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*J Immunol* 2000; 165:4505-4514; doi: 10.4049/jimmunol.165.8.4505
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Changing the Antigen Binding Specificity by Single Point Mutations of an Anti-p24 (HIV-1) Antibody

Karsten Winkler,* Achim Kramer,† Gabriele Küttner,* Martina Seifert,† Christa Scholz,*, Helga Wessner,* Jens Schneider-Mergener,† and Wolfgang Höhne**

The murine mAb CB4-1 raised against p24 (HIV-1) recognizes a linear epitope of the HIV-1 capsid protein. Additionally, CB4-1 exhibits cross-reactive binding to epitope-homologous peptides and polyspecific reactions to epitope nonhomologous peptides. Crystal structures demonstrate that the epitope peptide (e-pep) and the nonhomologous peptides adopt different conformations within the binding region of CB4-1. Site-directed mutagenesis of the fragment variable (Fv) region was performed using a single-chain (sc)Fv construct of CB4-1 to analyze binding contributions of single amino acid side chains toward the e-pep and toward one epitope nonhomologous peptide. The mutations of Ab amino acid side chains, which are in direct contact with the Ag, show opposite influences on the binding of the two peptides. Whereas the affinity of the e-pep to the CB4-1 scFv mutant heavy chain variable region Tyr^{32} Ala is decreased 250-fold, the binding of the nonhomologous peptide remains unchanged. In contrast, the mutation light chain variable region Phe^{84} Ala reduces the affinity of the nonhomologous peptide 10-fold more than it does for the e-pep. Thus, substantial changes in the specificity can be observed by single amino acid exchanges. Further characterization of the scFv mutants by substitutional analysis of the peptides demonstrates that the effect of a mutation is not restricted to contact residues. This method also reveals an inverse compensatory amino acid exchange for the nonhomologous peptide which increases the affinity to the scFv mutant light chain variable region Phe^{84} Ala up to the level of the e-pep affinity to the wild-type scFv. The Journal of Immunology, 2000, 165: 4505–4514.

Antibodies are developed during evolution as a sophisticated system for specific Ag recognition and are generally thought to make highly specific Ab-Ag interactions. In contrast, polyreactivity is a known feature of natural autoantibodies (NAA) found in sera of healthy humans and rodents as well as in lower phylogenetic species (1, 2). Affinity maturation of Abs can be considered a fast mini-evolution of a specific binding behavior and is well understood from the immunological point of view (3). Nevertheless, the structural features underlying the differences between specificity and polyreactivity are poorly understood at the molecular level (4, 5).

Ag binding of Igs is mediated by atomic interactions within complementary surfaces between Ab (paratope) and Ag (epitope). This high complementarity of the Ag combining site of an Ab is accomplished by residues from six hypervariable loops of complementarity-determining regions (CDRs), contributed by the heavy (\(V_H\)) and light chain (\(V_L\)) variable domains (6). Comparative structural analysis of the main-chain conformation of the CDRs showed that five of them (L1, L2, L3, H1, and H2) preferentially adopt distinct backbone conformations, also termed “canonical structures” depending on the length of each loop and the nature of a few key residues (7, 8). In the Ag binding region the major determinants of specificity and affinity for an Ag are as follows: 1) the canonical structures of the CDRs; 2) the size, shape, and chemical features of their surface exposed residues; 3) their position relative to each other; and 4) the length and conformation of the H3-loop (9–11).

Inspecting crystal structures of Ab-Ag complexes reveals usually 15–20 directed interactions between the individual residues in the contact interface, which bury a surface between 160 and 900 Å² (11). Nevertheless, the contribution of single amino acid positions to overall affinity usually becomes inappropriate in crystal structures. Only the substitution of residues within the paratope and epitope together with detailed binding studies allows the identification of the binding contribution of individual amino acid positions. Several mutational analyses of Ab binding regions were performed during the last years (12–18). They elucidate that Abs can use different strategies to accomplish high affinity and specificity, enthalpy mediated on the one hand or entropy mediated on the other hand (18–21). Furthermore, it became evident that four to six amino acids make the largest contribution to the free energy of binding (6, 18).

Despite the high affinity and selectivity typical for the interaction of Abs with their Ag, the phenomena of cross-reactivity (recognition of homologous structures) and polyspecificity (binding of epitope-unrelated structures) are observed quite frequently (5, 13, 22–27).

