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Revealing and Utilizing Receptor Recognition Mechanisms in a High-Throughput World

Irwin Chaiken

University of Pennsylvania, 909 Stellar Chance Labs, 422 Curie Drive, Philadelphia, Pennsylvania 19104-6100

Abstract Recent genomic mapping promises to identify essentially all of the proteins that underpin normal and aberrant biology in humans. What genomics leaves undone is to determine how these proteins interact and integrate into molecular pathways in health and disease. Specific molecular interactions provide the fundamental mechanism for selectivity in virtually every aspect of biological structure and function. The convergence of structural and mutational studies makes it possible to define what parts of a protein are important for recognition. Still, knowing what is important does not necessarily foretell how binding epitopes actually function. We have applied the approach of epitope randomization on phage to explore how structural elements in such receptor recruitment systems as interleukin-5 (IL-5) and HIV-1 function in receptor recognition. This work has led in the IL-5 case to differentiation of recognition and activation epitopes, and this in turn has potential to help in the design of non-activating mimetics that could stimulate development of therapeutic antagonists for allergic inflammations such as asthma. Whether it is possible to differentiate recognition and activation in designing inhibitors in cases such as HIV-1 cell attachment and infection remains a tantalizing, but unsolved goal at present. Overall, these studies portray advances as well as limitations in the effort to decipher protein recognition mechanisms and utilize the wisdom gained for mechanism-based antagonist design in an increasingly high throughput world stimulated by the advent of genomics and proteomics. J. Cell. Biochem. Suppl. 37: 126–135, 2001.

Key words: receptor; biosensor; mimetics; phage library; interleukin-5; HIV-1

A seminal event in biomedical sciences has been the outpouring of protein informatics from genomics [Howard, 2000]. We now can learn the primary amino acid sequences of virtually all proteins. In addition, we are discovering the three dimensional structures of an expanding number of proteins by direct analysis or by comparison with sequence-homologous proteins of previously known structure. Yet, there is a missing link. What is missing is how these proteins assemble together to drive biological function. The potential knowledge of recognition networks at a molecular and structural level promises to open the way to reveal mechanistic understanding of how proteins drive normal biology and pathogenesis of disease, and therefrom, the possibility to develop mechanism-based recognition mimetics for disease intervention and other biotechnological tools. Yet, while the vastness of protein informatics has stimulated high throughput methodologies to identify proteins, correlate them with disease and discover small molecule ligands, mechanistic study has been intrinsically slower. Being able to utilize high throughput methodologies in mechanistic study, and to develop new high throughput methods of use in mechanistic study, is a major challenge in the transition from genomics to proteomics.

In our own current research, we are interested in deconvoluting receptor recognition mechanisms and using the resulting understanding to design receptor antagonists and other recognition mimetics that could be useful leads to therapeutic or biotechnological tools. We focus on interleukin-5 receptor recruitment and HIV-1 host cell attachment, systems where

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Correspondence to: Irwin Chaiken, University of Pennsylvania, 909 Stellar Chance Labs, 422 Curie Drive, Philadelphia, PA 19104-6100.
E-mail: chaiken@mail.med.upenn.edu

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mechanistic understanding could help define therapeutic approaches to allergic inflammation and AIDS, respectively. We have used such high throughput methodologies as epitope randomization via phage display to explore recognition events. From such studies, we have been able to differentiate recruitment and activation epitopes in receptor recognition and have begun to utilize this understanding as a guide to design mimetic antagonists. The results so far show the promise of methods to identify selectively interacting molecules, but also highlight slow steps such as quantitative analysis of interaction properties for which high throughput methods could play a stimulating role in the future.

Interleukin-5 Receptor Recruitment and Activation

Human IL-5 is the major hematopoietin responsible for differentiation, proliferation, migration, and activation of eosinophils. IL-5 is a homodimeric protein dominated by 2 four-helix bundle units and acts on eosinophils through a cell surface receptor. The IL-5 receptor contains α and β_c chains, with α primarily responsible for ligand binding and β_c for signal transduction (Fig. 1, left). Recent major advances in our molecular understanding of this system, along with recombinant tools and methods for mutagenic, structural, molecular interaction, and cellular analysis, together provide an important opportunity to identify the structural determinants in IL-5 and receptor subunit ectodomains that drive recruitment of α and β_c receptor subunits into a functional complex.

