Use of a Peptide Mimotope to Guide the Humanization of MRK-16, an Anti-P-glycoprotein Monoclonal Antibody*

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A mimotope-guided strategy for engineering antibodies directed against orphan targets or antigens that are difficult to purify was developed and used to humanize the murine MRK-16 monoclonal antibody (mAb). MRK-16 recognizes a conformational epitope of a 170-kDa membrane protein, termed P-glycoprotein (P-gp). Elevated expression of P-gp on tumor cells is associated with resistance to cytotoxic drugs, a major obstacle in chemotherapy. Murine MRK-16 was used to enrich and screen a phage-displayed peptide library to identify reactive mimotopes. One peptide, termed ALR1, was enriched to a greater extent than others and subsequently was expressed as a fusion protein with glutathione S-transferase. ALR1 fusion protein bound MRK-16 specifically and inhibited binding of MRK-16 to cells expressing elevated levels of P-gp. To humanize MRK-16, the murine complementarity determining regions were grafted onto homologous human heavy and light chain variable region frameworks. Framework residues that differed between the murine MRK-16 and the homologous human templates were analyzed and subsequently, five framework positions potentially important for maintaining the specificity and affinity of MRK-16 were identified. A combinatorial library consisting of 32 variants encoding all possible combinations of murine and human residues at the five differing framework positions was expressed in a phage system. In the absence of purified P-gp, ALR1 fusion protein was used as surrogate antigen to screen the antibody library to identify the framework combination that most preserved the binding activity of the mAb. On the basis of the initial screening against the mimotope four antibody variants were selected for further characterization. The binding affinity of these variants for the ALR1 fusion protein correlated with their binding to cells expressing elevated levels of P-gp. Thus, peptide mimotopes which can be identified for virtually any antibody including those that recognize conformational or carbohydrate epitopes, can serve as antigen templates for antibody engineering.

The widespread success of murine hybridoma technology has resulted in the discovery of numerous well characterized monoclonal antibodies (mAbs) with unique specificities. Many of these mAbs display tremendous therapeutic potential both as vehicles for targeting cytotoxic agents (reviewed in Ref. 1) and as function blocking molecules (2–7). However, murine mAbs are generally recognized as foreign antigens by the human immune system preventing the administration of multiple doses (8, 9). As a result, there has been considerable effort devoted to circumventing the immunogenicity of murine mAbs, including the development of methods for discovering human antibodies. For example, human lymphocytes have been stimulated in vitro (10, 11), phage-expressed human antibody libraries have been synthesized (reviewed in Ref. 12), and transgenic mice expressing human Ig genes have been created (13). Although human mAbs have been discovered by these approaches there remains a need to fully exploit the potential clinical benefits of murine antibodies.

As an alternative to discovering human mAbs, antibody engineering approaches have been developed to reduce the potential immunogenicity of murine mAbs. For example, chimeric antibodies consisting of the murine variable region fused to a human constant region have been constructed (14–16). Antibody variants containing even fewer murine residues have been synthesized by grafting murine CDRs onto human variable region frameworks (17–19). Unfortunately, chimeric antibodies may still display immunogenic properties in humans (20, 21) and CDR grafting often diminishes the affinity of mAbs (18, 22). A general and simple approach for reducing the immunogenicity of murine mAbs without diminishing their affinity would permit a greater number of the existing well characterized mAbs to be evaluated as therapeutics.

The diminished affinity often observed with antibodies humanized by CDR grafting reflects the structural significance of certain framework residues. The relative importance of specific framework residues varies between different mAbs and consequently, identifying important residues and determining the optimal amino acid at those positions has proven difficult. Previously, we described an approach for the humanization of antibodies that uses phage expression of combinatorial antibody framework libraries and CDR grafting coupled with identification of the most active humanized variant by rapid screening methods (23–25). Phage expression of combinatorial framework libraries takes advantage of the efficiency of bacterial cloning systems and, when coupled with the appropriate assays, increases the likelihood of identifying fully active humanized variants. Examination of a wide range of framework structures reduces the requirement for structural modeling to predict precisely the framework residues critical for binding activity. Furthermore, the identification of fully active humanized variants from combinatorial libraries is straightforward.