We used the well-characterized murine mAb CB4-1 for investigations on the structure-function relationship of Ab-Ag interaction. The subtype of this mAb was immunochemically identified as IgG2a/k. The mAb CB4-1 recognizes the peptide epitope GAT PQDLNTML corresponding to the aa 46–56 of the HIV-1 capsid protein p24 (28, 29) and was used to analyze multiple binding capabilities of an affinity-matured mAb by means of synthetic combinatorial peptide libraries (24) and crystal structure analysis.
(30). From cellulose-bound positional-scanning combinatorial libraries, five peptides were selected that are able to compete with the natural epitope peptide (e-pep) for binding to CB4-1 (24, 31). One of these library-derived peptides has a sequence related (homologous) to the epitope; whereas the sequences of the other peptides are completely unrelated (nonhomologous). It was demonstrated that even an Ab with high affinity toward its epitope is able to bind completely different peptides by interaction with unequal sets of “key residues” with comparable affinities (22–24). The term key residues defines those positions in the peptide which cannot be substituted (or can only be exchanged by physicochemically related amino acids) without substantial loss of peptide binding. It was suggested to discriminate between the term cross-reactivity, which means that the binding of homologous molecules is based on the same key residues, and the term polyspecificity, which is characterized by specific interaction with nonhomologous molecules mediated by different sets of key residues (24). The CB4-1 Fab was crystallized without and in complex with four different peptides, and x-ray structural analysis was performed to a final resolution of 2.6 Å (30). The crystal structures demonstrate that unrelated peptides adopt different conformations within the Ab-Ag complex and also form their critical contacts with different Ab side chains. Only small movements are observed in the framework of the Fab upon binding.

Here we investigate the binding contribution of two contact residues and one noncontact residue of the CB4-1 binding region and their individual influences on the specificity of peptide recognition for an epitope-related and an unrelated (nonhomologous) peptide. The variable region of CB4-1 was cloned into a vector which permits periplasmic expression of the CB4-1 scFv in *Escherichia coli*. Three single amino acid exchanges were introduced in this scFv by site-directed mutagenesis. The binding behavior of the expressed and purified scFvs was compared with the unmutated scFv using competition ELISA and substitutional analysis for the two structurally unrelated peptides. The latter method also allowed the detection of an inverse compensatory amino acid substitution in the epitope nonhomologous peptide, which was additionally characterized by competition ELISA. The structural and immunological impact of the results is discussed.

**Materials and Methods**

**Reagents**

All chemicals were of analytical grade. Restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany). Oligonucleotides were obtained from TIB-MOLBIOL (Berlin, Germany). Peptides were synthesized according to standard Fmoc protocols using a multiple peptide synthesizer (Abimed Analyse-Technik, Langenfeld, Germany) and analysed by reverse-phase HPLC and matrix-assisted laser desorption ionization-time of flight mass spectrometry. In the e-pep, norleucine (= n) was introduced instead of methionine to prevent oxidation during peptide storage and handling in solution. It was shown earlier that this exchange has no influence on the peptide affinity to CB4-1 (29).

**Amplification, cloning, and sequencing the variable region of CB4-1**

The fusion of cells and the hybridoma selection were conducted as described earlier (28). The preparation of mRNA from the hybridoma cell line CB4-1/F7 and the reverse transcription of cDNA was performed as described (32).

According to Jones and Bendig (33), PCR reactions were set up using the mouse heavy chain variable region leader sequence primers and the Cγ constant region primer (Cy15 = 5'-GCCGACCTGGATAGAC; Pharmacia-LKB, Uppsala, Sweden) for the heavy chain amplification. PCR reactions were performed with mouse κ light chain variable region primers and the Cκ constant region primer (Ck17 = 5'-TGGATGTGGGAGAAGT; Pharmacia-LKB) for the light chain amplification. Amplifications were conducted as described (32). The PCR fragments were purified on a 1% agarose gel, extracted by Qiagen (Qiagen, Chatsworth, CA) and cloned into the PCR II vector according to the TA cloning system protocol (Invitrogen, San Diego, CA). The nucleotide sequences are stored at the European Molecular Biology Laboratory Nucleotide Sequence data base under the accession numbers Z50145 for the CB4-1 Vκ region and Z50146 for the CB4-1 Vγ region.

**Cloning and expression of the CB4-1 scFv in *E. coli***

For the expression of the CB4-1 Fv in *E. coli*, the variable regions were assembled by an oligonucleotide coding for a flexible linker fragment to form a scFv (Vγ(c-Gly-Ser)-Ala-Vγ) by PCR as described (34). After a second PCR, which introduced a Sfi site at the 5′ end and a NotI site at the 3′ end, the scFv construct was ligated into the Sfi-NotI-cleaved phagemid pHEN1, including the myc tag for detection and purification purposes (35). 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 Ab 9E10 (Boehringer Mannheim, Mannheim, Germany) recognizing the myc tag was applied.

Examination of expression conditions has shown in our case that expression levels mainly depend on low isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations and the choice of the *E. coli* strain. The most suitable *E. coli* strains were W3110, TGI, JM109, and XL1 (in the order of the expression level).