A growing body of evidence, in particular by site directed mutagenesis [Graber et al., 1995; Morton et al., 1995; Tavernier et al., 1995; Li et al., 1996a,b], argues for the involvement of residues in the C-terminal D helix region of human IL-5 (in particular Glu 110), and nearby residues in the turn between the C and D helices (the so-called CD turn), in particular Glu 89 and Arg 91, in receptor α chain interaction (Fig. 1, right). Glu 13, in the A helices at the distal ends of the IL-5 cylindrical dimer, has been shown important in receptor activation, putatively via β_c activation. This has led to the identification of a relatively limited Rα pharmacophore of E89, R91, and E110 and to the question of how these residues function in receptor recruitment vs. activation.

Functional display of scIL-5 on phage using the chimeric construction of single chain IL-5 has enabled asymmetric randomization mutagenesis of the IL-5 molecule and hence an examination of replaceability of residues in and around the Rα pharmacophore. We focused first on the CD turn, which contains two of the key residues, namely Glu 89 and Arg91. An asymmetric scIL-5 chimeric mutant [wt/A5] scIL-5 was constructed as a key intermediate to prepare scIL-5 libraries. This mutant was constructed of an N-terminal half containing the original five charged residues (88EERRR92) in the CD-turn combined with a C-terminal half

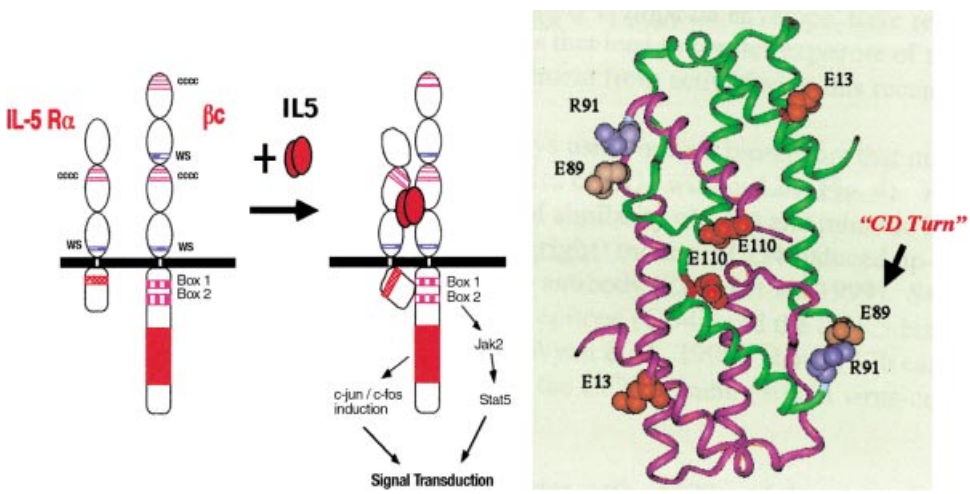

Fig. 1. Structural components of the IL-5-receptor system. Left: Receptor α and β_c subunits and their recruitment leading to activation and signaling. Right: Residues identified as key components in hIL-5 for binding α and β_c receptor subunits, including those in the ''CD turn'', the β-turn between helices C and D that appears key for Rα recruitment [Chaiken and Proudfoot, 1999].

containing a disabling CD-turn sequence (88A-AAAA92). The asymmetrically disabled [wt/A5] scIL-5 variant was used as a starting point to generate an asymmetric scIL-5 library in which the 88-92 N-terminal CD-turn was randomized in the N-terminal domain while it was disabled by replacement with an AAAAA sequence in the C-terminal half. Affinity selection of a phage-displayed library of scIL-5 mutants with randomized sequences in the CD turn allowed the search for hIL-5 sequence variants displaying hIL-5Rα binding activity [Wu et al., 1999]. Selection was done with beads containing immobilized IL-5Rα-Fc. Phage selectants (Fig. 2, left) were analyzed for receptor α binding using a competition ELISA assay. Representative results are in Figure 2, right. [wt/A5] scIL-5 phage as expected had significant activity compared with wild type scIL-5, and plate binding was similarly competed by soluble receptor. In contrast, many of the selectants showed marginal receptor binding activity at best. A notable exception was phage displayed [SLRGG/A5] scIL-5. This phage selectant, which showed preferential receptor affinity bead binding vs. all sequences except for the wild type, was detected to bind to shIL-5Rα-Fc immobilized on microtiter plates with a potency similar to that of [wt/A5] scIL-5. A second single R90 variant, [LPRCG/A5] scIL-5, showed partial potency.

