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Molecular Basis for the Binding Promiscuity of an Anti-p24 (HIV-1) Monoclonal Antibody

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Summary

Multiple binding capabilities utilized by specific protein-to-protein interactions in molecular recognition events are being documented increasingly but remain poorly understood at the molecular level. We identified five unrelated peptides that compete with each other for binding to the paratope region of the monoclonal anti-p24 (HIV-1) antibody CB4-1 by using a synthetic positional scanning combinatorial library XXXX[B_1,B_2, B_3,X_1,X_2,X_3]XXXX (14 mers; 68,590 peptide mixtures in total) prepared by spot synthesis. Complete sets of substitution analogs of the five peptides revealed key interacting residues, information that led to the construction of binding supertopes derived from each peptide. These supertope sequences were identified in hundreds of heterologous proteins, and those proteins that could be obtained were shown to bind CB4-1. Implications of these findings for immune escape mechanisms and autoimmunity are discussed.

Introduction

Protein–protein interactions play a crucial role in nearly all physiological processes. In order to maintain the numerous complex networks and functions of molecular recognition, specificity in most of these events is essential. For example, in immunity a sophisticated system has evolved to discriminate between self and foreign, in which specificity of recognition is a fundamental prerequisite. Dysfunction of this network might have serious consequences for the organism, such as the induction of autoimmune diseases (Kaplan and Meyeserian, 1962; Oldstone, 1990; Zhang et al., 1994; Wucherpfennig and Strominger, 1995). In these cases, antibodies or T cells, which are induced or activated in response to foreign pathogens, are also able to recognize autoantigens. How is such binding promiscuity (other terms used in this context are polyspecificity, heterospecificity, multispecificity, cross-reactivity, multireactivity, heteroclitic binding, molecular mimicry, mimotope, etc.) accomplished structurally? A detailed structural investigation of this phenomenon should be helpful in elucidating the molecular origin of autoimmunity, and it should also provide important contributions toward a deeper understanding of protein–protein interactions in general.

The term cross-reactivity originally referred to polyclonal antibodies reacting not only with the wild-type antigen but also with homologous compounds; however, it is also used for monoclonal antibodies. Polyspecificity and multispecificity are terms associated with low-affinity antibodies such as endogenous antibodies and IgM molecules whose variable regions are frequently of germ-line origin (Guilbert et al., 1982; Burastero et al., 1988; Baccala et al., 1989; Roggenbuck et al., 1994; Wing 1995). Conversely, high-affinity antibodies that have undergone antigen-driven somatic mutations are usually thought to be monospecific. Nevertheless, antibody cross-reactivity and polyspecificity have been observed since the earliest immunochemical studies, but the structural basis and biological relevance of this phenomenon are still open questions (Van Regenmortel, 1994; Webster et al., 1994; Wilson and Stanfield, 1994; Mariuzza and Poljak, 1995).

The development of biologically (phage display) and chemically generated peptide libraries has greatly facilitated systematic studies of the multiple binding capabilities of protein molecules, owing to the enormous number of peptides that can be tested. This led to several observations that even high-affinity binding monoclonal antibodies are able to recognize more than one peptide epitope (Christian et al., 1992; Needels et al., 1993; De Ciechi et al., 1995; Pinilla et al., 1995; Appel et al., 1996; Demangel et al., 1996; Schneider-Mergener et al., 1996; Valadon et al., 1996). Consensus motifs obtained from phage libraries point to important residues, although they do not necessarily have to be key residues for binding (Kramer et al., 1995; Schneider-Mergener et al., 1996). Moreover, from these studies it cannot be deduced whether the peptides binding to the same antibody truly use a different binding mode (polyspecificity) or display a sometimes hidden molecular similarity (cross-reactivity) by using the same few key residues (see also Results and Discussion). Furthermore, these studies did not investigate the implications of these findings with respect to the potential cross-reactivity and polyspecificity of the particular antibody with heterologous proteins, nor did they analyze the molecular and structural basis of these phenomena.

Using spot synthesis (Frank, 1992), we previously prepared different types of combinatorial peptide libraries bound to continuous cellulose membrane supports for the identification of protein, metal, and nucleic acid binding peptides (Kramer et al., 1994; Malin et al., 1995; Schneider-Mergener et al., 1996). Several antibody epitopes were detected using combinatorial libraries XXB_1B_2XX (20^2 mixtures) or $XXXXB_1B_2B_3XXXX$ (20^3 mixtures) (B = defined position, X = randomized position) (Kramer et al., 1994; Schneider-Mergener et al., 1996; Kramer and Schneider-Mergener, 1997). However, we

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were not able to detect the peptide epitope GATPQD LNTML recognized by the murine anti-p24 of HIV-1 monoclonal IgG2a antibody CB4-1 (Höhne et al., 1993) using the two types of libraries described above. For that reason, we designed a more complex positional scanning (Pinilla et al., 1992) combinatorial peptide library XXXX[B_1,B_2,B_3,X_1,X_2,X_3]XXXX (Schneider-Mergener et al., 1996) consisting of 68,590 peptide mixtures. The library comprises 10 sublibraries containing the different hexapeptide cores [$XXB_1B_2B_3X$], [$XB_1XB_2B_3X$], [$XB_1B_2XB_3X$], [$XB_1XXB_2B_3$], [$XB_1XB_2XB_3$], [$XB_1B_2XXB_3$], [$B_1XXXB_2B_3$], [$B_1XXB_2XB_3$], [$B_1XB_2XXB_3$], and [$B_1B_2XXXB_3$] corresponding to all possible distance patterns of three defined positions ($10 \times 19^3 = 68{,}590$ peptide mixtures (spots), cysteine omitted). This library takes into account the observation that linear epitopes usually contain only a few (2–5) key interacting residues, frequently not in adjacent positions, that cannot be substituted without substantial loss of binding (Geysen et al., 1988; Pinilla et al., 1993; Volkmer-Engert et al., 1994; Stigler et al., 1995).

Here, we report the use of the positional scanning library described above to analyze the binding specificity of the monoclonal anti-p24 antibody CB4-1. From this repertoire, four structurally unrelated CB4-1 binding peptides were identified. Moreover, one D-amino acid peptide was defined from a corresponding D-amino acid library. The molecular basis of the observed polyspecificity of CB4-1 was investigated by cross-competition binding experiments as well as synthesis and binding analysis of complete sets of substitution analogs of all library-derived peptides. The different involvement of CB4-1 residues in these recognition events was demonstrated via binding studies with antibody single chain Fv mutants and X-ray structural data of three peptide-antibody complexes. In addition, we present an approach to identify putative cross-reacting heterologous proteins, including autoantigens. For this, the substitutional analysis data from the library-derived peptides were used to delineate the binding supermotifs (supertopes) that reflect the structural requirements for binding at each position of the relevant peptide. Database searches for proteins that match the supertopes resulted in the identification of more than 6000 heterologous proteins. A substantial number (>16%) of those protein-derived peptides was able to bind CB4-1. Some of the corresponding proteins were obtained and analyzed for CB4-1 binding. The results are discussed with respect to structural and immunological implications.

Results

Identification and CB4-1 Binding Characteristics of Structurally Unrelated Peptides

Screening the cellulose-bound positional scanning combinatorial library XXXX[B,B_2,B_3,X_1,X_2,X_3]XXXX (Schneider-Mergener et al., 1996) for CB4-1 binding peptide mixtures resulted in the detection of 225 peptide mixtures, i.e., 0.33% of the total 68,590 spots. Subsequent iterative deconvolution (Geysen et al., 1986; Houghten et al., 1991; Kramer et al., 1994; Malin et al., 1995) of the randomized positions of the selected peptide mixtures led to the identification of the epitope-related peptide GATPEDLNQKLAGN and the three completely different peptides GLYEWGGARITNTD, RDFDKEWNLIEQNS, and FDEDSQPRRWQRLS (Table 1). The epitope-related peptide GATP**E**DLN**QK**LAGN contains three substitutions (bold) compared to the minimal epitope GATPQDL NTML (Höhne et al., 1993). In addition, one CB4-1 binding D-peptide efslkGpllqwrsG obtained from a structurally identical D-peptide library was defined (small letters are D-amino acids).