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The abbreviations used are: mAb, monoclonal antibody; Fab, antigen-binding fragment; H, heavy; L, light; CDR, complementarity-determining region; ELISA, enzyme-linked immunoassay; P-gp, P-glycoprotein; GST, glutathione S-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
using screening methods that permit the affinities of numerous (>10^6) soluble Fabs to be distinguished rapidly (24, 26). To date, the optimal screening methods have required soluble labeled antigen. However, therapeutic mAbs are often directed against cell surface antigens that are integral membrane proteins and therefore, the purification or expression of quantities of antigen sufficient to support screening of antibody libraries has been limiting in certain instances.

Antibodies recognize a restricted portion of protein targets, termed the antigenic determinant or epitope, which can consist of peptide, protein, carbohydrate, or other chemical moieties. Regardless of the precise chemical nature of the epitope, peptides can often be used to mimic the structure of the antigenic determinant (27). Moreover, the efficient synthesis of peptides or the display of random peptide libraries on the surface of phage (28) have greatly facilitated the identification of peptides reactive with a wide range of antibodies. For example, peptides reactive with mAbs that recognize linear (29), conformational (30), and carbohydrate (31) epitopes have been described. The ability of these mAb-reactive peptides to mimic the actual antigen epitope has been demonstrated by the use of the peptides as assay reagents and affinity matrices (32, 33), to provide structural information about the antigen (34, 35) or antibody (36), and as immunogens (37–40). The ability of peptides to effectively mimic a portion of the structure of the antigen suggests that it may also be possible to use peptide mimotopes as templates for screening libraries of humanized variants to identify clones that retain the conformation of the binding site of the murine mAb. As such, a readily available supply of soluble antigen would be available to direct the humanization of mAbs, regardless of the nature or availability of the actual antigen.

In the present study we wanted to determine if a mimotope could in fact be used to guide the successful humanization of an antibody. The murine mAb MRK-16, which is directed against an external domain of P-glycoprotein (41), was selected as a model system. P-gp is an integral membrane protein that putatively spans the plasma membrane 12 times (42–44), is expressed at elevated levels on many tumor cells (45), and acts as a drug efflux pump (reviewed in Ref. 46). The expression of elevated levels of P-gp on tumor cells is associated with multidrug resistance, a primary cause of failure of chemotherapy. However, MRK-16 can block the efflux of certain chemotherapeutic drugs in vitro (41) and treatment of athymic mice with MRK-16 inhibited the growth of drug-resistant human tumor cells in vivo (47). Thus, MRK-16 may increase the benefits of certain chemotherapeutic agents. Although a humanized version of MRK-16 is preferred for human clinical studies, the difficulty in obtaining sufficient levels of soluble P-gp has hindered progress.

In this study we describe the humanization of MRK-16 using a peptide mimotope as surrogate for the P-gp antigen for screening.

**EXPERIMENTAL PROCEDURES**

**Materials**—HCT-15 human colorectal carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). The human adriamycin-resistant K562/ADM cell line was established from the human myelogenous leukemia K562 cell line and was maintained as described previously (48). MRK-16 antibody and K562/ADM cells were obtained from Hoescht Japan.

**Identification of MRK-16 Mimotopes**—Poly styrene latex beads (1 μm diameter, Interfacial Dynamics Corp., Portland, OR) were washed twice and resuspended in 100 mM MES, pH 5.0, to the original volume. The beads were incubated at 45 °C for 5 min at which time 70 μl were combined with 150 μg of MRK-16 in 700 μl of 100 mM MES, pH 5.0, for 1 h at 45 °C. The beads were collected by centrifugation, resuspended twice with 1 ml MOPS, pH 7, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, 1% poly(vinyl alcohol), 0.1% sodium azide (MOPS, 1% poly(vinyl alcohol)) containing 3% BSA followed by two washes with MOPS, 1% poly(vinyl alcohol) alone. The beads were resuspended in 500 μl of MOPS, 1% poly(vinyl alcohol) and were stored at 4 °C until use.