We followed up identification of active CD turn sequences from phage-based randomization with more complete molecular analysis of the SLRGG/A5 variant. [SLRGG/A5] scIL-5 was expressed in *E. coli*, purified using immunoaffinity chromatography and both receptor α chain affinity and cell proliferation activities measured. Strikingly, the data confirm the efficacy of the [SLRGG/A5] scIL-5 molecule and hence the replaceability of the normal CD turn by SLRGG. Hence, while one-residue-at-a-time mutagenesis had argued that two specific charged residues Glu 89 and Arg 91 function in the IL-5 pharmacophore for α chain binding, we hypothesize that the key structural epitope provided by the CD turn is actually charge distribution and that the single Arg 90 in SLRGG provides the same type of distribution as does EERRR in wild type IL-5.

We further have used sequence variation through functional phage display of single chain human IL-5 to investigate the limits of replaceability of the E110 residues, which form a part of the receptor binding epitope. The results [Wu et al., 2000] reveal that there are both activating and non-activating modes of receptor α chain recruitment. Glu110 was varied in the A domain

C-D Turn Selectants from IL5Rα-Fc Panning

Clone	Residue position					frequency
	88	89	90	91	92	
wild type	E	E	R	R	R	
CD1a	L	P	R	C	G	1
CD1b	D	G	I	W	G	1
CD1c	S	C	A	D	V	1
CD1d	E	E	T	R	R	1
CD1e	A	S	L	V	W	1
CD1f	H	L	G	C	C	1
CD1g	K	E	R	R	R	1
CD1h	E	E	R	R	R	7
CD1i	C	L	F	S	S	1
CD1j	P	G	A	T	S	1
CD1k ***	S	L	R	G	G	3 ***

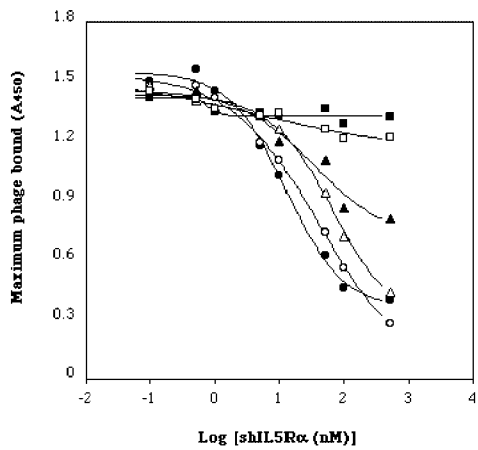

Fig. 2. Results of phage randomization of the CD-turn epitope in IL-5 for Rα recruitment [Wu et al., 1999]. Left: Sequences selected from the asymmetric CD-loop library, [X5/A5]scIL-5. The amino acid sequences of [wt/A5]scIL-5 variants identified after four rounds of affinity selection are listed. Residues that are the same as wild-type sequence are italicized. Right: Competitive phage ELISA evaluation of relative receptor binding affinities of loop library selectants. Phage selectants after four rounds of panning were analyzed for binding to shIL-5Rα-Fc. Different titers of phage samples that result in equivalent signal were incubated with different concentrations of sIL-5Rα protein, ranging from 0 to 500 nM, and then the incubated mixtures were added to shIL-5Rα-Fc-coated microwells. Phage amounts were 4×10^9 cfu for [wt/A5]scIL-5 (●), 2×10^9 cfu for scIL-5 (○), 9×10^8 cfu for [SLRGG/A5]scIL-5 (△), 4.5×10^9 cfu for [LPRCG/A5]scIL-5 (▲), 1×10^{10} cfu for [SRLGA/A5]scIL-5 (■) and 2×10^9 cfu for [DGIWG/A5]scIL-5 (□). The data shown are the mean of two independent experiments, each carried out in triplicate. Data taken from [Wu et al., 1999].