Table 1. Identification and CB4-1 Binding of Library-Derived Peptides

first screening step	deconvoluted peptides	K_I [ELISA] (M)	K_D [ET] (M)	binding site overlap
XXXXX**LN**XX**L**XXXX	(1) GATPEDLNQKLAGN	1.2×10^{-9}	1.4×10^{-9}	1.8 ± 0.9
XXXX**WXG**XX**I**XXXX	(2) GLYEWGGARITNTD	2.0×10^{-7}	6.1×10^{-8}	0.8 ± 0.4
XXXXX**EXN**X**I**XXXX	(3) RDFDKEWNLIEQNS	2.7×10^{-6}	1.9×10^{-6}	1.0 ± 0.5
XXXXX**PXRW**XXXXX	(4) FDEDSQPRRWQRLS	2.2×10^{-4}	n.d.†	n.d.†
xxxxx**Gp**x**l**xxxxx	(5) efslkGpllqwrsG	1.9×10^{-5}	n.d.†	n.d.†
	(6) GATPQDLNTnL§	1.3×10^{-8}	8.6×10^{-9}	1.0 ± 0.5
	(7) GATPQDLNTnLNTV§	1.0×10^{-8}	7.1×10^{-9}	1.4 ± 0.7

Columns: (1) The strongest CB4-1 binding peptide mixture for each motif (first screening step) obtained from the positional scanning combinatorial peptide library XXXX[B_1,B_2,B_3,X_1,X_2,X_3]XXXX. (2) Library-derived peptides 1-5 after iterative deconvolution of the X-positions plus epitope-derived peptides 6 and 7 (see below). (3) Inhibition constants (K_I) determined by competitive ELISA with solid phase-adsorbed p24 and N-terminally acetylated peptides 1-7. (4) Dissociation constants (K_D) calculated from fluorescence energy transfer (ET) titrations with anthranilic acid-labeled peptides 1-7. (5) Binding site overlap between the natural epitope (peptide 6) and peptides 1-7 (1.0 = complete overlap) calculated from ET competition experiments.

†n.d. = could not be determined due to high intramolecular energy transfer of the peptides.

§The epitope (peptide 6) and for comparison with the library-derived 14-mer peptides a C-terminally elongated p24-derived peptide 7 are shown. Substitution of the wild-type methionine by the isosteric norleucine (n) does not influence CB4-1 binding (Höhne et al., 1993).

Determination of binding affinities and competition experiments revealed that all library-derived peptides bind to the same region of this monoclonal antibody but with different affinities (Table 1). The three substitutions of the epitope-related peptide 1 resulted in an order of magnitude higher affinity compared to the p24-derived peptides 6 and 7, whereas the other peptides bound CB4-1 more weakly. Substitutional analyses of the peptides (Pinilla et al., 1993; Volkmer-Engert et al., 1994; Stigler et al., 1995; Schneider-Mergener et al., 1996), in which each position of the peptides was substituted by all other amino acids (cysteine omitted), allowed the identification of key residues for binding (Figure 1). For instance, for peptide 1 (Figure 1), leucine at position 7 is the most important residue and cannot be substituted at all. Leucine 11, as well as aspartic acid 6 and asparagine 8, are also very critical but can be substituted by physicochemically related amino acids without substantial loss of binding. For peptide 2, tryptophan 5, glycine 6, glycine 7, and isoleucine 10 are essential for antibody binding; for peptide 3, the most critical residue is glutamic acid 6; for peptide 4, proline 7 and arginine 9 are important; and in the D-peptide 5, the core motif lkGpl is essential.

From these analyses it became obvious that the molecular basis of antibody recognition is specific and

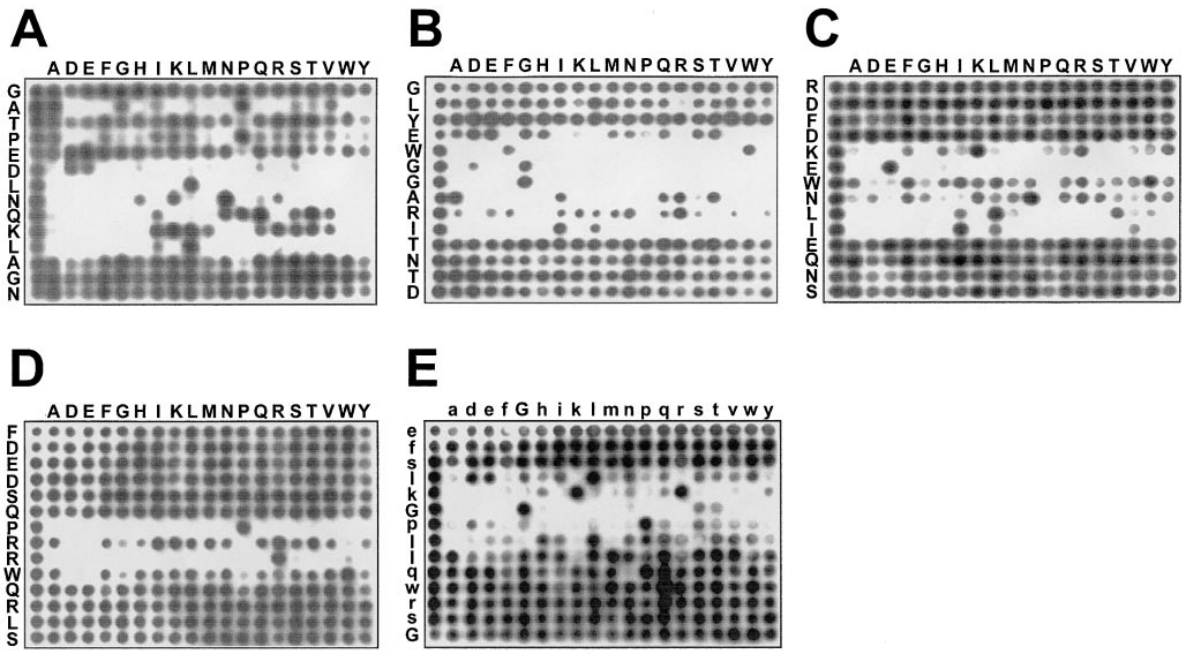

Figure 1. Substitutional Analyses of Library-Derived CB4-1 Binding Peptides

Each residue of peptides 1–5 (Table 1) was substituted (rows) by all other (cysteine omitted) L- or D-amino acids (small letters) and analyzed for CB4-1 binding. The sequences corresponding to the left columns are identical and represent the starting peptide. Other spots are single substitution analogs. The exposure time was adjusted to approximate equal intensities in the left control columns. The relative spot intensities correlate qualitatively with the binding affinities (Volkmer-Engert et al., 1994).

unique for each identified peptide. These findings are confirmed by X-ray structures of peptides 1, 2, and 5 in complex with CB4-1. Figure 4 displays an overall view of peptides 1, 2, and 5 binding to the same cleft formed by the six complementarity determining regions (CDRs) of CB4-1 (for details, see Keitel et al., 1997 [this issue of *Cell*]). For peptide 1, the key residues leucine 7 and 11 make hydrophobic contacts with phenylalanine 32 of the light chain and histidine 52 of the heavy chain. For peptide 2, the key residues tryptophan 5 and isoleucine 10 interact with tyrosine 49 and phenylalanine 94 of the light chain, respectively. The neighboring glycine residues 6 and 7 are required to prevent a sterical clash with two aromatic side chains of the light chain (Phe 32L and Tyr 91L) by fitting into a very tight pocket, as seen in Figure 4 (red peptide, left side). As in peptide 1, the two important leucine residues in the D-peptide 5 are separated by three residues, but the contacts of both aliphatic side chains to the antibody are completely different. In this case the D-leucines 4 and 8 make hydrophobic contacts to tyrosine 91 and phenylalanine 94 of the light chain, respectively (Keitel et al., 1997).