The phage-expressed peptide library was designed to randomly express cysteine residues at a 5-fold higher frequency and was synthesized as described previously (49). To amplify the peptide library, 300 nmol of amber suppressor tRNA for XL1-Blue (Stratagene, Inc., San Diego, CA) was grown at 37 °C until the cultures reached a density of 0.8 at OD₆₀₀ at which time isopropyl-thio-β-D-galactoside was added to a final concentration of 2 mM and the culture was infected with 100 μl of a high titer phage stock (10⁶–10¹¹ phage/ml). After 6 h of growth, bacteria were removed from 10 ml of culture media by centrifugation at 26,000 × g for 25 min.

For enrichment of phage, 100 μl of MRK-16-coated beads were added to a microcentrifuge tube which had been blocked with MOPS, 1% poly(vinyl alcohol) for 15 min at 25 °C. The beads were collected by centrifugation, resuspended in 1 ml of culture media containing the amplified peptide library for 16 h at 4 °C, and collected by centrifugation. The beads were washed six times with 1 ml of Tris-buffered saline, resuspended in 500 μl of Tris-buffered saline, and stored at 4 °C. The bound phage were amplified as described above, using 400 μl of the beads to infect a 50-ml culture of XL1-Blue. The cells were pelleted by centrifugation at 11,000 × g for 30 min and the amplified phage were precipitated from the culture supernatant following a 30-min incubation at 4 °C with 1.25% PEG 8000 in 220 mM ammonium acetate. The phage were collected by centrifugation at 11,000 × g for 30 min and resuspended in 2 ml of 1% BSA in PBS. Subsequently, 1 ml of the amplified phage was used for the next round of selection.

Following the second and third rounds of enrichment an aliquot of beads was screened by plaque lift, as described previously (50). The filters were incubated with 5 μg/ml MRK-16 in 5% nonfat powdered milk, 0.2% Tween 20, 0.01% anti-fade A emulsion, and 0.01% thimerosal in PBS and washed three times with PBS containing 0.1% Tween 20 (PBS-T). Reactive plaques were identified colorimetrically following incubation with goat anti-murine IgG-alkaline phosphatase conjugate (25).

**Expression and Biotinylation of GST-Peptide Fusion Proteins**—Three overlapping oligonucleotides encoding the reactive peptide, a six-proline linker and unique EcoRI and NotI restriction sites were synthesized. The overlapping oligonucleotides were annealed and polymerized by polymerase chain reaction. Following digestion with EcoRI and NotI and purification by agarose gel electrophoresis, the DNA fragment was ligated into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) and introduced into DH10B cells by electroporation. Expression and purification of GST-peptides was performed as described by the manufacturer (Amersham Pharmacia Biotech). For visualization of GST-domain-integrated protein in PBS and incubated with 10-fold molar excess sulfosuccinimidobiotin for 2 h at 25 °C. The reaction was terminated by addition of 15 mM ethanolamine and excess biotin was removed by dialysis against PBS.

**Design, Synthesis, and Characterization of Phage-expressed Humanized MRK-16 Fab**—Overlapping oligonucleotides (69–75 bases in length) encoding the framework regions of the H and L chain variable domains were synthesized and the murine MRK-16 CDRs, as defined by Kabat et al. (51, 52), were synthesized and used to construct a CDR-grafted version of MRK-16. Briefly, the H and L chain variable domains were synthesized separately by combining equimolar amounts of the overlapping oligonucleotides with Ffu DNA polymerase in a polymerase chain reaction. Subsequently, the variable domains were fused to human κ and constant region sequences in a modified M13X104 vector (53), termed M13IX104CS, using hybridization mutagenesis (23, 54, 55). The M13IX104 vector was modified by replacing cysteine residues at the end of the κ and constant regions with serine. Subsequently, all possible combinations of murine and human residues at five framework positions (Fig. 3, asterisks) were synthesized using site-directed mutagenesis, resulting in the expression of 32 unique variants.

