B the most potent. Peptides A and B were synergistic for inhibition, but peptide C was not. Peptides A and B also blocked IL-16 binding to CD4 in vitro, whereas peptide C did not. CD4, in addition to its known function as a receptor for major histocompatibility complex class II, contains a binding site for IL-16 in the D4 domain. The D4 residues required for IL-16 binding overlap those previously shown to participate in CD4-CD4 dimerization following class II major histocompatibility complex binding, providing a mechanistic explanation for the known function of IL-16 to inhibit the mixed lymphocyte reaction.

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The abbreviations used are: IL-16, interleukin-16; IL-16, recombinant IL-16; MHC, major histocompatibility complex; aa, amino acid; GST, glutathione S-transferase; HIV-1, human immunodeficiency virus-1; HIV-2, human immunodeficiency virus-2; FITC, fluorescein isothiocyanate; IL-2R, interleukin-2 receptor; PAGE, polyacrylamide gel electrophoresis.

sient increase of intracellular Ca2+ concentration (reviewed in Ref. 3).

Previous functional data suggest that CD4 is a receptor for IL-16. IL-16 induces chemotactic responses in CD4+ but not CD4− T lymphocytes (4). The T cell chemoattractant response to IL-16 is inhibited by co-incubation with Fab fragments of the anti-CD4 monoclonal antibody OKT 4, and the magnitude of the IL-16-induced cell migration by monocytes is directly proportional to the amount of CD4 expressed on the responding cells (5). Finally, transfection of human CD4 confers IL-16 responsiveness to an otherwise unresponsive L3T4+ murine hybridoma cell line as demonstrated by the induction of cell motility and rises in intracellular Ca2+ and inositol trisphosphate which are inhibited by OKT4 Fab (6). In addition to this functional evidence, there is direct physical evidence for an IL-16-CD4 interaction. IL-16 can be co-immunoprecipitated with recombinant soluble CD4, and rIL-16 partially displaces OKT4 bound to CD4 (7).

Data from several laboratories indicate a high degree of sequence and functional homology for IL-16 across different animal species (8, 9).2 Human IL-16 induces chemotaxis of human, rat, or mouse CD4+ T cells (1, 9). Murine IL-16 also induces motility and interleukin-2 receptor (IL-2R) expression in human and murine target cells. These data suggest that the site(s) on CD4 interacting with IL-16 are also likely to be conserved. Comparison of the predicted amino acid sequences of CD4 across several species led us to hypothesize that the D4 domain is critical for IL-16 bioactivity.

EXPERIMENTAL PROCEDURES

Cell Preparations—Murine spleens were harvested from healthy 8-week-old male BALB/c mice (Jackson Laboratory). Splenocytes were isolated by grinding spleens between frosted slides in M199 culture medium (BioWhittaker), supplemented with 0.4% bovine serum albumin, 22 mM HEPES buffer, 100 units/ml penicillin, 100 μg/ml streptomycin (M199-HPs).

Cells were washed and erythrocytes were lysed in Gey’s solution. Splenocytes were washed twice in M199 and resuspended to a final concentration of 2 × 10^6 cells/ml in RPMI 1640 medium (BioWhittaker) supplemented with 0.4% bovine serum albumin, 22 mM HEPES buffer, 100 units/ml penicillin, and 100 μg/ml streptomycin. By flow cytometry, 25% of the isolated splenocytes within the lymphocyte cloud were CD4+.

Recombinant Murine IL-16—Recombinant murine IL-16 was produced in Escherichia coli (strain HMS 174, Novagen) as a polystyrene fusion protein containing the 118 C-terminal residues encoded by the previously reported murine cDNA (9), using the bacterial expression vector pET16b (Novagen). The recombinant product corresponds to the secreted form of natural murine IL-16 following cleavage by caspase-3 (11). The rIL-16 was purified by metal chelation chromatography, and the polystyrene tag was cleaved with factor Xa (New England Biolabs).