of [wt/A5] scIL-5. The data obtained argued that Tyr and Trp are almost as effective in receptor binding as negatively charged residues (Glu and Asp). *E. coli*-expressed E110 variants containing E110W in the otherwise sequence-intact N-terminal half, including a variant with an E110A replacement in the sequence-disabled C-terminal half, were shown by circular dichroism to be folded into secondary structures similar to that of scIL-5. Biosensor kinetics analysis revealed that [E110W/A5] scIL-5 and [E110W/A6] scIL-5 had receptor α-chain binding affinities similar to that of [wt/A5] scIL-5. However, [E110W/A6] scIL-5 had a significantly reduced bioactivity in TF-1.28 cell proliferation compared to both [wt/A5] scIL-5 and [E110W/A5] scIL-5, and this activity reduction was disproportionately greater than the much smaller effect of E110 mutation on receptor binding affinity. The marked and disproportionate decrease in TF-1 proliferation observed with [E110W/A6] scIL-5 suggests a role for E110 in biological activity mediated by the signal transducing receptor β_c subunit of IL-5 receptor. The data from phage-based randomization data argue that the CD turn, and in particular charge distribution in that turn, are key in Rα recruitment but on their own will not activate receptor efficiently (Fig. 3, left). This conclusion, borne out by subsequent combined mutagenesis [Plugariu et al., 2000], leads to the possibility to form CD turn mimetics as non-activating antagonists of IL-5. Intriguingly, cyclic peptides have recently been reported [England et al., 2000], which were derived using a path including phage libraries, exhibit significant affinity and antagonist activity, and contain charged residues in a CD turn-like EXXR sequence (Fig. 3, right). Whether these latter sequence elements are responsible for the antagonist effect of such peptides is as yet unproven. Nonetheless, the use of phage epitope libraries has brought us to the point of being able to decipher receptor recruitment epitopes and to begin to use this understanding in IL-5 mimetics design.

Mechanism-Based Antagonism of HIV-1 Cell Attachment

Human immunodeficiency virus type 1 (HIV-1) infection, leading to acquired immunodeficiency syndrome (AIDS), is dependent on initial attachment of the virus to host cells by the viral spike envelope protein gp120 (Fig. 4). HIV-1 attachment leads to fusion of viral membrane with target cell membrane [Barre-Sinoussi et al., 1983; Gallo et al., 1984]. The fusion process can be triggered by interaction of HIV-1 Env gp120 with the T-cell antigen receptor CD4 glycoprotein [Allan et al., 1985; Robey et al., 1985; Maddon et al., 1986]. The second envelope protein, gp41, is a key mediator of virus–cell fusion. Several seven-transmembrane chemokine

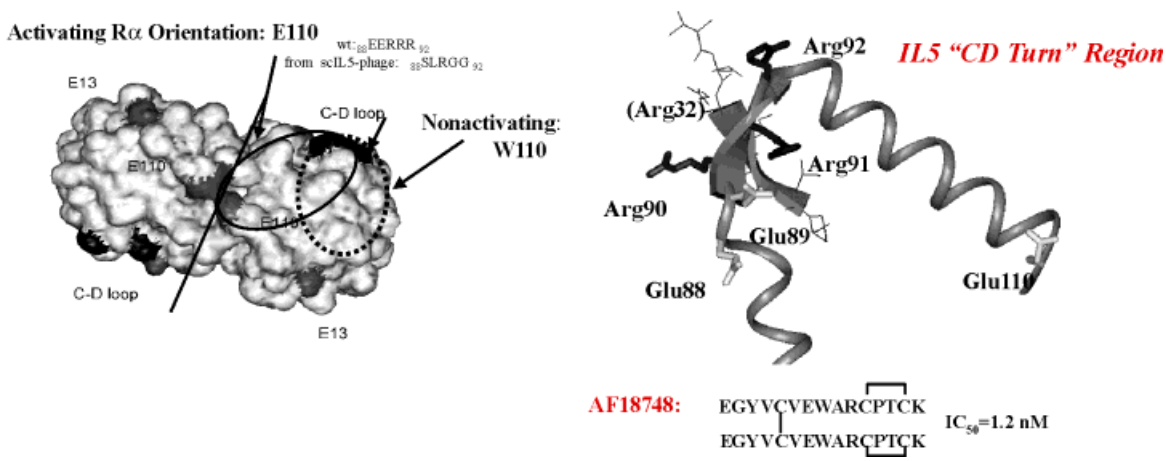

Fig. 3. Left: Structural model depicting the hypothesis of separable epitopes on IL-5 for activating and non-activating recruitment of receptor α. The scheme shows the hypothetical structural rationale for the finding that receptor α chain can bind in activating (solid line) vs. nonactivating (dotted line) modes, the latter of which would disable activating recruitment of the β_c chain at the hypothesized IL-5 site around E13 [Wu et al., 1999; Plugariu et al., 2000]. Right top: Ribbon cartoon of the CD-turn region of IL-5 highlighting the charged sequences of $_{88}$EERRR$_{92}$ and Arg 32 in the context of the β-sheet conformation in the region of these residues. Right bottom: Sequence of a recently identified IL-5 antagonist derived by phage randomization and subsequent synthetic variation [England et al., 2000].