Identification of CB4-1 Binding Peptides Present in Heterologous Proteins via Supertope Analysis

From the substitutional analyses it became obvious that CB4-1 is able to recognize not only unrelated peptides but also a broad spectrum of peptide sequences related to each library-derived peptide. This observation led to the construction of different CB4-1 supertopes. A supertope reflects the structural requirements for binding at each position of the relevant peptide. As an example supertope 1 XXXXX[DE]L[HKNR]XX[IL]XXX (derived from peptide 1 GATPEDLNQKLAGN) and supertope 2 XXXX[FW][DG]GXX[IL]XXXX (derived from peptide 2 GLYEWGGARITNTD) were delineated from the substitutional analyses (Figures 1A and 1B) (X = 20 amino acids). At the key positions each permitted amino acid was included, whereas X was assigned to those positions with more than three possible substitutions. A SWISSP ROT (release 31) database search using the program PESEARCH (software PCGENE, Oxford Molecular, Oxford, United Kingdom) for proteins matching these supertopes resulted in two sets of peptide sequences (5517 matches for supertope 1; 1219 matches for supertope 2) present in heterologous proteins. These numbers are in the statistically expected range (calculations not shown). All of the identified peptides were prepared by spot synthesis and analyzed for CB4-1 binding. CB4-1 was able to recognize a substantial number of the synthesized peptides, i.e., more than 1000 binding peptides were detected for supertope 1 (data not shown) and more than 200 peptides for supertope 2 (Figure 2). For each supertope the fifty strongest binding peptides and their corresponding proteins are given in Table 2.

Database scans for protein regions matching peptides 1 and 2 using the alignment programs SCANSIM and FASTA (software PCGENE) revealed almost no correlation with those identified via supertope analysis. For peptide 1, only three of the 50 supertope-derived proteins listed in Table 2 were among the 50 best matches identified by these alignment programs (Gag polyprotein

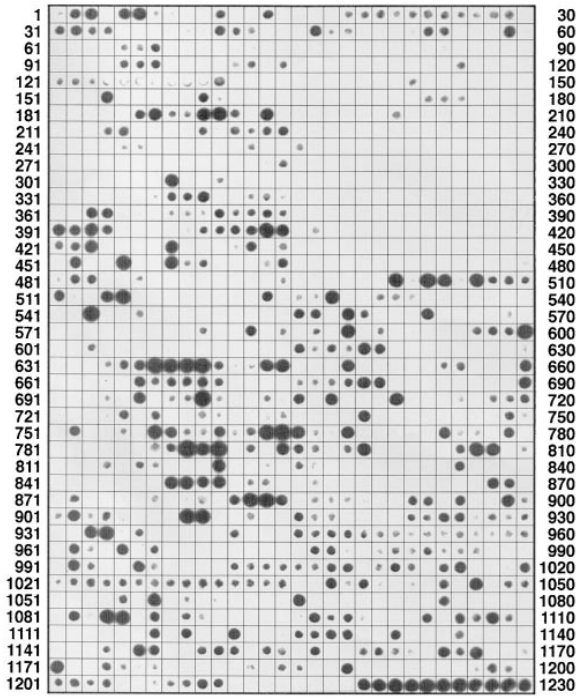

Figure 2. CB4-1 Binding Studies with Database-Derived Peptides Matching Supertope 2

Peptide sequences (1219) matching supertope 2 were synthesized by spot synthesis in a 30 × 41 array and tested for CB4-1 binding. For comparison, the spots 1220–1230 correspond to peptide 2. Binding was visualized as described. The exposed X-ray film was scanned and quantified using the software IMAGEQUANT (Molecular Dynamics, Sunnyvale, California). The sequences of the 50 strongest binding peptides are given in Table 2.

of different HIV-1 strains, myosin II heavy chain of A. castellanii, and probable ATP-dependent permease of bakers yeast). For peptide 2, none of the supertope-derived proteins listed in Table 2 were predicted. For a practical comparison between these two approaches, the peptides corresponding to the 50 best scores of the SCANSIM search using peptide 2 were synthesized on cellulose membranes and tested for CB4-1 binding. Only one of them was able to bind CB4-1 with an affinity comparable to those of the supertope-derived peptides listed in Table 2 (data not shown).

Are the Identified Heterologous Proteins Recognized by CB4-1?

Small amounts of 11 heterologous proteins containing CB4-1 binding supertope sequences, such as alcohol dehydrogenase (E. coli), UmuD (E. coli), candidapepsin (candida albicans), or X-Pro-dipeptidase (human) were obtained (see Acknowledgments) and tested for antibody binding. All of them were recognized by CB4-1 (Table 2 and Figure 3) in denatured and/or native form using a solid phase enzyme-linked immunosorbant assay (ELISA). Several control proteins (see Table 2 legend) did not bind. In addition, it had already been shown that frequently reported putative antigens of multireactive antibodies, such as ssDNA, dsDNA, tetanus toxin, diphtheria toxin, and others do not bind to CB4-1 (Jahn et al., 1995).

For the UmuD protein of E. coli (supertope 1-derived) and candidapepsin (C. albicans; supertope 2–derived), sufficient material was obtained for competitive ELISA studies with the soluble proteins. Both proteins were able to inhibit CB4-1 binding to solid-phase adsorbed p24 (Figure 3A). The inhibition constants K_I were 1.1×10^{-5} M for UmuD, 6.4×10^{-6} M for candidapepsin, and 2.4×10^{-6} M for the natural antigen p24. Furthermore, in the case of UmuD, enough material was available to determine the dissociation constant (2×10^{-6} M) for the UmuD–CB4-1 interaction via BIAcore measurements (Figure 3B).

CB4-1 Binding Proteins Exhibit Differential Binding Modes

To answer the question whether the CB4-1 binding to the identified proteins was accomplished via a direct interaction of CB4-1 with the defined protein regions, we compared the binding of peptides 1 and 2 with that of alcohol dehydrogenase (E. coli) and candidapepsin (C. tropicalis) to two different CB4-1 single chain Fv mutants. Alcohol dehydrogenase contains the sequence YPLISELKQILLDT (amino acids 847–860), which matches supertope 1, whereas amino acids 157–170 of candidapepsin (DTVGFGGISIKNQQ) correspond to supertope 2. In the CB4-1 single chain Fv mutants, phenylalanine 94 of the light chain and tyrosine 32 of the heavy chain are substituted by alanine residues (V_L:Phe94Ala and V_H:Tyr32Ala; K. W., unpublished data). The influence of these mutations on the binding to the peptides and the proteins is illustrated in Figure 3C. The single chain Fv mutant V_L:Phe94Ala bound more weakly to peptide 1 compared to the wild-type CB4-1 single chain (wt) and showed almost no binding to peptide 2, whereas V_H:Tyr32Ala reacted weakly with peptide 1 but considerably better with peptide 2, even compared to the wild-type single chain antibody. Alcohol dehydrogenase behaved like peptide 1, and candidapepsin behaved like peptide 2, indicating that CB4-1 binding to these proteins is in fact mediated by the completely different key residues of both supertopes.

Discussion

The data presented provide a clearly defined functional and structural description of the cross-reactivity and polyspecificity of a monoclonal antibody. Although some data dealing with the structural aspects of antibody cross-reactivity have been reported (Arevalo et al., 1993; Chitarra et al., 1993 [for review see Mariuzza and Poljak, 1993; Webster et al., 1994; Wilson and Stanfield, 1994]), we present an example concerning the interaction of several unrelated peptides with the same antibody molecule, thus giving structural insight into how the observed polyspecifity is accomplished.