Production of scFvs was performed using the pHEN-4-1 vector in the *E. coli* strain W3110 in 1 L FM medium (20 g yeast extract, 8 g casamino acids, 1.55 g MgSO4, 1 g sodium citrate, 0.2 g CaCl2, 0.5 ml trace elements solution (36), 3 g NaH2PO4, 6 g KH2PO4) containing 1% glucose and 100 g/ml ampicillin. Cells from overnight culture were collected by centrifugation (15 min, 5000 rpm, 25°C; Kontron, Zürich, Switzerland) and resuspended in the 4-fold volume of the overnight culture in FM medium + 100 µg/ml ampicillin without glucose. The culture was induced with 0.05 mM IPTG for 20 h at 25°C. After cell harvesting by centrifugation and preparation of periplasm by osmotic shock (30 min at 0°C in 200 mM sodium borate (pH 8.0), 160 mM NaCl, 10 mM EDTA), the scFv was present in both the soluble and the insoluble fraction.

**Purification**

For Ag affinity chromatography, a modified e-pep (affi-pep = GPGGGAT PQDLNTrc; n = norleucine) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotec, Uppsala, Sweden). For Ag-independent affinity chromatography, biotinylated anti-myc tag mAb 9E10 was immobilized to streptavidin-Sepharose (Sigma, München, Germany). After filtration through a 0.2-µm membrane filter, the soluble fractions of periplasm and culture supernatant (the latter being concentrated 1:10 with a 10-kDa membrane) were directly applied to a 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, and 1 mM EDTA, followed by a third wash step with 0.2 M glycine (pH 5.0) and 0.2 M NaCl. Homogeneous scFv fractions were eluted with 0.2 M glycine (pH 2.0) and 0.2 M NaCl and immediately neutralized with 2 M Tris-HCl, pH 9.0. After dialysis against PBS-buffer, scFv proteins were concentrated to 0.1–0.3 mg/ml by ultrafiltration using Centricron 10 concentrators (Amicon, Beverly, MA). All scFvs were characterized by SDS-PAGE, Western blot, and ELISA. In the Western blot analysis, a mixture of 2 µg/ml anti-myc tag mAb 9E10 (37) and 1:500 v/v HRP-labeled anti-mouse Ab (Amersham, Braunschweig, Germany) was used for specific detection of the CB4-1 scFvs.

The eluted scFvs were at least 95% pure as judged by SDS-PAGE and were used without further purification.

**Site-directed mutagenesis**

The site-directed mutagenesis was performed by the method of Deng and Nickoloff (38), which uses two primers, the first one introducing the mutation and the second one eliminating a unique selection site in the vector. The following mutation primers were used: Vγ:Y32A, 5'-Phos-GACTTGGTTGACGCGTCACCAGTCACAG. The resulting mutants were selected by restriction site analysis, ELISA screening, and Western blot analysis. Finally, the desired exchanges were confirmed by control sequencing.

**Determination of binding constants: Fab competition ELISA**

In the Fab competition ELISA, the HRP-labeled CB4-1 Fab competes with the Ab probe (Ab, Fab, and scFv) for binding to the native Ag p24 (HV1-1).
immobilized to the solid phase. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg/ml rp24 (39) in 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 20 h at 4°C. After washing three times with PBS/0.1% Tween 20, 0.1 μg/ml HRP-labeled CB4-1 Fab was added with unlabelled mAb, Fab, or scFv 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 h 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 H₂O₂ 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 as reference at 620 nm, using an ELISA reader (Anthos, Köln, Germany). Affinity constants were calculated according to Friguet et al. (40).

**Determination of binding constants: peptide competition ELISA**

For the peptide competition ELISA two kinds of solid-phase ligands were used to capture free Ab, Fab, or scFv. The first capture molecule was rp24, immobilized as described above. A second kind of capture molecule was N-terminally biotinylated peptides immobilized via streptavidin. Microtiter plates (Nunc) were coated with 5–0.5 μg/ml streptavidin (Sigma) in 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 20 h at 4°C. After washing three times with PBS/0.1% Tween 20, 10 μg/ml biotinylated peptides were added in PBS/0.1% Tween 20 containing 6% Gelifundol S (Biotest) and incubated for 2 h at 25°C. After an additional three washes with PBS/0.1% Tween 20 decreasing amounts of peptides were mixed in a total volume of 50 μl with constant concentrations of the Ab, Fab, or scFv probe and incubated for 20 h at 4°C. CB4-1 mAb and Fab were detected with HRP-labeled anti-mouse Ab (1:500 v/v; Amersham) and scFv-fragments were detected with a mixture of 1 mg/ml anti-myc tag mAb 9E10 (37) and 1:2000 v/v HRP-labeled anti-mouse Ab. After washing three times with PBS/0.1% Tween 20, the detection of bound enzymatic activity and calculation of affinity constants was performed as described above. Optimal concentrations for streptavidin coating of microtiter plates and for binding were selected by cross titration in direct binding assays.

**Substitutional analysis of peptides**

The peptides were synthesized on a β-Ala-β-Ala matrix bound to cellulose sheets at a spot according to Frank and Overwin (41). Each single position of the epitope was substituted by all other 19 amino acids resulting in the analysis of 209 epitope mutants.