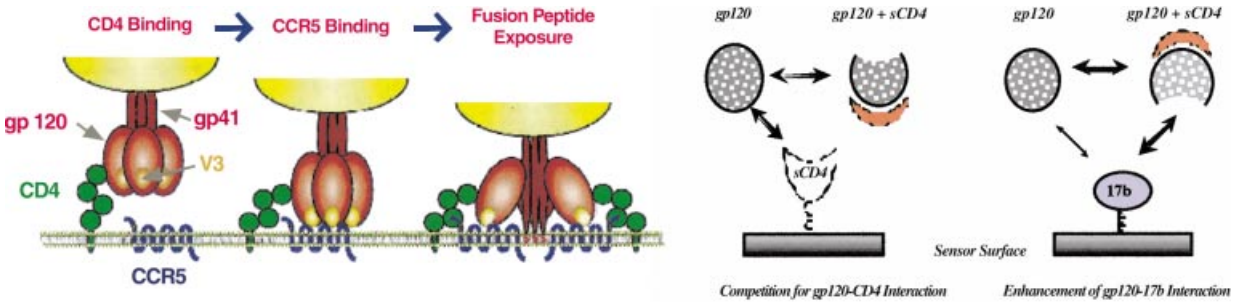

Fig. 4. Left: Model depicting the organization of the HIV-1 envelope proteins gp120 and gp41 and the cell receptors that are involved in virus–cell attachment leading to fusion [adapted from Doms and Peiper, 1997]. Right: Configurations of gp120-17b Fab-sCD4 3-component biosensor assays [Zhang et al., 1999] for effects of CD4 on the binding of gp120 analyte to immobilized CD4 and effects of CD4 on binding of gp120 analyte to immobilized 17b.

receptors, in particular CCR5 and CXCR4, have been identified as obligate co-receptors for virus cell entry [Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996]. The virus-host cell attachment process that occurs in HIV-1 infection appears to induce a staged rearrangement of the envelope components that leads to enhanced viral entry. As depicted in Figure 4, CD4 binding is believed to induce conformational changes in gp120 which are postulated to promote subsequent steps in the fusion process, such as co-receptor binding, dissociation of gp120 from gp41, exposure of fusigenic components of gp41 to enhance virus–cell fusion and consequent viral entry [Sattentau and Moore, 1991; Sattentau et al., 1993].

Soluble CD4 has been shown to dramatically increase the affinity of the env gp120 of macrophage-tropic (M-tropic) HIV-1 for co-receptor CCR5 [Choe et al., 1996]. Further, CD4 has been shown to induce a greater affinity of gp120 for co-receptor surrogates such as the antibody 17b [Wyatt et al., 1995; Zhang et al., 1999]. That CD4 binding to viral envelope increases exposure of co-receptor binding, along with the observed variable affinity of CD4 for the envelope of clinical isolates, may explain at least in part why soluble CD4 has had poor efficacy as a therapeutically active antagonist in human clinical trials.

The above suggests the possibility that gp120 binding antagonists can be useful as potential therapeutic antagonists by blocking fusion-triggering initial steps of virus cell attachment, including CD4 binding and conformational isomerization of the viral envelope. It further suggests that for CD4-gp120 inhibition, antagonists that might be the most effective therapeutically may, while recognizing the CD4-binding epitope on envelope, have reduced capacity for inducing the conformational rearrangements that lead to greater exposure of the co-receptor site. Thus the benefits of differentiating recruitment from activation in this receptor system.

We configured kinetic interaction analysis assays using optical biosensors that monitor both recruitment and cooperative interaction properties of CD4 with gp120 (Fig. 4). The competition assay (Fig. 4, middle) is a convenient monitor of similarity of CD4 and mimetic binding sites on gp120. In contrast, the 17b effect assay (Fig. 4, right) monitors CD4-induced upregulation of gp120 binding to the co-receptor surrogate, 17b antibody [Zhang et al., 1999]. Several observations argue for the proximity of the 17b-epitope (CD4i) and the CCR5-binding site [Thali et al., 1993; Wyatt et al., 1995; Moore and Sodroski, 1996]. Hence, 17b can be used as a reporter to detect molecular events that occur at the CCR5-binding site in virus–cell docking and fusion.