The identification of five nonhomologous peptides (including one D-peptide) that bind to the same antibody region demonstrates the potential of this type of synthetic cellulose-bound positional scanning library XXXX $[B_1,B_2,B_3,X_1,X_2,X_3]$XXXX for discovering novel binding

Table 2. CB4-1 Binding Peptides and Corresponding Proteins Identified by Supertope Analyses

Supertope XXXX[DE]L[HKNR]XX[IL]XXX

Sequence	Region	Protein	Species	CB4-1 Binding
ENLSE**ELN**QKLSTL	511-524	Kinesin heavy chain	L. pealeii	
GTTPE**DLK**TLLSMK	258-271	Anthranilate synthase comp. II	P. chrysosporium	
GIHII**DLN**QTLSYL	216-229	Mitoch. 40S ribos. protein MRP4	S. cerevisiae	
GATPQ**DLN**MMLNIV	183-196	Gag polyprotein	HIV-1 (MAL)	
GATPQ**DLN**MMLNVV	173-186	Gag polyprotein	HIV-1 (U455)	
GATPQ**DLN**TMLNTV	177-190	Gag polyprotein	HIV-1	+++/+++
HPKTY**DLK**QILLEI	173-186	Protein BGLF1	EBV	
CRHNH**ELN**QRLSQS	106-119	Smh class II histocompatibility antigen, beta-1 chain	S. leucodon, S. ehrenbergi	
GATEI**DLN**TKLLHD	718-731	Probable ATP-dependent permease	S. cerevisiae	
YELRV**ELN**NTLGNH	157-170	Fibrinogen-like protein A	P. parvimensis	
VEQRI**DLN**QLLIQH	34-47	UmuD protein	E. coli	+++/+++
YPLIS**ELK**QILLDT	847-860	Alcohol dehydrogenase	E. coli	+++/+++
ANLAK**ELN**TKLEGR	130-143	Hypoth. 44.9 kD protein in erg7-nmd2 intergen. reg.	S. cerevisiae	
GSVYR**DLK**QSLIDM	397-410	Mitochondrial transpoter ATM1	S. cerevisiae	
YVMPS**DLR**TELSKR	173-186	Integrin VLA-4 alpha subunit	M. musculus	
KAMPE**ELK**QKLKLT	287-300	Myosin II heavy chain, non muscle	A. castellanii	(+)/(+)
CTLPQ**DLR**QSLQIH	169-182	Glucan synthase-1	N. crassa	
LARKE**DLN**QQIISV	210-223	Hypoth. 37.5 kD protein in dur1,2-ngr1 intergen. reg.	S. cerevisiae	
MTRIE**DLK**TVLGQT	382-395	Put. clathrin-coated vesicle/ synapt. ves. proton pump subunit	C. elegans	
NKLTV**DLN**TKLKEQ	903-916	Myosin heavy chain 95F	D. melanogaster	
YRFVC**DLH**NLLGYT	443-456	C-ets-2a protein	X. laevis	
SPSQE**DLN**CIINLK	244-257	Extracellular signal-regulated kinase 2	H. sapiens, R. norv., M. musculus	
VLAED**DLK**QLLSIN	37-50	Probable transcription initiation factor IIE-alpha chain	S. cerevisiae	
IDELK**DLN**QKLQLE	728-741	Kinesin heavy chain	M. musculus	
YSRRG**DLH**VTLTSA	515-528	Neuroendocrine convertase 1	R. norv., M. mus., M. cookii, H. sap.	
KDLIK**DLK**SELSGN	211-224	Annexin VII	H. sapiens, M. musculus	
CLDLN**DLK**PVLQRS	386-396	Laccase	N. crassa	
ITGTY**DLK**TVLGHL	308-321	Alpha-1 antitrypsin	P. anubis	
CRLQV**DLN**TTLPQE	317-330	Hypoth. 48.1 kD protein co2F5.6 in cH.some III	C. elegans	
SPSQE**DLN**CIINLK	249-262	Mitogen-activated protein kinase	X. laevis	
SASAE**ELR**QRLAPL	257-270	Apolipoprotein A-IV	H. sapiens, M. fascicularis	++/++
DALCQ**DLN**NILRNL	299-312	Salivary glue protein Sgs-3	D. erecta	
YIAAV**DLN**NKLTVW	289-302	DNA polymerase α-binding protein	S. cerevisiae	
FFDPR**DLK**PTLESK	347-360	Glucose inhibited devision prot. A	E. coli	
IPCKD**DLK**THLYTL	159-172	Calreticulin	S. mansoni	
RISAK**DLK**NMLSQV	171-184	1-Phosphatidylinositol-4,5-bis-phosphate phosphodiesterase γ 1	H. sapiens, R. norv., B. taurus	
PKLAA**DLN**TVLTRY	397-410	Transcript. Regulatory protein TyrR	E. coli	
YLATI**DLN**SLLYKY	518-531	Probable trehalase	S. cerevisiae	
CHYSD**DLN**TVLSHD	4864-4877	HC-Toxin synthetase	C. carbonum	
LMKVQ**DLK**QLLLNV	1043-1056	Hypoth. 134.4 kD protein in efb1-tfc3 intergen. reg.	S. cerevisiae	
APYSD**DLR**QRLTAR	187-200	Apolipoprotein A-I	B. taurus	
IKSVT**ELN**GDIITN	98-111	Fatty acid-binding protein, liver	H. sapiens	
APYSD**ELR**QRLAAR	188-201	Apolipoprotein A-I	M. fascicularis, H. sapiens	
PSLPN**DLK**TLLFRA	17-30	14.7 kD (put. nucleic acid-BP)	ShVX	
RRLLR**DLN**IKINQV	1-14	Protochlorophyllide reductase chlB subunit	Z. fischeri	
YLATI**DLN**SLLYKY	431-444	Neutral trehalase	S. cerevisiae	
TASLC**DLK**SRLDSR	402-415	Hydroxymethylglutaryl-CoA synthase, cytoplasmatic	H. sapiens, R. norv., C. griseus	
YKFVC**DLK**TLIGYS	397-410	GA-binding protein alpha chain	H. sapiens, M. musculus	
NGLIE**ELN**QALETF	63-73	Enoyl-CoA hydratyse, mitochondrial	R. norvegicus	++/+
ELYVG**DLN**TKLITR	1130-1143	RNA polymerase	TV	