Murine CD4 Peptides—Synthetic oligopeptides based on murine CD4...
D4 sequences were purchased from Research Genetech. Lyophilized oligopeptides were resuspended in double-distilled H₂O at 2 mg/ml as stock solutions and subsequently diluted in M199-HPS for experiments. The following peptides corresponding to the indicated residues of murine CD4 were prepared: peptide A (aa 351-362), VSEEKQVVQVVA; peptide B (aa 367-376), TGLWQCLLSEG; and peptide C (aa 374-385), EGDVKVKMDSRIQ. A random scrambled peptide designated peptide D (LSQKQMVSREGT) synthesized for use as a negative control was designed to be neutral in charge, hydrophillic, and devoid of cysteine to avoid possible dimer formation. Four 6-amino acid peptides were synthesized based on the native murine CD4 D4 domain sequence as follows: Accepted against VSEEKQV (aa 327-338); VVQKVA (aa 333-338); WQCLLS (aa 344-349); and VAPETG (aa 337-342). Five 6-amino acid peptides were derived based on the above peptides but with selected non-conservative amino acid substitutions as follows: VKQKVA (aa 334, V;K); VVQKVA (aa 336, V;K); WACLLS (aa 345, QA); WQALLS (aa 346, CA); WQ-CELS (aa 347, LE).

Chromatography—Cell migration was assessed by a modified Boyden chamber as described (1,2). Murine splenocytes (5 x 10⁶ cells/ml) in M199-HPS were loaded into the top wells of a microchemotaxis chamber, and 10⁻³ m murine rIL-16 in M199-HPS (or M199-HPS alone) was added to the bottom wells. For blocking experiments, 10⁻⁶ m murine rIL-16 with various concentrations of oligopeptides was mixed and placed in the lower well. The upper and lower wells were separated by an 8-m pore size nitrocellulose filter membrane (Neuroprobe). Following incubation (3 h, 37 °C), the membranes were removed, stained with hematoxylin, and dehydrated. Cell migration was quantified by counting the number of cells migrating beyond a depth of 40 μm. Counts were compared with control cells exposed to M199-HPS alone, which was normalized to 100%. Cell migration is expressed as the mean percent control migration. All samples were tested in duplicate, with five high power fields counted in each duplicate. Results from multiple experiments were analyzed using Student's t test for paired variables and Tukey's test for multiple variables. A p value < 0.05 was considered significant.

Flow Cytometry—Induction of IL-2R (CD25) on rIL-16-stimulated murine splenocytes was detected by staining with FITC-conjugated anti-mouse IL-2R Ab (PharMingen). Cells were fixed with 10% formalin in the presence of 2 mM dithiothreitol. Native GST fusion protein purification protocol using glutathione-conjugated Sepharose 4B beads in the presence of 2 mM dithiothreitol. Native GST fusion protein, or GST alone, was bound to the glutathione-conjugated Sepharose 4B beads. Five 6-amino acid peptides were released from the beads by boiling in SDS gel loading buffer, and the supernatant was analyzed by 12% SDS-PAGE. The presence of 35S-labeled mrIL-16 was detected by autoradiography of the dried gel. Specificity was determined by competition with an excess (10⁻⁵ M) of unlabeled rIL-16, and the effects of the three 12-residue peptides on rIL-16 binding was tested by adding each peptide (10⁻⁶ M) to the binding reaction time 0. In other preliminary experiments (data not shown), neutralizing anti-IL-16 antibody blocked rIL-16 binding to GST-D3D4, and an irrelevant recombinant protein (luciferase) failed to displace rIL-16 bound to GST-D3D4.

Structural Modeling—The crystal structure of the D3 and D4 domains of rat CD4 (Brookhaven Protein Data Bank code 1cid.pdb) was chosen to model domain 4 of murine CD4 that contains the oligopeptide sequences VSEEKQVVQVVA, TGLWQCLLSEG, and EGDVKVKMDSRIQ. The program MolMol (13) was used to create ribbon diagrams and solvent accessible surface maps.