Knowledge of the structure of CD4 and its complex with gp120, and the availability of biosensor assays to detect interactions of CD4 with HIV-1 envelope protein, stimulated an effort to identify structure-based antagonists. Previous mutagenic results have shown the importance of Phe 43, Arg 59, and sequences around these residues for gp120 binding. Phe 43 has been shown to be particularly dominant in stabilizing the gp120 interaction. Further, Phe 43 is present in a β-turn of the CDR2 region of CD4. Most importantly, the revealing of the three dimensional structure of the CD4-gp120-17b complex showed that the Phe 43 β-turn is

preserved in the CD4-gp120-17b structure and the Phe 43 side chain makes an intimate contact in cavity 2 of gp120. We and others have focused on this residue in a β-turn conformation in the design of miniprotein mimetics of CD4.

Scorpion toxin scaffolds such as charybdotoxin have provided a useful miniprotein scaffold of CD4 mimicry [Drakopoulou et al., 1998; Zhang et al., 1999]. The native charybdotoxin molecule possesses a small antiparallel β-sheet connected to a short α-helix by a β-turn [Vita et al., 1993, 1998]. The molecule is stabilized in this α/β/β structure motif by three disulfide bonds. This structure motif is well conserved in all known scorpion toxins and tolerates a large number of amino acid mutations in the molecule without loss of folding [Bontems et al., 1991; Vita et al., 1998]. The β-turn in this scaffold was used as an initial starting point for transplanting Phe 43 β-turn residues from CD4.

In our own work, we designed and synthesized a charybdotoxin-based miniprotein to attempt to mimic CD4. The underlined residues in the CD4 mimetic TXM1, NH_2-GCTTSKECWSVCQRLHNTSRGGCQGSFCTCGP-OH, are non-charybdotoxin sequence elements that were incorporated to mimic structural features of CD4, in particular Phe 24 to mimic Phe 43 of CD4. In addition to the Phe residue, we also attempted to mimic the position of Arg 59, a second residue shown to be important for gp120-CD4 affinity mutagenically [Wu et al., 1996], by the Gly 1 α-amino group of amino terminal shortened toxin sequence. The energy-minimized structure of TXM1 and its comparison with CD4 crystal structure are shown in Figure 5, left. It also denotes that we made a control miniprotein, which we called TXM0, that had the residues in positions 26 and 27 reversed, FS instead of SF in TXM1.

Overall, TXM1 behaves exactly as one would expect for sCD4, competing for gp120 binding to immobilized CD4 and enhancing the binding of gp120 to immobilized 17b (Fig. 5, right). Neither effect was observed with TXM0. The affinity of TXM1 for gp120 was estimated to be low, approximately 20 μM. Nonetheless, that it possesses CD4-like interaction properties encourages further synthetic variation in scorpion toxin scaffolds. Importantly, toxin-scaffolded variants with relatively high affinity (Kd < μM) have been identified [Vita et al., 1998].

Results with peptides such as TXM1, along with concurrent studies in our group [Li et al., 2001] and elsewhere [Drakopoulou et al., 1998; Vita et al., 1998], have shown that CD4 mimicking miniproteins could be made in an αββ fold of scorpion toxin. The working hypothesis here is that a key structural element in each case is the Phe-containing β-turn. Phage epitope randomization of a β-turn loop of a charybdotoxin-based miniprotein scaffold was used to identify variations of these peptides that can bind gp120 and block the gp120-CD4 interaction.