The membrane-bound libraries were blocked overnight with blocking buffer (i.e., blocking reagent; Cambridge Research Biomedicals, Northwich, U.K.) in TBST containing 1% sucrose. After washing with 1 μg/ml CB4-1 in blocking buffer was added and incubated for 3 h at room temperature. For the substitutional analyses, a concentration of 0.1 μg/ml scFv was applied. After three times washing with TBST, the anti-myc tag mAb 9E10 and a peroxidase-labeled anti-mouse Ab (Sigma; both Abs 1 μg/ml in blocking buffer) were applied for 2 h at room temperature. For detection, a chemiluminescence system (Boehringer Mannheim) was applied using standard x-ray films. The relative spot intensities correlate with the binding affinities (42).

**Structural modeling of the mutations**

The modeling of the amino acid substitutions in the CB4-1 binding site was performed on the basis of the corresponding CB4-1 Fab/peptide x-ray structures (30). After the exchange of the mutated site chains using the modeling software Quanta (Micron Separations, San Diego, CA), a local energy minimization was performed with the CHARMM force field (Quanta) (43).

**Results**

**Cloning, expression, and characterization of the scFv in E. coli**

For a mutational analysis of the Ab binding region consisting of V₃H and V₃L, we cloned the corresponding scFv constructs with a (Gly⁴Ser)₃ Ala linker into the pHEN I vector, which includes the pelB signal peptide for periplasmic expression in E. coli and the myc tag as carboxy-terminal fusion peptide for affinity purification of the expression products (Fig. 1). The best yield was achieved with the E. coli strain W3110, cultured in a fermentation medium

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**FIGURE 1.** Amino acid sequence of the complete scFv CB4-1 with signal peptide and affinity tag. Numbering and CDR assignment according to Kabat et al. (44). →ϕ. The cleavage site of the signal peptidase. Residues deviating from the original Ab sequence due to primer requirements are underlined. The three positions where mutations to Ala were introduced are boxed.
and induced with very low amounts of the inducer (0.05 mM IPTG). Following the optimized expression conditions described in Materials and Methods, usually 1–3 mg soluble CB4-1 scFv per liter cell culture were obtained.

Affinity chromatography was applied for purification of recombinant protein from soluble fractions of periplasm and culture supernatant using at the solid phase either a modified e-pep (affi-pep, for wild-type (wt) scFv) or the anti-myc tag mAb 9E10 (for mutant scFvs). Generally, fluorescence emission spectra of the mutants were inspected to assure that also the corresponding scFvs with low or lacking affinity are folded into the native conformation. Their emission spectra were not distinguishable from those of the wt scFv (data not shown). Furthermore, for wt scFv CB4-1 we know that on the one hand misfolded material is insoluble and that on the other hand there is no difference in affinity constants for soluble scFv batches independently, whether the affinity purification was performed using the anti-myc tag 9E10 Ab or an Ag peptide at the solid phase. Thus the copurification of substantial amounts of misfolded scFv material after affinity purification with anti-myc 9E10 can be excluded.

Fast protein liquid gel chromatography and ultracentrifugation revealed that the purified CB4-1 scFv is mainly dimeric (data not shown). The dimer-monomer ratio ranges from 70 to 90% depending on the expression and purification procedure applied.

The binding behavior of wt scFv-myc tag fragment was compared with that of the CB4-1 Fab and the complete mAb by two different competition ELISA experiments as shown in Fig. 2. The Fab competition assay (Fig. 2A) provides a measure of the avidity against the solid-phase Ag p24; the peptide competition assay (Fig. 2B) determines the affinity against the peptide in solution. The scFv of CB4-1 exhibits nearly the same binding behavior to p24 as the parental IgG Ab CB4-1, whereas the affinity of the Fab is significantly lower. This underlines the predominantly dimeric nature of the scFv. In contrast, the affinities of both scFv and Fab toward the e-pep are very similar to that of the mAb CB4-1 (Fig. 2B), which is to be expected because affinity constants derived from a competition assay should not be influenced by avidity effects. Therefore, the CB4-1 scFv expressed in E. coli is suitable for mutagenesis studies of the mAb CB4-1 binding region.

Mutagenesis of CB4-1 binding region

For mutational analysis of the Ab binding region, those amino acid side chains are of particular interest which potentially provide different contributions to the binding of the structurally unrelated peptides. By inspecting all CDR residues for which Ag contacts can be observed in the corresponding crystal structures of the two peptide/Fab complexes (30), and assisted by the results from the corresponding peptide substitutional analyses (31), such amino acids can be identified if they interact with a peptide key position in one peptide and with a nonkey position in the structurally unrelated peptide. The Ab residues VH :Tyr32 and VL :Phe94 are such residues, both in hydrophobic contact with the two unrelated peptides e-pep and epitope unrelated peptide (u-pep; Ref. 30). VH :Tyr32 interacts with key positions in e-pep but not in u-pep, whereas it is vice versa with VL :Phe94. Nevertheless, those peptide residues identified as key positions must not necessarily reflect the

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**FIGURE 2.** Affinity of the CB4-1 scFv to the Ag p24 (A) and to the e-pep GATPQLNnL (n = norleucine) (B) in comparison with CB4-1 mAb and the corresponding Fab, as measured by Fab competition ELISA (A) and by peptide competition ELISA (B). The principles of the two ELISA systems are presented schematically on the left.