RESULTS

Sequence Comparison of the CD4 from Different Species—Analysis of predicted amino acid sequences derived from IL-16 cDNA clones of different species indicates a high degree of homology (8, 9). Furthermore, Keane et al. (9) reported cross-species functional activity of murine and human IL-16 using target cells from both species and demonstrated that an oligopeptide based on the human IL-16 sequence inhibited murine IL-16-stimulated migration of murine splenocytes. It is postulated that IL-16 exerts bioactivity through an interaction with CD4 (14), and together with the structural and functional conservation of IL-16, we hypothesized that an IL-16-binding site on CD4 might also be conserved across species. The predicted amino acid sequences of the four extracellular immunoglobulin-like domains of CD4 from different species were compared by the method of Lipman and Pearson (15), using Lasergene software (DNASTAR). Each of these four domains (designated D1 through D4) of cat (16), dog (17), rabbit (18), rat (19), and mouse (20) were compared with the predicted human (21) CD4 sequence (Fig. 1). In each case, the D4 domains were found to have a higher similarity index than the other three domains.

Inhibitory Activity of D4 Sequence Peptides—Based on the CD4 sequence comparison, we hypothesized that IL-16 interacts with residues in the CD4 domain. To test this hypothesis, synthetic oligopeptides based on murine D4 domain sequences with the highest homology to human CD4 (Fig. 2) were tested for their ability to block murine rIL-16-stimulated murine splenocyte migration. Initial experiments identified three 12-residue D4-based oligopeptides designated A, B, and C that had IL-16 inhibitory activity. Peptide B (aa 341–352) includes the sequence WQCLLS (residues 344–349) which is 100% conserved in all the species examined. Peptide A (aa 327–338) and peptide C (aa 351–362) correspond to the N-terminal and C-terminal flanks of peptide B, respectively. A random scrambled 12-residue peptide (LSQKQMVSREGT) designated peptide D was used as a negative control for nonspecific effects of peptide administration on splenocyte motility. Criteria for the design of peptide D included overall hydrophilicity, neutral charge, and no cysteine residues (to prevent possible dimer formation).

The effect of cotreatment with the 12-residue peptides on rIL-16-stimulated splenocyte migration is shown in Fig. 3. In the absence of added peptides, rIL-16 (10⁻⁹ M) activated murine splenocytes. As shown in Table I, rIL-16 increased IL-2R induction significantly (P < 0.05) in all the experiments. Peptide B at 10⁻⁹ M significantly inhibited IL-16. Peptides A and C were not inhibitory at 10⁻⁹ M, but both demonstrated significant inhibitory activity at 10⁻⁷ M. In contrast, peptide D failed to inhibit IL-16-stimulated splenocyte migration. None of these peptides (at 10⁻⁹ to 10⁻⁵ M) inhibited murine splenocyte migration in response to an optimal concentration of the murine chemokine JE (10⁻⁹ M, data not shown). These results indicate that certain D4 domain peptides specifically inhibit IL-16-stimulated splenocyte motility.

Peptide Inhibition of IL-2R Induction—To demonstrate that peptide inhibition is not limited to the chemotactic activity of IL-16, the D4 sequence peptides were tested for their capacity to inhibit IL-16-stimulated induction of IL-2R on resting murine splenocytes. As shown in Table I, rIL-16 increased basal IL-2R expression by 50%, and this was completely inhibited by peptide B at 10⁻⁶ and 10⁻⁷ M. Peptides A and C were inhibitory at 10⁻⁷ M but not 10⁻⁶ M. The negative control peptide D (10⁻⁶ M) had no effect on rIL-16-stimulated IL-2R...
expression. The D4 region peptides are equally potent inhibitors of two distinct IL-16 bioactivities, the induction of motility and the up-regulation of IL-2R.