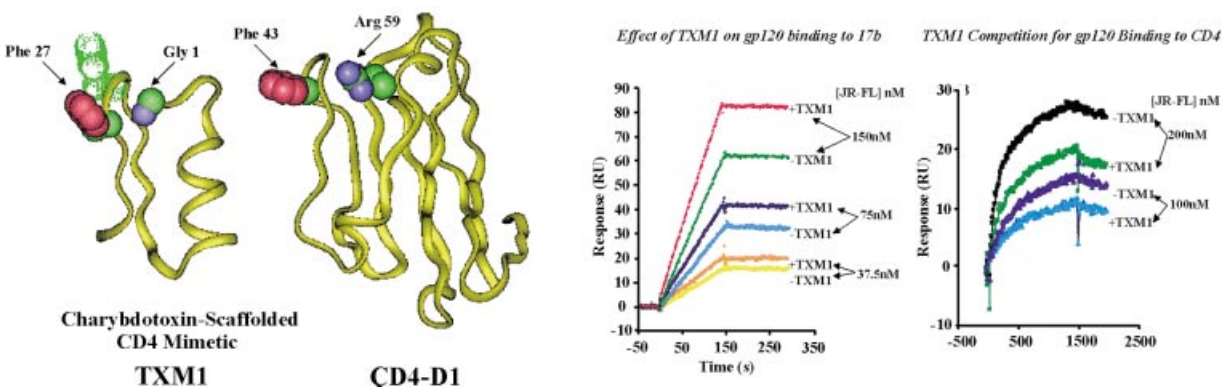

Fig. 5. Left: Structural comparison of CD4 D1 domain (right structure) and charybdotoxin analog TXM1 (left structure). The latter contains Phe 27 in a loop configuration believed to mimic Phe 43 in CD4, while it contains an adjacent α-amino group believed to mimic the Arg 59 side chain of CD4. The TXM1 structure also shows in dotted space filling the position of Phe in an alternative mimetic, TXM0, made as a control for TXM1. (TXM1 and TXM0 structures taken from Zhang et al. [1999], CD4 D1D2 from Kwong et al. [1998]). Right: Sensorgram overlays for the JR-FL protein binding to immobilized 17b Fab (left graph) and immobilized sCD4 (right graph) in the absence and presence of TXM1. Surface density: 1600 RU (left) or 150 RU (right). Buffer: 10 mM phosphate, pH 7.4, 300 mM NaCl, 0.005% Tween. JR-FL protein concentrations are shown at the right of each sensorgram. Flow rate: 30 (left) or 4 μl/min (right). The concentration of TXM1 and TXM0 in each mixture is 50 (left) or 20 times (right) that of each JR-FL concentration. Data taken from Zhang et al. [1999].

We displayed the charybdotoxin scaffold on the filamentous phage fUSE5 and constructed a β-turn library. The phage library formed through this construction was affinity selected on immunotubes coated with immobilized JR-FL gp120. After three rounds of selection, individual phage clones were prepared and sequenced. Using the above library and panning with immobilized Env gp120, we identified a set of selectants shown in Figure 6, left.

Competition enzyme-linked immunosorbent assay (ELISA) identified high frequency phage selectants for which specific gp120 binding was competed by sCD4 (Fig. 6, right). Surprisingly, several of these selectants contain hydrophobic residues in place of the Phe that occurs in the gp120-binding β-turns of both CD4 and previously identified scorpion toxin CD4 mimetics. One of these selectants, denoted TXM [$_{24}$ GQTL$_{27}$], contains the GQTL in place of the CD4 β-turn sequence $_{40}$QGSF$_{43}$. TXM [$_{24}$GQTL$_{27}$] peptide was prepared by solid phase chemical synthesis, its binding to gp120 demonstrated by optical biosensor kinetics analysis and its affinity for the CD4 binding site of gp120 confirmed by competition ELISA. The results of this project demonstrated that aromatic-less loop containing CD4 recognition mimetics can be formed with detectable envelope protein binding within a β-turn of the charybdotoxin miniprotein scaffold. This work established a methodology for phage display of a charybdotoxin miniprotein scaffold and pointed to the potential value of phage-based epitope randomization of this miniprotein to identify novel CD4 mimetics. The latter are potentially useful in deconvoluting structural determinants of CD4-HIV envelope recognition and possibly in designing antagonists of viral entry.

The above results of phage randomization revealed the dominance of nonaromatic residues in and around the β-turn position initially occupied by Phe in TXM1. This finding evoked the questions: To what extent do these molecules recapitulate the binding properties of CD4 and what is the role of the β-turn Phe in envelope interactions? We have examined the binding properties of the most frequently selected sequence from phage display, EGLV, and the corresponding CD4 D1D2 variant, F43V D1D2. This work, currently in progress (C. Dowd et al., unpublished communication) promises to help delineate the role of Phe43 in the mechanism of HIV entry and may help facilitate the development of HIV entry antagonists.