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importance of their interaction with individual residues in the Ag binding site, but may as well be the result of conformational restraints or requirements within the peptide (26). To investigate the binding contribution of VH:Tyr\(^32\) and VL:Phe\(^94\), we substituted these residues with alanine. Additionally, we intended to check the influence of a noncontact CDR residue that is highly conserved in the germline-encoded \(\kappa\) light chain variable region genes. One of the most significant residues without direct Ag contact is the proline in position 95 of the light chain that stabilizes the CDRL3 loop in a conformation corresponding to the canonical structure 1 (8). In contrast, there is a structure reported for an anti-CD5 Ab which lacks this conserved proline in CDRL3, leading to a variant of the canonical structure (45). Thus, a mutation of this position in CB4-1 could show different influences on the binding of epitope related and unrelated peptides. The VL:Pro\(^95\) was again mutated to alanine.

The site-directed mutagenesis was performed by the method of Deng and Nickoloff (38) and the resulting mutants were selected by restriction site and Western blot analyses. Finally, the desired exchanges were confirmed by control sequencing for at least two mutant clones. After expression, the mutated scFv proteins were purified from the soluble fractions of periplasm and culture supernatant as described above.

Characterization of binding behavior of the scFv mutants

The binding behavior of wt and mutant scFvs of CB4-1 were compared by the two different ELISA mentioned above (see Fig. 2), using either the Fab of CB4-1 or the e-pep GATPQDLNTrL and the u-pep GLYEWWGGARITNTD for scFv competition. The influence of the mutations on the binding of the natural Ag p24 is measured in the Fab competition ELISA (Fig. 3A). The alanine exchange of VH:Tyr\(^32\) reduced the binding to immobilized p24 250-fold, that of VH:Phe\(^94\) about 10-fold, and that of VL:Pro\(^95\) to a nondetectable level (\(K_D > 2 \times 10^{-8}\) M). Hence it follows that for the detection of free scFv mutants in the peptide competition ELISA it became necessary to modify the solid phase, because in competition assays, the solid phase requires high affinity to the captured molecule. Therefore, in parallel to p24 as the capturing molecule, we used biotinylated e-pep and u-pep which were immobilized on streptavidin-coated microtiter plates. In the cases where the peptide competition could be performed with both the biotinylated peptide and p24 as solid-phase Ag (scFv mutants VH:Tyr\(^32\)Ala/u-pep and VL:Phe\(^94\)Ala/e-pep), the values agree well, demonstrating the independence of the measured affinity on the nature of solid phase capturing molecule (data not shown). Peptide competition assays for the scFv mutants VH:Tyr\(^32\)Ala with e-pep and for VL:Phe\(^94\)Ala with u-pep can only be measured with biotinylated e-pep or u-pep at the solid phase. Because the affinity constants of wt CB4-1 to e-pep and u-pep differ by one order of magnitude (\(K_D\) [e-pep] = 1.3 \times 10^{-8}\) M; \(K_D\) [u-pep] = 2.0 \times 10^{-7}\) M) for better comparison of the values with their mutational impact, the affinity constants are shown as relative affinity constants (rel.\(K_D\) = \(K_D\)scFv(wt)/\(K_D\)scFv(mutant)) in Fig. 3B. The influence of the mutations on the e-pep was completely the same as on the natural Ag p24 as measured in the Fab competition ELISA. For the u-pep, an opposite effect became obvious; whereas the mutation VH:Tyr\(^32\)Ala results in an unchanged or even slightly enhanced binding, the affinity of the scFv mutant VL:Phe\(^94\)Ala is 50-fold reduced. Thus, the single point mutation VH:Tyr\(^32\)Ala causes a changed Ag binding specificity if taking into account the maintained u-pep binding and significantly reduced e-pep binding. The differing mutational effects were characterized in more detail by substitutional analysis of the two peptides with respect to the binding contribution of each amino acid residue in the peptides.

Substitutional analyses with scFv mutants

In comparison to the wt scFv (Fig. 4A) the substitution matrix for the e-pep with scFv mutant VH:Tyr\(^32\)Ala revealed a loss of selectivity in position proline 4 and a slightly increased selectivity in the positions alanine 2, aspartate 6, and asparagine 8 of the e-pep (Fig. 4B). Especially, the two latter residues cannot be substituted furthermore by any other amino acid. One new spot became visible in position 9 where the threonine can be substituted preferentially by aspartate which was not allowed for the wt scFv (Fig. 4, A and B). From inspection of the corresponding x-ray structure, it becomes evident that this aspartate may interact electrostatically with the heavy chain residue lysine 99. Similar to the VH:Tyr\(^32\)Ala exchange, the scFv mutant VL:Phe\(^94\)Ala displays with the e-pep substitution matrix an increased selectivity in the positions alanine 2, aspartate 6, and asparagine 8 (Fig. 4C). Opposite to the effect observed for the VH:Tyr\(^32\)Ala mutation, the selectivity of the scFv mutant VL:Phe\(^94\)Ala is additionally increased in position proline 4, whereas the selective binding in position methionine 10 is slightly decreased.