Identification of Residues in Peptide B Critical for IL-16 Inhibition—Because peptide B was active at 10-fold lower concentration than peptides A or C, we next sought to define residues within peptide B critical for IL-16 inhibition. Within peptide B, the six residues WQCLLS are highly conserved between species. A synthetic WQCLLS oligopeptide inhibited IL-16 at 10^{-7} M (Fig. 4), comparable to the activity of the 12-residue peptide B. Preliminary experiments with truncated peptides indicated that the Trp344 and Ser349 residues are dispensable with respect to IL-16 inhibition (data not shown). The substituted peptide WQALLS was equally as effective as the native WQCLLS, suggesting that Cys346 is also dispensable. In contrast, the substituted peptides WACLLS and WQCELS failed to block IL-16, suggesting that the D4 domain residues Gln345 and Leu347 might be involved in IL-16 binding or activation via CD4. The peptide VAPETG which bridges sequences between peptide A and peptide B lacked inhibitory activity, indicating that the two C-terminal residues of peptide A, the two intervening residues between peptides A and B, and the two N-terminal residues of peptide B are dispensable.

Synergistic Inhibitory Activity between Peptides—Since peptides A and C inhibit rIL-16-induced splenocyte migration at 10^{-6} M, it is possible that the corresponding regions in the D4 domain of CD4 are involved in cell activation by IL-16. We hypothesized that a combination of IL-16-inhibiting peptides would be synergistic if they simultaneously blocked IL-16 binding at distinct touch points on CD4, or if contact with discrete binding and activating domains was blocked. This was investigated using combinations of peptides A, B, and C at sub-optimal inhibitory concentrations. As shown in Fig. 3, none of the three peptides alone at 10^{-8} M inhibited IL-16 activity. However, the combination of peptide A and peptide B at a final concentration 10^{-8} M significantly inhibited chemotaxis (Fig. 5). The combination of peptide A plus peptide C, or peptide B plus peptide C, did not inhibit IL-16 at this concentration. These data suggest that sequences within both peptide A and peptide B may be important for IL-16 activation via CD4 but do not indicate whether this involves contiguous sequences or discrete domains within the combined 24 residues covered by the peptides. The failure of VAPETG to block IL-16 favors a model with discrete touch points.

Identification of Critical Residues in Peptide A—To identify the residues that are responsible for the synergistic effect of peptide A, two 6-residue peptides were prepared as follows: VSEEQK and VVQVVA represent the N-terminal half and the C-terminal half of peptide A, respectively. The C-terminal half-sequence VVQVVA inhibited rIL16-induced splenocyte migration at 10^{-6} M comparably to peptide A, whereas the N-terminal VSEEQK demonstrated no inhibitory activity (Fig. 6).
To identify residues in VVQVVA required for IL-16 inhibition, and for synergy with peptide B, non-conservative substitutions were tested. Sequence comparison across species shows that Val334 and Val336 are conserved in all cases studied except for rat whose sequence is VIQVQA. The substituted oligopeptide VKQVVA was compared with the native sequence peptide VVQVVA for inhibition of IL-16-stimulated splenocyte motility. Each peptide was separately combined at various concentrations (0.5 × 10⁻⁶ M to 0.5 × 10⁻³ M) with a sub-optimal concentration of peptide B (fixed at a constant 0.5 × 10⁻⁶ M). As shown in Fig. 7, the substituted peptide VKQVVA was weaker than the native sequence VVQVVA at 10⁻⁶ and 10⁻⁷ M. However, the inhibitory activity of VKQVVA was weaker than the native sequence VVQVVA, which (in combination with peptide B) significantly inhibited motility of stimulated splenocytes at 10⁻⁶ M. These data suggest that the D4 region residues Val334 and Val336 may be important for IL-16 stimulation via CD4.