Prospects for Determining Receptor Recognition Mechanisms and Designing Receptor Antagonists in a High Throughput World

Using phage epitope randomization in mechanistic studies. High throughput methodologies are being increasingly used to identify protein and other macromolecular correlates of disease through tissue and genomic screening

TX Selectants from gp120 JR-FL Panning

Sequence	*Selection frequency*
E G L V	12/35
G Q T L	8/35
Q G S F	4/35
S T K A	3/35
P Q L E	2/35
G Q K V	1/35
P Q R L	1/35
P Q L L	1/35
T Y G G	1/35
T Y V G	1/35
L R L L	1/35

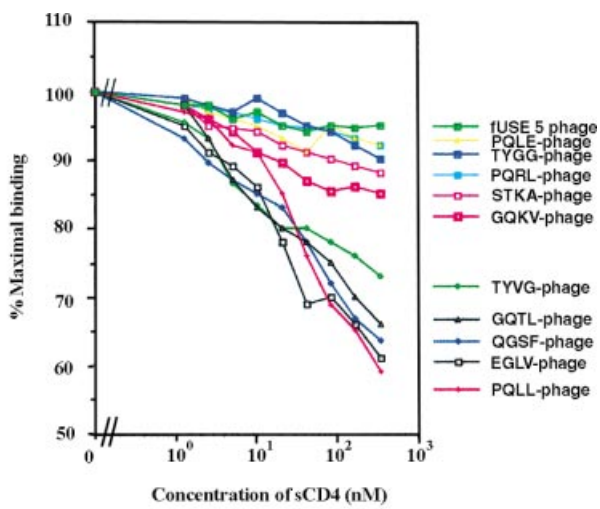

Fig. 6. Left: Phage displayed charybdotoxin turn sequences from the library selected after panning against immobilized gp120 [JR-FL]. Right: Soluble CD4 competition ELISA of CT phage selectants. Phage and sCD4 (0–250 nM) were premixed before addition to the plates. Data taken from Li et al. [2001].

and to identfy antagonists of those putative disease targets through combinatorial search. In contrast, more rational connections between target identification and antagonist discovery often involve deliberate and ultimately slower steps including revealing location of active sites and structural elements in those active sites. As exemplified in this article, phage epitope randomization can be used to speed up revealing how recognition sequences work, by going beyond the information from one-residue-at-a-time mutagenesis to identify sequence themes that satisfy requirements for recognition and function. Often, the initial results of library screening can lead to unexpected combinations of functional sequences. Deconvoluting the meaning of sequence selectants is a major challenge in epitope randomization studies. Yet, understanding the unexpected can lead to enlightenment, generally helping to learn about rules of recognition and specifically teaching not just what groups are important, but how they may function, and enlightenment may lead to something useful, for example, learning what structural elements of a protein to utilize in order to form a specifically targeted antagonist.

Differentiating recruitment and activation in receptor recognition mechanisms and design of receptor antgonists. Our studies on recognition epitopes argue that it is possible to differentiate structural elements driving receptor recruitment from those needed for receptor activation. This is most evident in the results with interleukin-5, for which we have found that charge balance in the CD-turn of this cytokine drives receptor α recruitment but not, at least by itself, activation of the receptor α/β_c complex. This conclusion enables a promising structural focus for investigating existing antagonist leads and for future antagonist design. In contrast, differentiating recruitment and activation epitopes in the CD4 /HIV-1 envelope system is not yet achieved. Mimetic antagonists made so far, at least through miniprotein design and randomization studies, are both recruiting and activating. Nonetheless, the potential that non-activating CD4 mimetics could lead to effective antagonism of HIV-1 cell attachment and infection is likely to stimulate further work by researchers in this field, including the use of combinatorial search methods to speed the screening of possible CD4 mimetic and other envelope ligand structures.

Development and convergence of other high throughput methods, such as in interaction analysis, to accelerate mechanistic and design studies. The forces of increasing genomic and proteomic data, improved opportunities for structure-based investigation and the demands for biotechnological output make it likely that other high throughput methods will be introduced into mechanistic and design studies of protein targets. One area of need is interaction analysis. The advent of optical biosensors for real time kinetics analysis has set new standards for identifying interaction properties of protein targets and mimetic molecules. We have integrated this methodology into almost all aspects of our own studies in the IL-5/IL-5 receptor and CD4/HIV-1 envelope systems. Nonetheless, as the number of protein variants and antagonist candidates increases, the multiple flow cell throughput allowed by current instrumentation will likely lead to technology enabling even greater interaction analysis throughput. High throughput quantitation of interaction kinetics will also help map and understand the dynamics of the extensive recognition networks being revealed in biology, for example signal transduction cascades. For those interested in mechanism and design, the ability to incorporate high throughput methodologies will likely have a transforming effect on experimental pace and style and will pay enabling dividends for the types of questions that can be asked and reasonably answered.

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