Supertope XXXX[FW][DG]GXX[IL]XXX

Spot#	Sequence	Region	Protein	Species	CB4-1 Binding
1171	TVAN**FGG**ARITGQI	136-149	Purescine-ornithine antiporter	E. coli	
404	RLAC**WGG**KTIGTDQ	274-287	DNA polymerase	DHBV (S5)	
789	EAKD**FGG**IAIDKHG	39-52	3-Isopropylmalate dehydrogenase	L. lactis	
700	MRTA**FGG**ARIVDML	318-331	Hydrogenase expression/ formation protein HypE	H. capsulatus	
765	NVVS**FGG**AIIGKLL	102-115	Galactose-6-phosphate isomerase LacB subunit	S. mutans	
1084	RFRG**FGG**VRIEEDV	441-454	X-Pro dipeptidase	H. sapiens	-/+++
637-40	KPGR**FGG**KRIQQAI	360-373	Hemagglutinin-neuraminidase	NDV	
600	TAAA**FGG**ATIGALS	420-433	Protein transport protein Sec61 alpha subunit	S. cerevisiae	
909	GRCS**FDG**AQIALLP	35-48	Nitrogenase iron-molybdenum cofactor biosynthesis protein NifE	A. vinelandii	
791	EVFP**FGG**AAIDASA	36-49	3-Isopropylmalate dehydrogenase	T. aquaticus	
543	KACL**FGG**VNISGIN	177-180	Glutamine synthetase	H. vulgare	
712	LLCL**FGG**VSIASWN	654-667	Synaptic vesicle protein 2	R. norvegicus	
504	RLSD**FGG**RSLSVLS	373-386	Replication protein A 70 kD DNA-binding subunit	X. laevis	
191	DTVG**FGG**ASITKQV	147-160	Candidapepsin 3	C. albicans	
910	AGCA**FDG**AQITLLP	35-48	Nitrogenase iron-molybdenum cofactor biosynthesis protein NifE	K. pneumoniae	
515	SLFT**WGG**ICICHIR	460-473	General amino acid permease	S. cerevisiae	
564	NVIG**FGG**RIIGELL	102-115	Galactose-6-phosphate isomerase LacB subunit	S. aureus	
1122	LVFD**FGG**KTLVSLA	7-20	DNA polymerase α-binding protein	S. cerevisiae	+/(+)
455	GRWC**FGG**KSIKTPT	374-387	Fasciclin III	D. menalogaster	
557	QDGY**FGG**ARIQTGK	590-603	Phosphorylase B kinase alpha regulatory chain, skeletal muscle	O. cuniculus	n.d./+++
807	EQLR**FGG**KTITVQD	45-58	Usg-1 protein	E. coli	
308	SRAY**FGG**VSILIGL	303-316	Cytochome C oxidase polypeptide I	L. tarentolae	
1085	ALKP**FGG**IRIEDNV	413-426	X-Pro dipeptidase	E. coli	
800	LGPQ**FDG**ATIIRNT	1915-1928	N-end-recognizing protein	S. cerevisiae	
1066	LCSN**FGG**KILVPRE	239-252	Hypoth. 39.3 kD protein in gcn4-wbp1 intergen. reg.	S. cerevisiae	
644-45	RRLD**FGG**CRLSLAT	97-110	ATP phosphoribosyltransferase	S. typhimurium, E. coli	
1047	VARW**FGG**AHIGPDR	226-239	Hypoth. 29.0 kD protein in pwp2-sup61 intergen. reg.	S. cerevisiae	
1167	CGRR**FGG**KLLRRFS	62-75	Hypoth. 21.4 kD protein in dld-cdd intergen. reg.	E. coli	
884	LQKR**FGG**KRIQIYL	132-145	Sodium/glucose cotransporter	O. cuniculus	
514	IGCA**FGG**IILSKGG	130-143	Galactose transporter	S. cerevisiae	
190	DTVG**FGG**VSIKNQV	153-166	Candidapepsin 2	C. albicans	+++/+++
187	GELT**FGG**KSIPSHY	203-216	Chaperone protein Caf1M	Y. pestis	
502	RVSD**FGG**RSLSVLS	382-395	Replication protein A 70 kD DNA-binding subunit	H. sapiens	
884	LRKR**FGG**KRIQVYL	75-88	Sodium/glucose cotransporter	S. scrofa	
507	SLVV**FGG**VSINPQM	106-119	Putative ATP-dependent RNA helicase RhlE	E. coli	
6	AMVS**WGG**VSIPNSP	745-758	Endothelial actin-binding protein	H. sapiens	
584	CPAL**FDG**ARLRCCA	452-465	Surface membrane glycoprotein GP46/M-2	L. amazonensis	
1108	YVVI**FGG**RSLVKSC	73-86	Hypoth. 36.2 kD protein in prp21-cdc6 intergen. reg.	S. cerevisiae	
405	RMAC**WGG**QTIGVNQ	274-287	DNA polymerase	DHBV (S31)	
527	TKFS**FGG**KNIHSCL	228-241	Ribonucleoside-diphosphate reductase large chain	HVS	
769	TSSR**FGG**VVIGSNG	53-66	Phosphatidylinositol 3-kinase TOR1	S. cerevisiae	
696	IGCA**FGG**LTLGRLG	115-128	High-affinity glucose transporter HXT2	S. cerevisiae	
559	YGTC**FGG**SKLEDMS	975-988	DNA-directed RNA polymerase III130 kD polypeptide	S. cerevisiae	
194	DTVG**FGG**ISIKNQQ	157-170	Candidapepsin	C. tropicalis	+++/+++
934	RIPL**WGG**VLITIVD	164-177	Natural resistance-acciociated macrophage protein	M. musculus	
154	AHSP**FGG**VSITVEL	443-456	Sensor protein BaeS	E. coli	
1016	EENG**FGG**VCIRTAG	101-114	Hypoth. 20.6 kD early proetin	Ad7	
505	RVTD**FGG**KSLSMGF	381-394	Replication factor-A protein 1	S. cerevisiae	
848	VYED**WGG**IWIGRLG	309-322	Methane monooxygenase comp. A alpha chain	M. capsulatus	(+)/(+)
1132	QSCA**FGG**PKLLNIS	784-797	Putative 128.2 kD transcriptional regulatory protein in ptm1-ixr1	S. cerevisiae	

The sequences of the 50 strongest CB4-1 binding peptides of each supertope analysis (numbers (#) refer to the spots shown in Figure 2) and their location within the corresponding proteins are listed in the order of binding intensity. The bold letters correspond to the identified key positions for CB4-1 binding. Binding of obtained proteins (greyed) to CB4-1 was investigated by coating the proteins on microtiter plates (100 µg/ml) without (symbol before the oblique) and after treatment with 1 % SDS/0.1 % mercaptoethanol (30 min, 95 °C) (symbol behind the oblique) and subsequent incubation with peroxidase-labelled CB4-1 (2 µg/ml). After 2.5 hours, the bound enzymatic activity was determined using *o*-phenylenediamine as chromogenic substrate. The optical density (OD) was measured at 492 nm and 620 nm using an ELISA-reader (Anthos, Köln, Germany). Symbols indicate: +++ OD > 2.0; ++ OD > 1.0; + OD > 0.5; (+) OD > 0.25; - OD < 0.25. Several control proteins (bovine serum albumin, bovine pancreas chymotrypsin and hen egg albumin) did not bind (OD < 0.15, not shown). n.d. = not determined (native protein was not available).

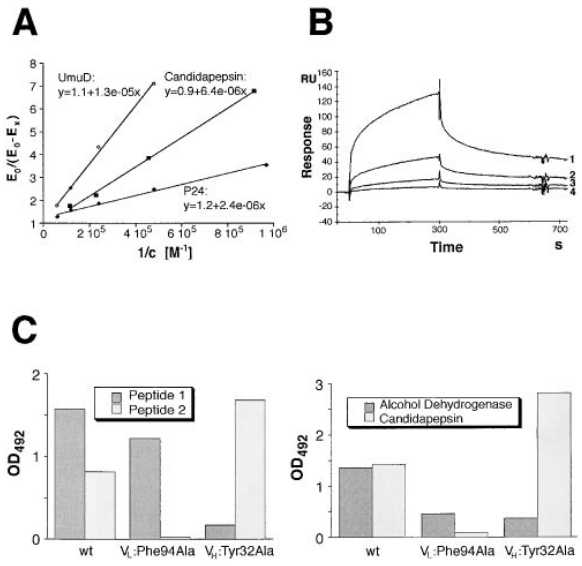

Figure 3. Dissociation Constants of CB4-1 Interacting with UmuD and Candiapepsin and Influences of CB4-1 Mutations on Peptide and Protein Recognition

(A) Inhibition of CB4-1-p24 interaction by p24, UmuD, and candidapepsin determined by competitive ELISA. Soluble proteins compete with solid phase-adsorbed p24 for CB4-1 binding. 1/c (c = concentration of the inhibitors) is plotted versus $E_0/(E_0-E_x)$ (E = extinction with no inhibitor (0) and various inhibitor concentrations [x]). The slope of the fitted curves correspond to the inhibition constants (Friguet et al., 1985).