The substitution matrix of the u-pep with the scFv mutant VH:Tyr\(^32\)Ala reveals an increased selectivity in comparison to the wt scFv (Fig. 4D) in the positions tryptophan 5, alanine 8, arginine 9, and isoleucine 10, as well as for the contact residue leucine 2 (Fig. 4E). Simultaneously, two increased signals in position 8 of the
u-pep indicate that the alanine can be preferentially exchanged against basic residues. In the corresponding x-ray structure there is the possibility that these positively charged side chains form a salt bridge with the carboxyl group of VL:Asp 93. The substitutional analysis of u-pep with the scFv mutant VL:Phe 94 Ala (Fig. 4) demonstrates that a single substitution, that of alanine 8 to phenylalanine, which is inverse to the mutation in the binding region, is able to restore the binding. The peptide with this compensatory substitution was called u-pep:Ala 8 Phe and was synthesized as a soluble peptide for affinity measurements in solution.

Analysis of the u-pep:Ala 8 Phe

The affinities of the wt and mutant scFvs to the peptide u-pep:Ala 8 Phe were measured by peptide competition ELISA (Fig. 5). The affinity of the wt scFv to u-pep:Ala 8 Phe is $1.3 \times 10^{-6}$ M and, therefore, in the same order of magnitude as the affinity of the scFv mutant VL:Phe 94 Ala to u-pep ($K_D = 5.9 \times 10^{-6}$ M). In comparison to the wt scFv, the affinity of the VH:Tyr 32 Ala mutant to u-pep:Ala 8 Phe is slightly decreased by a factor of about 4 ($K_D = 3.5 \times 10^{-6}$ M) whereas the affinity of the scFv mutant VL:
Ala8Phe, whereas e-pep binding is discriminated by the factor of 80.

FIGURE 5. Influence of the scFv mutations on the binding of u-pep: Ala8Phe, which is probably germline encoded (data not shown).

Phe94Ala was increased 80-fold to a $K_D$ of $1.6 \times 10^{-8}$ M. This increase not only restores the binding of the mutant scFv to u-pep: Ala8Phe, but it results in a nearly 10-fold higher affinity than the original wt scFv/u-pep interaction, thus increasing the affinity of u-pep-Ala8Phe to the value of the e-pep/wt scFv interaction. As a result, the preferential binding of wt scFv to e-pep has switched for the scFv mutant V_L :Phe94Ala, which shows the highest affinity to u-pep: Ala8Phe, whereas e-pep binding is discriminated by the factor of 80.

Discussion
Polyreactivity and germline genes

Polyreactive Abs, which are frequently also designated as NAA in the literature, form a substantial part of the normal B cell repertoire (1, 2). A characteristic shared by many of these Abs is their binding to various dissimilar Ags such as proteins, nucleic acids, and polysaccharides (5, 46, 47). This broad specificity may be responsible for a major role of these kinds of Abs in primary defense against invading agents before higher specific Abs are produced by the immune system (2). It has been found that similar V genes can encode both natural polyautoactive and Ag-induced Abs (48). However, the most significant difference between NAA and Ag-induced Abs from normal immune response is that NAA do not undergo Ag-dependent affinity maturation, their variable regions being always in a close germline configuration (5, 49, 50). In contrast to this, an Ag-induced Ab passes through multiple rounds of somatic hypermutation and selection in germinal centers. With respect to the polyclonal binding capability of the CB4-1, the question arises whether the variable region sequences of mAb CB4-1 are nearly identical with germline-encoded V genes, which would point to a “natural” polyreactivity, or whether they show typical features of somatic hypermutation normally found in T cell-dependent affinity maturation. To address this, we performed multiple sequence alignments with known putative germline V genes.3 Inspecting the somatic mutations of CB4-1 V_H and V_L with respect to the number of mutations, the hot spots, the frequency of transitions vs transversions, and the N-region addition, they show all typical features of an Ag-dependent affinity maturation with multiple rounds of diversification and selection (51, 52). Therefore, despite its polyclonal binding behavior toward a number of nonhomologous peptides, the mAb CB4-1 can be considered a normal Ag-specific Ab. This is to be expected because the Ab was derived from secondary immune response after repeated administration of the Ag HIV-1 capsid protein p24 (28).