Inhibition of IL-16 Binding to CD4 in Vitro—The ability of D4 oligopeptides sequences to block the chemotactrant activity of IL-16 is postulated to be mediated by structural mimicry of binding sites for IL-16 on its receptor. To test this hypothesis, and to corroborate previously published evidence that IL-16 interacts directly with CD4, an in vitro binding system was developed. A recombinant chimeric protein consisting of GST fused to the N terminus of the murine CD4 D3D4 region was expressed in E. coli as detailed under “Experimental Procedures.” Radiolabeled murine rIL-16 produced by in vitro transcription and translation in the presence of [³⁵S]methionine was incubated with GST-D3D4 bound to glutathione-conjugated Sepharose 4B beads. Native GST was also tested for each condition to control for nonspecific binding. Following incubation of [³⁵S]labeled rIL-16 with GST-D3D4 or GST-coated beads, complexes were washed three times and then boiled in SDS-PAGE sample buffer prior to electrophoresis and autoradiography. Although some background binding of rIL-16 to native GST was observed, binding to GST-D3D4 was much more intense and was specifically competed by an excess of unlabeled rIL-16 (Fig. 8). Nonspecific binding of [³⁵S]labeled rIL-16 to GST alone was not competed with cold rIL-16, and specific binding of rIL-16 to GST-D3D4 was not competed with an excess of irrelevant protein (recombinant luciferase; data not shown). IL-16 binding in this system was reduced to background in the presence of either peptide A or peptide B alone and with peptide A and B combined. In contrast, peptide C failed to reduce IL-16 binding. These data indicate that peptide A and peptide B function as competitive receptor antagonists.
for IL-16. The ability of peptide C to inhibit IL-16-stimulated splenocyte motility at 10^{-6} M appears to be mediated by a different mechanism.

**Structural Modeling of D3D4**—The locations of the three 12-residue oligopeptide sequences, and of those amino acids critical for IL-16 inhibition, were evaluated in the context of the three-dimensional structure of rat CD4 and previous studies demonstrating CD4-CD4 dimer formation (reviewed in Ref. 22). The sequence homology between rat and mouse CD4 domains 3 and 4 is 87%, and it is expected that the tertiary structure of murine CD4 is similar to the rat. Furthermore, the sequences of the 12-residue murine peptides used in our experiments are conserved in relationship to the corresponding sequences of rat CD4, with the exception of Val337 which is Gln in rat. This residue is located in a \( \beta \)-strand and is solvent-exposed in the rat structure, suggesting that this amino acid difference would not affect the core packing of domain 4, thus preserving the tertiary structure.

Fig. 9A is a ribbon diagram of domains 3 and 4 of rat CD4. \( \beta \)-Sheets are depicted as flat ribbons on domain 4 only. The sequence corresponding to peptide A (colored blue in the figure) is located in strands C', E, and the EF loop. Peptide B (colored red) spans the C-terminal half of the EF loop, all of the F strand, and a portion of the FG loop. Peptide C (colored green) corresponds to the FG loop and G strand. The residues lying between peptide A and B are colored gold, and those shared by peptides B and C are colored orange. The two faces of the \( \beta \)-sandwich form an apex at the EF loop. Residues in peptides A and B that are proximal to the EF loop might constitute a binding site, as can be seen in Fig. 9A. The modeling results indicate that peptide A does not lie within the predicted dimerization region of domain 4 (23). Portions of peptide B lie within the dimer interface, particularly Gln345 (corresponding to Gln346 in human CD4) that is in the center of the interface zone. Peptide C also partially overlaps the suggested dimerization region of D4.

A solvent-accessible surface map was generated (Fig. 9B), again displaying the residues corresponding to peptides A in blue, peptide B in red, and peptide C in green. Our functional studies indicated that Gln^{345} and Leu^{347} of peptide B and Val^{334} and Val^{336} of peptide A are required for inhibition of IL-16. The structural modeling indicates that all of these residues are solvent-accessible and therefore might contribute to binding interactions with IL-16. Fig. 9C shows the solvent-accessible residues Gln^{345} and Leu^{347} in red and orange, respectively, forming part of a potential binding site.
DISCUSSION

CD4 is a co-receptor with the T cell receptor complex (reviewed in Ref. 24). It facilitates the association between T cells and antigen-presenting cells by interaction with the non-polymorphic region of MHC class II and contributes to signal transduction through its cytoplasmic association with the lymphocyte tyrosine kinase p56\(\text{Lck}\). CD4 is also the major receptor for human immunodeficiency virus-1 (HIV-1), HIV-2, and human herpesvirus-7 (25–27). Originally identified as a differentiation antigen on T lymphocytes, CD4 was later found to be expressed on a variety of cell types including monocytes, macrophages, eosinophils, hematopoietic progenitor cells, neurons, and spermatozoa (28). Expression of CD4 by these non-lymphocytic cells indicates that it mediates functions independent of the T cell antigen receptor, although the nature of these putative functions remains to be defined. In addition to binding MHC class II proteins, there is strong evidence that CD4 can serve as a receptor for other soluble ligands.