**ELISA Screening**—The relative affinities of the humanized MRK-16 variants expressed in small-scale (<1 ml) bacterial cultures were assayed by ELISA (24). Briefly, microtiter plates were coated with 5 μg/ml goat anti-human κ antibody (Southern Biotechnology) and blocked with 3% BSA in PBS. Next, 50 μl of Fab from Escherichia coli cell lysates were incubated with the plate for 1 h at 25 °C. The plate was then washed three times with PBS-T, and 1 μg/ml biotinylated GST-peptide in PBS containing 1% BSA was added for 2 h at 25 °C. The plate was washed three times with PBS-T and NeutrAvidin-alkaline phosphatase conjugate diluted 1000-fold in PBS-T was added for 30 min at 25 °C. The plate was washed three times with PBS-T and binding was quantitated colorimetrically (24). Purified Fab and Fab in the periplasmic space fraction from bacterial lysates was quantitated as described (24).
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**RESULTS**

**Mimotope-guided Humanization of mAbs**—The goal of the present study was to humanize the murine MRK-16 mAb by screening a combinatorial framework library for antigen binding. Unfortunately, the target antigen P-gp, a large integral membrane protein with multiple transmembrane domains, was not readily available as a soluble protein. Consequently, we evaluated an alternative strategy for humanizing MRK-16 and other antibodies in the absence of labeled soluble antigen. The approach, outlined in Fig. 1, uses a peptide mimotope to serve as surrogate antigen. Initially, the murine antibody is used to identify reactive peptides that mimic the antigen (step 1). Next, the mimotope is expressed as a fusion protein with GST to ensure solubility and permit efficient purification and biotinylation (steps 2 and 3). The biotinylated GST mimotope is then used to screen the phage-expressed combinatorial antibody libraries, leading to the identification of the most active humanized construct (step 4). Finally, the murine and humanized antibodies are characterized for binding to cells expressing the antigen to verify that the mimotope-guided process does not alter the specificity of the antibody (step 5).

Identification and Characterization of MRK-16-reactive Peptides—A phage-expressed peptide library was synthesized using codon-based mutagenesis (49, 56) such that peptides 20 amino acids in length were fused to the pVIII major coat protein of M13 filamentous phage. Subsequently, the library was incubated with immobilized MRK-16 to enrich phage displaying reactive peptides. After three rounds of selection, MRK-16-reactive phage were identified by a plaque lift assay. DNA sequencing of randomly selected reactive phage identified multiple unique peptides of which one, termed ALR1, was the predominant species. MRK-16 is not reactive with P-gp under denaturing conditions, suggesting the mAb recognizes a conformational epitope. Nonetheless, ALR1 displayed a small degree of homology with a region of P-gp predicted to be the fourth extracellular loop (Fig. 2A). MRK-16 reactivity has previously been mapped to this region of P-gp using overlapping synthetic peptides (57). Interestingly, a peptide corresponding directly to the linear sequence 737–756 of the extracellular P-gp loop expressed as a GST fusion protein was not reactive with MRK-16 either by ELISA or Western blot analysis (data not shown). The reason(s) for this apparent discrepancy are not clear but may reflect differences in conformation adopted by the free peptide used in the previous study (57) versus the fusion peptide described herein.

To permit further characterization of ALR1 the peptide was expressed as a fusion protein with GST. Bacterial lysates containing GST-ALR1 or an unrelated GST-peptide were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blot analysis. Although MRK-16 is reactive with Western blots of P-gp, it bound GST-ALR1. MRK-16 was unreactive with other bacterial lysate proteins as well as the unrelated GST-peptide, providing evidence for the specificity of the MRK-16/peptide interaction (data not shown). ALR1 contains a single cysteine residue and therefore, is unlikely to be constrained by disulfide bonds. Nonetheless, purified GST-ALR1 protein was reduced with 10 mM dithiothreitol in the presence of 0.75% SDS and subsequently, the sample was heated to 85 °C for 10 min, cooled, and treated with freshly prepared 0.04 M N-ethylmaleimide for 60 min on ice. GST-ALR1, reduced GST-ALR1, and reduced and alkylated GST-ALR1 were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Little or no higher molecular weight bands were observed under nonreducing conditions, demonstrating the lack of disulfide bridges between molecules. Furthermore, MRK-16 reacted with non-reduced, reduced, and reduced and alkylated GST-ALR1 in monomer form, as determined by Western blot. Although these studies do not rule out dimerization of the peptide through noncovalent interactions under non-denaturing conditions, the Western blot data suggest that dimerization is not essential for its activity.