(B) BIAcore measurements of UmuD interacting with solid phase-linked CB4-1. Overlay plot of various concentrations of UmuD (50 μM, 12.5 μM, 3.1 μM, and 0.78 μM [sensograms 1–4, respectively]) captured by CB4-1-coated chips. Samples were injected at t = 0 s. Dissociation started at t = 300 s. The association rate constant k_{on} was determined as $3.0 \times 10^3\ M^{-1}s^{-1}$, the dissociation rate constants k_{off} as $6.0 \times 10^3\ s^{-1}$ resulting in $K_d = 2.0 \times 10^{-6}$ M.

(C) CB4-1 single chain mutants V_L:Phe94Ala and V_H:Tyr32Ala as well as the wild-type CB4-1 single chain Fv fragment (wt) were tested for their ability to recognize immobilized peptide 1 (GATPEDLNQKL AGN) and peptide 2 (GLYEWGGARITNTD) as well as alcohol dehydrogenase (supertope 1-derived) and candidapepsin (supertope 2-derived). Binding of the CB4-1 single chains carrying the c-*myc*-tag were detected using the anti-c-*myc* antibody 9E10 and a peroxidase-conjugated anti-mouse antibody.

peptide sequences. Each peptide mixture (spot) theoretically contains 20^{11} single peptides with three defined and 11 undefined positions. Using combinatorial peptide libraries of the type XXB_1B_2XX or $XXXXB_1B_2B_3XXXX$ (Kramer et al., 1994; Schneider-Mergener et al., 1996), none of those peptides would have been isolated. This is explained by the results of the first screening step (Table 1) where those amino acids identified at the defined positions of the library turned out to be key residues in antibody binding (Figure 1). The peptide mixtures XXXXX**LN**XX**L**XXXX, XXXX**W**X**G**XX**I**XXXX, XXXXX**E**X**N**X**I** XXXX, XXXXX**P**X**RW**XXXX, and xxxx**Gp**x**I**xxxxx, which correspond to the best binding mixture for each peptide identified in the first screening step, do not contain their defined residues (bold) in adjacent positions but distributed within a window of up to six amino acids. With phage display techniques it would have been very difficult to identify the different L-peptide motifs displaying a broad range of affinities, because the selection strategy (panning) favors the isolation of molecules with similar binding affinities.

The binding affinities of the identified peptides varied by four orders of magnitude. Nevertheless, all peptides were able to compete with recombinant p24 and the epitope peptide for CB4-1 binding, demonstrating that the binding regions of all peptides are at least overlapping and are located at the wild-type epitope binding site. The increased affinity of peptide 1 compared to the wild-type epitope is due to three functionally important substitutions of residues, which were relatively unimportant in the wild-type epitope (Volkmer-Engert et al., 1994) but have become more crucial in the context of peptide 1 (Figure 1A).

As investigated by Jahn et al. (1995), CB4-1 is not a typical (Guilbert et al., 1982; Baccala et al., 1989) polyreactive antibody, because it does not react with a variety of antigens usually recognized by polyreactive antibodies (see also Results and Table 2). Furthermore, the V genes of the variable regions of CB4-1 do not display homology to known murine germ-line genes greater than 85% for V_H and 95% for V_L, supporting the antigen-driven affinity maturation of this antibody (K. Winkler, unpublished data). However, it cannot be excluded that CB4-1 is encoded by an as-yet-unknown V gene.

The fact that more than 1000 single substitution analogs derived from five different peptides bound to CB4-1 (Figure 1) raised the question whether CB4-1 also exhibits cross-reactivity and polyspecificity for different proteins. The delineation of supertopes from substitutional analysis followed by database searches turned out to be an excellent tool for the rapid identification of proteins that are potentially recognized by the antibody. We were able to identify a huge number of peptide sequences recognized by CB4-1 that are present in completely unrelated proteins of different species. All of the corresponding proteins obtained so far were bound by CB4-1 in a solid-phase ELISA demonstrating that this antibody is able to recognize not only different peptides but also heterologous proteins. Besides the natural antigen p24, enough material could be obtained to determine the dissociation constants for two supertopes containing heterologous proteins UmuD and candidapepsin in solution (Figures 3A and 3B).

In the solid-phase ELISA experiments, absorption to microtiter plates might cause partial unfolding of the proteins, although differences in binding were observed between native and denatured proteins (Table 2). For candidapepsin, UmuD, and the control protein p24, dissociation constants were determined with soluble proteins by competitive ELISA and BIAcore. For p24 and candidapepsin, denaturation of the protein samples can be excluded (see Experimental Procedures and Keitel et al., 1997), whereas unfolding is very unlikely for UmuD, since no difference in binding was observed for native (Figures 3A and 3B) and denatured protein (5×10^{-6} M, data not shown). For an explanation of CB4-1 binding to the broad variety of different structures, it can be assumed that that the antibody causes at least partial unfolding of the interaction partners upon binding. For the biological implications of cross-reactivity and polyspecificity discussed below, however, it is unimportant

whether a protein is bound in its native or partially denatured conformation.

The CB4-1 binding affinities for the nonhomologous proteins were within a biologically significant range (K_d = 10^{-5}–10^{-6} M), since those dissociation constants have been described for primary immune responses (Sharon, 1990; Cumano and Rajewsky, 1986). With respect to the induction of autoimmune deseases, it is important to notice that the available pool of B cells, which is capable to interact with antigen, also includes affinity-maturated memory cells. Thus, these B cells are able to reenter the cycle of hypermutation and selection in response to a new antigenic challenge (Berek and Milstein, 1988). Few mutations can dramatically alter affinity and specificity of antibodies. In our case, a single mutation in the CB4-1 heavy chain (tyrosine 32 to alanine) considerably increases the affinity to candidapepsin (Figure 3C), which in principle demonstrates the ability of CB4-1 to affinity-maturate in response to a novel antigen.

For the supertope delineation, all possible amino acids at the key positions, defined as those tolerating less than four substitutions, were included, whereas X was assigned to all other positions. As an example, 1219 matches were detected for supertope 2 XXXX[FW][DG]G XX[IL]XXXX, which was derived from the results of substitutional analysis of peptide 2 GLYEWGGARITNTD (Figure 1B). An alternative, more constrained supertope might take into account only the strongly binding substitution analogs ending up with, for example, X[ADEFGHI KLMNPQSTVWY]X[ADEGHMNQST][FW][DG]G[AIQ RS] [AEFIKLMNQRSVY][IL]XXXX as peptide 2–derived supertope. However, a database search using this supertope resulted in merely 126 sequences, only 10 of which were among the 50 best binding peptides listed in Table 2, indicating that simultaneous substitutions of several amino acids in peptides can alter their binding behavior in an unpredictable manner. Therefore, it seems advisable to consider only the key peptide positions for supertope delineation, allowing the identification of as broad a spectrum of putative cross-reacting peptides as possible. At the same time, multiple substitutions might also cause complete loss of binding, which probably explains our results of isolating nonbinding supertope-derived sequences.