Surface expression of CD4 is required for cells to respond to IL-16, and a direct interaction between IL-16 and CD4 was observed in co-immunoprecipitation experiments (3, 7). The CD4 ligand HIV-1 envelope glycoprotein gp120 and certain anti-CD4 antibodies mimic some of the bioactivities of IL-16 (29–31). Certain chemokine receptors are known to function as co-receptors with CD4 for HIV-1 infection (32, 33), but it is unknown whether co-receptors are utilized by IL-16. Another soluble CD4 ligand was reported by Autiero et al. (34, 35) who isolated a human seminal plasma glycoprotein, gp17, that binds to recombinant soluble CD4 coupled to Sepharose beads as well as to CD4+ Jurkat cells. The physiological role of gp17 is presently unknown. Together, these findings indicate that CD4 is a multi-functional receptor.

CD4 is a 55-kDa type I integral cell surface glycoprotein with four extracellular immunoglobulin-like extracellular domains (D1–D4), a single membrane-spanning region, and short intracytoplasmic tail which interacts with intracellular tyrosine kinases such as p56\(\text{Lck}\). The extracellular domains appear to form two rigid structures consisting of D1D2 and D3D4, with a flexible connection between D2 and D3 (36). Expression of CD4 by these non-lymphocytic cells indicates that it mediates functions independent of the T cell antigen receptor, although the nature of these putative functions remains to be defined. In addition to binding MHC class II proteins, there is strong evidence that CD4 can serve as a receptor for other soluble ligands.

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MHC class II binding site (37). The seminal plasma gp17 binding site on CD4 is also located in the D1 domain, close to but distinct from the gp120-binding site (38).

Until recently, no functions have been attributed to the D4 domain of CD4. Wu et al. (23) reported the x-ray crystallographic structure of recombinant soluble human D3D4 which spontaneously dimerizes at high concentrations in solution. They found that domain 4 mediates CD4 dimerization and that the interface between dimers involves D4 domains exclusively. At the center of the interface is a pair of conserved glutamine residues (Gln345 and Gln345) separated by a hydrogen bonding distance. In their model, the level of CD4 expression when evenly distributed on a cell surface (estimated at $10^7$ m$^{-2}$) would favor monomers. During antigen recognition, CD4 recruited by cooperative interactions at the cell-cell adhesion junction would lead to an increased local concentration favoring dimer formation. They proposed that CD4 dimerization-mediated trans autophosphorylation is required for CD4-associated kinase activation, and subsequent intracellular signaling. In support of this model, Satoh et al. (39) found that D4-based peptides were capable of inhibiting a mixed lymphocyte reaction (MLR). The activity of these peptides was postulated to result from competitive binding to CD4, thus inhibiting CD4 dimerization. Oligomerization of CD4 dimers, mediated by domain 1 interactions, has also been proposed to occur in the course of CD4 interactions with MHC class II (22).

Our laboratory has shown that the cytokine IL-16 may be a soluble ligand for CD4. In the present study, we attempted to identify domains within CD4 required for IL-16 interaction and bioactivity. Previous studies demonstrating a high degree of sequence and functional homology of IL-16 across different animal species implied reciprocal conservation of a putative binding site on CD4. Sequence comparison across several species indicated that the D4 domain might contain such a conserved site. Accordingly, we tested the ability of oligopeptides based on highly conserved D4 sequences in murine CD4 to neutralize biological activity of murine IL-16. By using this approach, we found that one 12-residue oligopeptide (peptide B) which inhibited IL-16 at $10^{-7}$ M and two 12-residue peptides based on N-terminal and C-terminal sequences flanking peptide B (peptides A and C, respectively) which inhibited IL-16 but only at a 10-fold higher concentration. The activity of peptide B in this system was exclusively associated with the residues WQCLLS that are completely conserved across six species examined. Within WQCLLS, the single amino acid substitutions Gin$^{345}$-Ala or Leu$^{347}$-Glu completely eliminated inhibitory activity, suggesting that Gin$^{345}$ and Leu$^{347}$ play an important role in CD4 interactions with IL-16.