Cloning, expression, and characterization of the scFv

Cloning and expression of the variable region of Abs as an scFv in E. coli is a widely used method to exploit the specific binding capacity of a certain Ab and offers the possibility to use site-directed mutagenesis to investigate the binding contribution of distinct amino acid residues (53–56). The binding behavior of wt scFv CB4-1 was characterized by two different competition ELISA experiments. The Fab competition ELISA measures the affinity (or avidity) to the solid-phase immobilized rp24. Ligands with two binding sites can bind the solid phase adsorbed Ag with a higher functional affinity (avidity). This is the reason for the lower $K_D$ values of the mAb and the mainly dimeric wt scFv as compared with the corresponding Fab in the Fab competition ELISA (Fig. 2A). The peptide competition ELISA determines the binding constant for the peptides in solution. We obtained no significant differences in the $K_D$ values between complete mAb and its fragments in the peptide competition ELISA. This is supported by earlier comparisons of fluorescence quenching measurements with peptides in solution, and peptide ELISA (29). Therefore, there was no need to analyze the dimer-monomer ratio for the mutated scFvs as well. Taken together, despite the primer-encoded differences in the terminal sequences of framework region 1 or framework region 4 in comparison with the original V_H and V_L sequences, the scFv of CB4-1 exhibits the same affinity as the parental Ab indicating that these sequence deviations, the fragmentation, the dimerization, or the myc tag fusion do not influence the binding behavior at all.

Mutation of the CB4-1 binding site

Usually, the polyreactivity of germline-encoded Abs is structurally accomplished by a higher flexibility which allows the binding of a wide range of Ags, but with low affinity (4). Somatic mutations introduced into the hypervariable CDR loops (but also sometimes into adjacent framework residues) during Ag-dependent affinity maturation result in a combining site with improved complementarity to the Ag which in contrast to the germline-derived Ab binds the Ag in a preorganized fashion. In addition to enthalpic effects, entropic restriction of residues in the combining site plays a key role in the increase of binding affinity (4). Nevertheless, a limited number of molecules which may be structurally related (crossreactivity) or unrelated (polyreactivity) can fit with high affinity in a more rigid binding site of affinity matured Abs (22–24, 30). In the case of CB4-1, the conformation and binding mode of the u-pep drastically differ from the e-pep (30). The contribution of single amino acid residues to the binding energy cannot be derived easily from crystal structure analysis but can be revealed by substitutional analysis of the peptide itself (31) and by mutagenesis experiments at the Ab binding site (13, 18). For the latter case usually alanine exchange was chosen because it minimizes the side chain without altering the main-chain conformation and does not impose extreme steric or electrostatic effects.

The measurement of the $K_D$ values by competition ELISA displays decreases in affinity to the peptides by factors in the range from 10 to 1000 for the CB4-1 scFv mutants. The strongest effect was obtained for the alanine mutation of the canonical residue V_L :Pro95 which is not in contact with the corresponding peptides.4

It has to be mentioned that not all murine germline genes are sequenced so far. A possible way to complete the sequence information is the delineation of a consensus sequence deduced from rearranged V genes with the highest homology. Following this strategy, we identified a cluster of four sequences (MM1M09596, MMU09590, and S73896) with a consensus sequence which is probably germline encoded (data not shown).
In reasons of maintained native folding of the mutated scFv proteins as revealed by an unchanged fluorescence emission spectra, the alanine mutation of VL:Pro95 obviously results in a conformational change of the light chain CDR3 which is unfavorable for both e-pep and u-pep binding despite that this CDR exhibits much more critical contacts with u-pep than with e-pep (30). Thus, this may point to some functional cooperativity between different CDRs within the binding site. Nevertheless, despite that this Pro95 is largely conserved between Ab κ-chains, this does not necessarily mean that an exchange of proline at this position generally leads to a loss in Ag affinity, as it is seen with an anti-CD5 Ab which lacks this conserved proline in CDRL3 (33).

The structurally unrelated peptides e-pep and u-pep interact with different sets of peptide residues to a similar set of amino acid side chains in the Ab binding region. Crystal structure analysis of the CB4-1 peptide/Fab complexes displays for the VH:Tyr32 residue hydrophobic contacts to e-pep proline 4 and to u-pep leucine 2. For the latter, the x-ray data show extensive hydrophobic stacking with u-pep tryptophane 5 and CB4-1 VH:Tyr94 (Fig. 6). Additionally, a hydrogen bound for e-pep was observed between VH:Tyr32 and the carbonyl oxygen of alanine 2. Despite that both peptides exhibit distinct hydrophobic contacts to the VH:Tyr32 in the unmutated binding region, the scFv mutant VH:Tyr32Ala displays a 250-fold reduced binding to the e-pep, whereas the binding to u-pep remains unchanged. Modeling of alanine substitution of VH:Tyr32 shows that there is no side chain orientation for Leu2 which allows a reconstitution of the u-pep:Leu2/VH:Ala32 contact (Fig. 6). Hence, it must be concluded that the wt Leu2/VH:Tyr32 contact does not contribute much to the free energy of u-pep binding. This is supported by the substitutional analysis of u-pep with wt scFv which demonstrates that leucine 2 can be substituted by all other residues (Fig. 4D). In contrast, the loss of the contact with the “key residue” Ile10 for the VH:Phe94Ala mutant leads to a drastic decrease in affinity, affirming that this key position indeed reflects a thermodynamically important residue interaction. The same conclusion can be drawn for the behavior of the mutants toward e-pep; there is much more loss of affinity for the VH:Tyr32Ala mutant without the contact to the key residue Pro4 than for the VL:Phe94Ala mutant without the contact to the nonkey residue Met10.