MRK-16 binding to GST-ALR1 was also evaluated by ELISA under non-denaturing conditions and was concentration-dependent and saturable (Fig. 2B, filled circles) while binding to an unrelated GST-peptide fusion was undetectable (Fig. 2B, open circles). In order to further demonstrate the specificity of
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the mimotope for MRK-16, GST-ALR1 was titrated with 5 μg/ml MRK-16 and incubated with fixed monolayers of P-gp-expressing HCT-15 cells. GST-ALR1 inhibited the binding of MRK-16 to the HCT-15 cells expressing elevated levels of P-gp while an unrelated GST-peptide did not affect MRK-16 binding (Fig. 2C, filled circles). Collectively, these results are consistent with ALR1 binding at or near the combining site of MRK-16.

Next, GST-ALR1 was labeled with biotin to permit the screening of phage-expressed MRK-16 by a previously described rapid approach (24). MRK-16 and an unrelated mAb were immobilized on a microtiter plate and subsequent incubated with varying concentrations of MRK-16. No detectable difference in binding of MRK-16 to either form was detected, consistent with biotinylation occurring at sites distal to the site of antibody/peptide interaction.

Synthesis of Humanized MRK-16 Variants—CDR grafting, or the transfer of murine CDRs onto a human framework, is the most straightforward approach to humanizing murine mAbs (17–19). However, the resulting humanized mAbs often display diminished affinity because certain murine framework residues serve a critical role either in maintaining the conformation of the CDRs or in antigen interactions. Previously, we have described a phage expression combinatorial approach to humanizing mAbs (23). Briefly, the murine variable region sequence is used to identify the most homologous human framework to serve as a template. Framework positions where the amino acids differ between the murine mAb and the human template are assessed individually and residues that are potentially important for maintaining the full binding activity of the mAb are characterized by synthesizing a combinatorial antibody library that examines all possible combinations of amino acids found at these locations in the murine parent mAb and the human template. Thus, the complexity of the combinatorial library is 2^n, where n is the number of differing framework positions that potentially contribute to the binding activity of the mAb.

The amino acid sequences of murine MRK-16 H and L chain variable regions were used to identify homologous human variable region sequences to serve as templates for humanization (Fig. 3). CDR residues, as defined by Kabat et al. (51, 52), are underlined and were excluded from subsequent analysis. The H chain template L38 was missing the first three amino acids of framework 1 and was 88% identical to MRK-16 at the remaining 84 framework residues, differing at 10 positions (Fig. 3, differences indicated by vertical lines). The L chain template KV2D was 84% identical to the MRK-16 framework, differing at 13 of 80 framework residues (Fig. 3).

Multiple parameters were considered in identifying framework positions to include in the combinatorial library. For example, surface residues not normally found on human antibodies are likely to contribute to the immunogenicity of the humanized mAb. Thus, the majority of murine MRK-16 framework residues predicted to be located on the surface based on solvent exposure (58) were changed to the corresponding human template amino acid. In addition, differing residues were analyzed for potential to contact the opposite variable region domain in the V_{H},V_{L} interface, for predicted importance in modulating CDR activity as defined by Studnicka et al. (59), and for similarity of amino acids based on the charge and size of side chain.

Assessment of the potential importance of the residues at all framework positions differing between murine MRK-16 and the homologous human templates resulted in five positions being selected for further characterization; L chain residues 12 and 100 and H chain residues 3, 44, and 77 (Fig. 3, asterisks). L chain residue 12 and H chain residue 77 are predicted to be buried and the amino acids found on the murine MRK-16 and human templates are dissimilar while L chain residue 100 and H chain residue 44 are solvent exposed but potentially involved in contacting the variable region of the other chain. Finally, H chain residue 3 was included in the combinatorial library because the sequence of the first three amino acids of the human H chain template was unknown and residue 3 is typically glutamine in human germline sequences. The remaining H and L chain framework positions were identical to the human tem-
plates. Because all murine/human residue combinations at five sites were tested the combinatorial framework library contained 32 unique variants.