The substitutional analyses for each of the five identified peptides revealed a unique key residue pattern indicating five different binding modes to the same protein surface. Three of these binding modes were further elucidated by X-ray crystallographic studies of peptides 1 and 2 as well as the D-peptide in complex with the antibody (Keitel et al., 1997; Figure 4). These peptides do not share any sequence homology but do bind to the same region between light and heavy chain of CB4-1. They use overlapping binding sites but adopt different backbone conformations (Figure 4). Furthermore, the most critical contacts were made by different antibody residues, although the antibody residues participating in overall binding are usually the same (Keitel et al., 1997). Different binding modes of phenylalanine 94 of the light chain and tyrosine 32 of the heavy chain may be deduced from the crystal structures but were also confirmed using CB4-1 single chain Fv mutants (Figure 4). Although the backbone conformation of the peptides

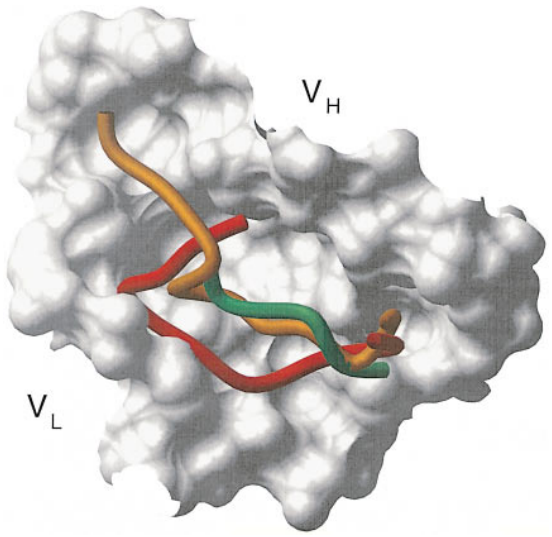

Figure 4. Conformations and Relative Orientations of Library-Derived Peptides 1, 2, and 5 in Complex with CB4-1

An overall view of the peptides GATPEDLNQKL (orange), GLYEWGG ARIT (red), and the D-polymer IkGpl (green) binding to the cleft formed by the six complementarity determing loops of the monoclonal antibody CB4-1 (V_H = heavy chain, V_L = light chain; the left side of the peptides represent the N termini) is shown using the program ICM (Cardozo et al., 1995). In comparison to sequences given in Table 1, peptides 1, 2, and 5 are C- and/or N-terminally deleted, since no electron density could be seen for these residues (Keitel et al., 1997).

differed significantly, only marginal changes in the hypervariable loops of CB4-1 were observed, in comparison to the free Fab fragment as expected for peptide antigens (Wilson and Stanfield, 1994). Indications for the recognition of different conformational elements at the protein level can also be deduced from binding to UmuD of E.coli (supertope 1-derived) as well as to candidapepsin of C.albicans (supertope 2-derived). The conformation of the interacting region of UmuD is helical (Peat et al., 1996) and differs significantly from that of candidapepsin-homologous human pepsin 3a (the most homologous protein with known three-dimensional structure), which forms a turn structure in the corresponding region.

From these results it became obvious that the term molecular mimicry would not be an applicable description of the binding promiscuity of CB4-1, since the peptides do not mimic each other with respect to sequence, conformation, and binding mode. To clarify the confusing terminology in this area of research (see Introduction) we suggest discriminating between the terms cross-reactivity and polyspecificity in the following operational way: cross-reactivity should be used for interactions based on wild-type-derived key residues, whereas polyspecificity should be applied to unrelated specificities, which means interactions caused by different binding modes. Using this definition, CB4-1 cross-reacts with peptide 1 and supertope 1-derived peptides and proteins but is polyspecific toward peptides 2–5 as well as supertope 2-derived peptides and proteins.

Due to the increasing number of studies that report

the identification of unrelated sequences via peptide library screening, it seems likely that structurally different binding motifs can be obtained for any monoclonal antibody. This could be important for the elucidation of the molecular basis of autoimmune diseases, since the search for autoantigens that are recognized by pathogen-triggered antibodies or T cells is usually restricted to sequence similarity searches. In the case of CB4-1 binding protein regions, only three out of the hundred sequences would have been predicted using alignment methods. The detection of structurally different binding motifs using peptide libraries combined with supertope analyses would help to identify new classes of putative autoantigens. Moreover, as a therapeutical approach, autoreactive B cells or T cells could be exposed recurrently to identified binding peptides (especially D peptides, which are much more stable toward proteolytic digestion), which might lead the cells to an anergic state (Cornall et al., 1995).

The hypothesis of a general polyspecific and cross-reactive potential of antibodies and T cells might further support speculations about possible immune escape mechanisms of foreign pathogens (Rossler et al., 1995). An effective immune response to certain epitopes of these pathogens might fail due to autoantigen-triggered elimination or inactivation of the corresponding cross-reactive or polyspecific B cells or T cells. Here, however, there is no evidence that HIV-1 has adopted such an immune escape mechanism, since a statistical overrepresentation of the identified supertopes in the SWISS PROT database was not observed.

Experimental Procedures

Peptide Synthesis

Cellulose-bound peptides and peptide mixtures were prepared by automated spot synthesis (Abimed, Langenfeld, Germany; Software DIGEN, Jerini BioTools GmbH, Berlin, Germany) using Whatman 50 (Whatman, Maidstone, United Kingdom) cellulose membranes (Frank, 1992), as described before in detail (Kramer et al., 1994; Kramer and Schneider-Mergener, 1997). All peptides were N-terminally acetylated.

The peptides for the solution phase binding experiments (ELISA and fluorescence energy transfer) and for cocrystallization with CB4-1 were prepared according to standard Fmoc machine protocols using a multiple peptide synthesizer (Abimed, Langenfeld, Germany) and analyzed by reversed-phase high-pressure liquid chromatography and MALDI-TOF mass spectrometry. N-terminal acetylation was carried out using acetanhydride and diisopropylethylamine; anthranilic acid coupling using Fmoc-anthranilic acid (Neosystem, Frankfurt, Germany) activated with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetra-fluorobo rate ([TBTU], Novabiochem, Bad Soden, Germany) and diisopropylethylamine; biotinylation using biotin (Fluka, Neu-Ulm, Germany) activated by benzotriazole-1-yl-oxy-tripyrrolidinophosphoniumhexa-fluorophosphate (PyBOP, Novabiochem) and *N*-methylmorpholine. The N-terminally biotinylated peptides contained two β-alanine residues as spacer between biotin and the peptide.

Antibody Binding Studies of Cellulose-Bound Peptides and Peptide Mixtures

The membane-bound libraries were blocked overnight with blocking buffer, i.e., blocking reagent (CRB, Northwich, UK) in Tris-buffered saline/0.05% Tween-20 (T-TBS) containing 1% sucrose. After washing with T-TBS, 1 μg/ml CB4-1 in blocking buffer was added and incubated for 3 hr at room temperature. For the substitutional analyses as well as the peptides derived from the database analyses, a CB4-1 concentration of 0.1 μg/ml was applied. After washing with T-TBS three times, a peroxidase-labeled anti-mouse antibody (Sigma, Munich, Germany; 1 μg/ml in blocking buffer) was applied for 2 hr at room temperature. For detection, a chemiluminescence system (Boehringer Mannheim GmbH, Mannheim, Germany) was applied using standard X-ray films.

Competitive ELISA Studies

N-terminally acetylated peptides 1–7 (Table 1) as well as the UmuD protein of E.coli (generous gift from A. Guzzo), candidapepsin of C.albicans (generous gift from P. Sullivan), and the natural antigen p24 were tested for their ability to compete with the CB4-1-p24 interaction in solution. Tryptophan fluorescence spectroscopy did not reveal any detectable denatured protein in the candidapepsin (data not shown) and p24 samples used (Hausdorf et al., 1994; Ehrhard et al., 1996). Since UmuD does not contain tryptophan residues, the protein could not be analysed by this method. Due to lack of material, CD spectroscopy could not be applied for UmuD. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg/ml recombinant p24 (Hausdorf et al., 1994) in 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 20 hr at 4°C. After washing three times with phosphate-buffered saline (PBS)/0.1% Tween-20, 0.1 μg/ml horseradish peroxidase-labeled CB4-1 was added with peptides or proteins in various concentrations (depending on the respective inhibition constants) in PBS/0.1% Tween-20 containing 6% Gelifundol S (Biotest, Dreieich, Germany) in a total volume of 50 μl for 20 hr at 4°C. After washing three times with PBS/0.1% Tween-20, the bound enzymatic activity was determined by adding 5.5 mM *o*-phenylenediamine hydrochloride (Fluka, Buchs, Switzerland) and 8.5 mM H_2O_2 in 0.1 M citrate buffer (pH 5.0). The reaction was terminated after 10 min by adding 1 M sulfuric acid containing 0.05 M sodium sulfite. The absorbance was measured at 492 nm and 620 nm, as reference, using an ELISA reader (Anthos, Köln, Germany). Inhibition constants were calculated according to Friguet et al. (1985).