Generally, substitutional analyses of peptides where each position of the peptides is substituted by all 19 other amino acids is a powerful method to analyze how mutations in the Ab combining site can change the preferentially interacting key residues of Ags in the context of polyspecificity (31). The less amino acid substitutions are accepted in a certain position the more stringent are the sterical and energetical constraints for maintaining the Ab binding, thus reflected by an increased selectivity in that position. For the whole range of potential ligand molecules an increased selectivity would result in a smaller ensemble of binding peptides, which means an increased Ab specificity.

The analysis of the u-pep substitution matrix incubated with scFv mutant VH:Tyr32Ala reveals that changes in position-specific binding patterns occur not only in the contact position leucine 2 (and tryptophane 5, which is involved in a hydrophobic stack with

![FIGURE 6. Structural consequences of VH:Tyr32Ala mutation and their influences on the binding of e-pep (red) or u-pep (blue). Residues from the Ab are in green; interaction between VH:Tyr32 and e-pep (A) or u-pep (B) as seen in the crystal structure, and mutation VH:Ala32 demonstrating the loss of van-der-Waals contacts to e-pep (C) and u-pep (D). The rel.KD given reflect the changes in binding constants in comparison to the binding of the unmutated scFv as measured with the peptide competition ELISA. Modeling and representation of the mutation was generated using Quanta software.](http://www.jimmunol.org/)

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Leu^2; Fig. 6). As illustrated in Fig. 4, changes in the substitution pattern are obvious in nearly all positions of u-pep. Similar effects were observed for the other peptide-scFv mutant combinations as well. In general, the selective binding is reduced in contact positions and increased in all noncontact positions. From this it may be concluded that independently of the affinity changes small rearrangements over the whole peptide are necessary to compensate the structural alterations caused by the mutation. Comparably complex structural changes are described for the affinity maturation of the Ab combining site for the catalytic Ab 48G7 (57).

For the scFv mutant V_L:Phe^94Ala, the substitutional analysis of u-pep reveals the inverse compensatory peptide mutation Ala^8Phe. Fig. 7 illustrates the structural changes which are responsible for the observed effect. The modeled structure shows that there is free space now around the side chain of V_L:Ala^94, so that large hydrophobic side chains can now be accepted in the corresponding peptide contact position without sterical hindrance (Fig. 4F). The affinity of u-pep:Ala^8Phe to the scFv mutant V_L:Phe^94Ala is not only restored, but it is increased up to the range of that for the e-pep/wt scFv interaction.

Considering that mutations during the process of hypermutation will be introduced randomly and thereafter selected via receptor engagement (Ag affinity), our results thus represent an experimental example for the possibility of affinity maturation in the absence of a target (auto) Ag. This becomes obvious if one compares the affinity of the V_L:Phe^94Ala mutation to u-pep:Ala^8Phe with that to the native Ag, e-pep.

General conclusions

Single amino acid substitutions in an Ab binding region can change the specific binding of a certain functional epitope by two orders of magnitude or even more, as seen for other examples (57). At the same time, the affinity to another functional epitope may remain unchanged or even be increased. This results in a switch of

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**FIGURE 7.** Influence of V_L:Phe^94Ala mutation on the hydrophobic interaction with u-pep or u-pep:Ala^8Phe. A, Van-der-Waals contact between V_L:Phe^94 (green) and u-pep:Ala^8 (blue) as seen in the crystal structure. B, Loss of the contact between the mutated light chain residue Ala^94 (green) and u-pep:Ala^8 (blue). C, Restored interaction between V_L:Ala^94 (green) and the substituted u-pep:Ala^8Phe (blue). The rel.K_D given reflect the changes in binding constants in comparison to the binding of the unmutated scFv as measured by the peptide competition ELISA. Modeling and representation of the mutation was generated using Quanta software.
the preferentially bound Ag and therefore, represents a change in Ag binding specificity. On the one hand, from the immunological point of view this means that each time when an affinity matured B cell introduces a new somatic mutation a possible autoantibody specificity can arise. This may be the initial step for the proliferation of an autoimmune B cell clone. On the other hand, for Abs used in therapy, such selective change of Ag binding specificity offers the possibility of reducing a harmful side specificity against self Ags, but without changing the desired target binding specificity, just by introducing single point mutations.

Acknowledgments
We thank Dr. G. Gruetz for advice and critical reading of the manuscript; C. Landgraf, B. Hoffmann, and M. Affeldt for peptide synthesis; and H. Tannetz for CB4-1 Ab preparations.

References