DNA from 20 randomly selected clones from the combinatorial framework library was sequenced to characterize the mutagenesis efficiency at each of the framework library sites. As expected, sequencing of random clones demonstrated representation of both the murine and human residues at all five framework positions (Table I), although a modest overrepresentation of variants expressing the human proline residue at L chain position 12 (75%) and the murine asparagine residue at H chain position 77 (75%) was observed.

Screening of Humanized MRK-16 Variants with Mimotope Fusion Protein—The precise combination of murine/human framework residues that most closely preserves the conformation of the CDR residues involved in antigen binding was identified based on affinity screening of the combinatorial framework library. Randomly selected MRK-16 variants from the library were expressed as soluble Fabs in the periplasmic space of small scale (1 ml) bacteria cultures grown in a 96-well format. Although variable amounts of MRK-16 Fab were released from the periplasmic space, uniform quantities were captured on a microtiter plate coated with a limiting (saturable) quantity of goat anti-human k chain antibody, as described previously (24). Subsequently, the Fabs were incubated with biotinylated GST-ALR1 and binding was detected with NeutrAvidin-alkaline phosphatase. The relative affinities of the humanized variants were compared with bacterially expressed chimeric MRK-16 and an irrelevant Fab based on the colorimetric signal generated in the ELISA.

Approximately 200 randomly selected clones were screened individually for mimotope binding. Eleven clones that displayed the strongest binding based on the colorimetric signal were selected for further characterization. DNA sequencing of the clones identified seven unique framework sequences and two clones, 4 and 12, were identified multiple times (n = 2 and 4, respectively), as summarized in Table I.

Although DNA sequencing of 20 clones randomly selected from the library demonstrated the diversity of the framework library, the frequency at which certain clones were identified (clones 4 and 12, for example) relative to other variants might reflect an abundance of these variants in the library. Therefore, in order to more fully characterize the mimotope binding, additional Fab was isolated from larger cultures of clones 4 and 12, as well as from a clone which did not display appreciable mimotope binding. Of the 32 distinct members of the combinatorial framework library, one contains an entirely human framework. Presumably, this CDR-grafted variant was not identified during the screening of the mimotope because it binds the mimotope weakly or because it is present in the library at an abnormally low frequency. Nonetheless, because the simplest approach to antibody humanization consists of CDR grafting, phage-expressed Fab consisting of the murine MRK-16 CDRs grafted to an entirely human framework was characterized in addition to clones 4 and 12. The variants were titrated against immobilized GST-ALR1 fusion protein (Fig. 4A). Clones 4 (open circles), 12 (filled circles), and the CDR-grafted variant (filled squares) all bound the immobilized mimotope in a concentration-dependent fashion while the nonreactive clone (open squares) and irrelevant clone (open triangles) did not bind appreciably. Based on the titration profile, each clone displayed different affinity for the mimotope with clone 4 binding with the highest affinity, followed by clone 12, and the CDR-grafted variant.

To determine if the strength of the binding interaction between GST-ALR1 and MRK-16 variants correlated with binding strength to the actual antigen, the same variants were titrated on cells expressing elevated levels of P-gp. Human HCT-15 colorectal carcinoma cell monolayers were fixed in 96-well microtiter plates, incubated with varying amounts of

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**Fig. 3. Alignment of MRK-16 variable region and human template sequences.** The amino acid sequences of the murine MRK-16 H and L chain variable region were used to identify homologous human variable regions. The numbering of residues and the definition of CDRs (underlined) were based on Kabat et al. Differences in sequence are indicated by vertical lines and framework positions characterized in the combinatorial expression library are marked with an asterisk.
The premise of this study was that optimization of MRK-16 binding to a peptide mimotope would lead to optimization of MRK-16 binding toward the antigen, P-gp. The success of mimotope-guided humanization relies on the ability of peptides to mimic the epitope recognized by mAbs and consequently, peptide mimicry of the antigen must be reflected by the specificity of peptide binding to the target mAb. Several lines of evidence demonstrate the specificity of ALR1 binding to murine MRK-16. First, using immobilized murine MRK-16 mAb ALR1 was specifically enriched from a phage-expressed peptide library containing as many as 10^9 unique peptides. In addition, the binding of GST-ALR1 fusion peptide to MRK-16 was concentration-dependent and saturable while binding of unrelated GST-peptide fusions was undetectable. Finally, GST-ALR1 inhibited the binding of MRK-16 to fixed and live cells expressing elevated levels of P-gp. Collectively, these results are consistent with ALR1 binding at or near the CDR of MRK-16.