Fluorescence Energy Transfer Experiments

The energy transfer (Förster, 1946) from tryptophan residues of the CB4-1 Fab fragment-binding region to anthranilic acid-labeled binding peptides was determined (excitation: 280 nm, detection: 426 nm) using a fluorescence spectrometer (Perkin-ELMER, Buckinghamshire, United Kingdom). The concentration of the CB4-1 Fab fragment was in the range of the dissociation constant of the peptide-Fab interaction. Increasing amounts of peptide were added to CB4-1 Fab fragment in 20 mM Tris/HCl (pH 7.5) and the energy transfer determined using peptide alone as reference. The slope of B/p as a linear function of B/(F × p) corresponds to the dissociation constant K_d of peptide-CB4-1 Fab complex (B = concentration of bound peptide, F = concentration of free peptide, p = protein concentration). The values for the binding site overlap and correspond to ($K_{D(p6)}$: $K_{D(p1-7)}$): ([p6]: [p1–7]) (p1–7 = peptides 1–7; [p] = concentration of peptides) at 50% binding competition of bound peptide 6 (anthranilic acid-labeled) with acetylated peptides 1–7.

CB4-1 Single Chain Fv Mutants

For the expression of the CB 4-1 Fv-fragment in E.coli, the variable regions were assembled by a flexible linker fragment to form a single chain Fv-fragment (V_H-$(Gly_4Ser)_3$-V_L) by polymerase chain reaction (Huston et al., 1988; Orlandi et al., 1989). After a second polymerase chain reaction, which introduced a *SfiI*-site at the 5′-end and a NotI-site at the 3′-end, the scFv construct was ligated into the SfiI-NotI cleaved phagemid pHEN1 including the *myc*-tag for detection and purification purposes (Hoogenboom et al., 1991). The resulting vector *pHEN 4-1* was used for the expression of soluble scFv into the periplasm of E. coli using the PelB signal peptide. For detection and purification, the antibody 9E10 (Boehringer, Mannheim, Germany) recognizing the *myc*-tag was applied. The site-directed mutagenesis was performed using two primers for introducing the mutation and eliminating a unique selection site in the vector, as described by Deng and Nickoloff (1992). The desired mutations were confirmed by sequence analysis. ScFvs were produced in E. coli strain W3110 in 2× TY-Medium containing 1% glucose and 100 μg/ml ampicillin.

Overnight cultures were induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside for 20 hr at 25°C. After cell harvesting and preparation of periplasm by osmotic shock (30 min at 0°C in 200 mM $NaBO_3$, [pH 8.0], 160 mM NaCl, 10 mM EDTA), the scFv4-1 fragments were present in both the soluble and the insoluble fraction of the supernatant and periplasm. For purification, biotinylated anti-c-*myc*-tag antibody 9E10 was immobilized to streptavidin-sepharose (Sigma, Munich, Germany). After filtration through a 0.2 μm membrane, the soluble fractions of periplasm and culture supernatant were applied directly to the 9E10 column equilibrated with 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl. The column was first washed with the same buffer followed by a second wash with 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA. Homogeneous scFv fractions were eluted with 0.2 M Glycine (pH 2.0), 0.2 M NaCl and immediately neutralized. After dialyzation against PBS-buffer, scFv proteins were concentrated to 0.1–0.3 mg/ml by ultrafiltration using Centricon 10 concentrators (Amicon, Berverly, MA). All scFv fragments were characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and ELISA. The purified wild-type scFv fragment occurs mainly in its dimeric form. Compared to the parental antibody, it has the same binding affinity to p24 as determined by ELISA and fluorescence quenching measurements (K. W., unpublished data).

Binding Studies with CB4−1 Single Chain Fv Mutants

Candidapepsin (generous gift from M. Monod; 10 μg/ml) and alcohol dehydrogenase (generous gift from D. Keßler; 100 μg/ml) were coated on microtiter plates in 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 20 hr at 4°C. After washing three times with PBS/0.1% Tween-20, mutants V_L:Phe94LAla, V_H:Tyr32HAla, and CB4-1 wild-type single chain Fv fragment (10 μg/ml each) were added in PBS/0.1% Tween-20 containing 6% Gelifundol S (Biotest, Dreieich, Germany) in a total volume of 50 μl for 2.5 hr at room temperature. After three washing steps, 10 μg/ml murine anti-c-*myc* antibody 9E10 was added and incubated for 1 hr at room temperature. After washing three times, 1 μg/ml horseradish peroxidase–labeled anti-mouse IgG (Sigma, Munich, Germany) is incubated for 1 hr at room temperature. Binding was visualized as described for the competitive ELISA. N-terminally biotinylated peptides (1 μg/ml in aqua dest.) were immobilized on streptavidin-coated (20 μg/ml) and blocked (1% bovine serum albumin for 1 hr) microtiter plates for 2 hr. The single chain antibodies (1 μg/ml) were incubated for 2.5 hr at room temperature. The anti-c-*myc* antibody 9E10 and the peroxidase-labeled anti-mouse IgG were added at concentrations of 1 μg/ml for 1 hr each. Binding was visualized as described above.

Affinity Measurements with BIACORE[TM]

Equipment and reagents: BIACORE 2000 system, sensor chips CM5, buffer HBS (10 mM Hepes with 0.15 M NaCl, 3.4 mM EDTA, and 0.005 % surfactant P20 [pH 7.4]), amine coupling kit, and BIA evaluation software were obtained from Biacore AB (Uppsala, Sweden). Sodium (meta-) periodate, sodium cyano borohydride, and carbohydrazide were purchased from Fluka (Deisenhofen, Germany).

Immobilization

The monoclonal antibody CB4-1 was immobilized on CM5 chips using the aldehyde coupling procedure described in the BIAapplication handbook (O'Shannessy and Wilchek, 1990). This method proved to be superior to the amine coupling procedure (described in the same manual) that is commonly used for the immobilization of proteins. A gradient surface was prepared in order to improve the monitoring of low affinity interactions by multispot sensing (Karlsson and Ståhlberg, 1995). The amount of immobilized CB4-1 corresponded to an increase of the SPR signal of 0, 1200, 2800, and 3800 resonance units (RU) for flowcell 1, 2, 3, and 4, respectively. Appropriate amounts of nonspecific monoclonal control antibody Tab2 were injected after the CB4-1 immobilization to give a total amount of covalently coupled protein corresponding to approximately 4000 RU in all four flowcells.

Binding Experiments

All binding experiments were performed at 25°C with a flow rate of 10 μl/min; UmuD, bovine serum albumin (negative control), and p24 (positive control) were used at concentrations of 0.78 μM, 3.1 μM, 12.5 μM, and 50 μM. The injection volume was 50 μl. Various HCl solutions were tested for complete regeneration of the sensor chip. Complete regeneration was obtained by injection of 10 μl 14 mM HCl (pH 1.85). After 100 cycles of injection/regeneration at this pH, the p24 binding capacity of the chip was reduced by about 50%.

Data Analysis

Transformation of data and kinetic analysis was performed with BIA evaluation software. The control sensorgram (flowcell 1) was subtracted from the sensorgrams obtained with flowcells 2 to 4. The association rate constant k_{on} was calculated by fitting the association part of the corrected curves; the dissociation rate constant k_{off}, by fitting the dissociation part for the highest protein concentration.

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