Peptides A and C also inhibited IL-16, albeit at a higher concentration than peptide B. We hypothesized that a putative IL-16-binding site on CD4 might span the sequences covered by one or more of three 12-residue peptides tested (from aa 327 to 362) or that the D4 domain might contain multiple contact points for binding and/or activation by IL-16. This was investigated in experiments evaluating potential synergism between the three peptides. The combination of peptide B plus peptide A was found to inhibit IL-16 activity at a 10-fold lower concentration than the minimal inhibitory concentration of peptide B alone. In contrast, synergy could not be demonstrated between peptide C and either peptide B or peptide A. Analysis of 6-residue peptides comprising two halves of peptide A revealed that only the C-terminal half-sequence VVQVVA inhibits IL-16. Experiments with amino acid substitutions in VVQVVA identified two valine residues (corresponding to Val$^{350}$ and Val$^{352}$ of murine CD4) as critical for inhibitory activity. These results suggest a model where IL-16 interacts at two discrete touch points in the D4 region of CD4 and that this interaction can be cooperatively inhibited by two specific peptides. Peptide A and peptide B also blocked binding of IL-16 to soluble D3D4 in an in vitro assay, consistent with their proposed mechanism as receptor antagonists.

Although peptide C inhibited IL-16 chemoattractant activity at $10^{-7}$ M, there was no synergism with the other D4 domain peptides for inhibition of bioactivity, and peptide C did not block rIL-16 binding to D3D4. The mechanism whereby peptide C inhibits IL-16 bioactivity is uncertain; our experimental data suggest that it does not compete with CD4 for IL-16 binding. An alternative hypothesis to account for the inhibitory activity of peptide C is that the peptide interacts with CD4 itself, preventing CD4-CD4 interactions that may be required for signal transduction. In support of this model, the structural studies of Wu et al. (23) indicate that the D4 residues contained in peptide C are located within the interface zone of soluble D3D4 dimers. Previous data from our laboratory indicate that multimerization of IL-16 is required for its biological activity, suggesting that IL-16 cross-links CD4 on the cell surface to generate activating signals (reviewed in Ref. 3). If peptide C competes with membrane-associated CD4 for D4-D4 interactions, then univalent IL-16 binding might not be inhibited but receptor cross-linking by multimeric IL-16, and subsequent
signal induction, would be prevented by the presence of peptide C in the D4-D4 interface zone. Peptide C would therefore block IL-16 chemoattractant activity but not IL-16 binding and would not be expected to act synergistically with compounds that directly blocked IL-16 binding.

The location of the conserved Gln$^{345}$ at the center of the CD4-CD4 dyad interface is also intriguing since this residue lies within the peptide B sequence and is demonstrated to be critical for the inhibitory function of the peptide WQCLLS.

Computer modeling of the rat D4 domain (which is highly homologous to mouse) shows that the region covered by peptide B contains a potential binding pocket with Gln$^{345}$ and Leu$^{347}$ residues displayed, and peptide B blocked IL-16 binding to D3D4 in vitro. These data explain the known property of IL-16 to inhibit the MLR (10) and suggest that CD4-CD4 interactions and downstream activation events mediated by IL-16 cross-linking differ in key respects from those that may occur when CD4 dimerizes in the context of MHC class II binding. The potential contribution of the critical valine residues within peptide A is presently unclear, but modeling indicates that they are solvent-accessible and thus may contribute to interactions with IL-16. Our data clearly indicate that a CD4 domain required for IL-16 binding and activation lies in close proximity to a domain involved in CD4 dimerization. The oligopeptide inhibitors that we described will be useful tools for investigating a range of IL-16 functions and provide information pertinent to drug discovery.

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