Additional evidence that ALR1 mimicked the P-gp epitope closely was provided by the screening of the library of humanized variants. The primary amino acid sequences of the 32 combinatorial framework variants are highly homologous, differing by five or fewer amino acids. Nonetheless, the five framework positions examined in the combinatorial humanization library were selected based on their potential to affect the CDR structure and thus, the antigen binding of MRK-16. Despite the high degree of primary sequence similarity between the 32 variants, ALR1 binding to the humanized MRK-16 clones was variable. Differences in mimotope binding were observed both in the primary screening of random clones as well as in the titrations performed on select variants. Thus, similar to antigen-antibody interactions the affinity of the mimotope-antibody interaction was dependent on the framework structure of the variable region.

Screening of 200 randomly selected clones identified seven distinct framework sequences that appeared to bind the mimotope with higher affinity. Of these, two variants, clones 4 and 12, were identified on multiple occasions providing further evidence that the enhanced signal observed during the initial screening of these particular clones was not an artifact. Sequence analysis of the 11 most active variants suggests that L chain residue 12 is a framework residue important for maintaining the affinity of humanized MRK-16. DNA sequencing of 20 randomly selected clones revealed a potential bias at L chain position 12 in the library synthesis with 15/20 (75%) clones expressing the human proline residue. However, DNA sequenc-
ing of the 12 clones selected on the basis of mimotope reactivity revealed that 10/11 (91%) clones expressed the murine serine residue. Because the method described herein does not use affinity selection/enrichment methods the results suggest that serine is favored over proline at L chain position 12. Preferences for either murine or human residues at the other four framework library positions were not observed.

Several lines of evidence suggest that certain combinations of framework residues other than serine at L chain position 12 serve critical roles in preserving the binding activity of humanized MRK-16. First, the combinatorial library contains 16 distinct members that express a serine residue at L chain position 12 yet only six of these were identified based on binding assays. In addition, one of the active variants, clone 21, expressed proline at L chain position 12. Finally, the clone non-reactive with the mimotope expressed serine at L chain 12 and bound P-gp less effectively than other clones.

Upon more thorough characterization one of the humanized versions of the murine mAb MRK-16 identified multiple times by screening with ALR1, clone 4, displayed higher affinity for P-gp than did the CDR-grafted construct, demonstrating that peptide mimicry of epitopes can be exploited to generate reagents that enable humanization of mAbs. Furthermore, although a very small number (n = 4) of variants from the combinatorial framework library were characterized fully by titration on both the mimotope and cells overexpressing P-gp there was good correlation between the relative affinities of these four variants for the mimotope as compared with P-gp.

Humanization of MRK-16 used a combinatorial framework library that examined the contributions of five framework positions to the overall affinity of the antibody. However, it is often necessary to construct larger combinatorial libraries to characterize a greater number of potentially important framework residues. Larger libraries increase the likelihood of identifying fully active humanized variants because characterization of a greater number of framework positions diminishes the requirement for completely accurate structural modeling. However, larger libraries are screened more efficiently if soluble labeled antigen is available, which is often not the case. The ease with which mimotopes are identified and expressed as soluble GST fusion proteins enables the synthesis of large combinatorial antibody libraries by removing screening restrictions. Although mimotope screening may not be capable of identifying the optimal clone from large libraries it is envisioned that it can be used as an initial screen to permit the identification of a smaller number of variants for further characterization. Thus, large combinatorial libraries can be reduced to a few clones which can subsequently be screened by virtually any assay.

The use of surrogate antigen for antibody humanization is of greatest utility if the mimotopes can be easily identified, are soluble, and can be readily purified and labeled for use as a screening reagent. In the present study, phage display peptide libraries were used for the rapid identification of a mimotope and subsequently, the peptide was expressed as a fusion protein with GST to enhance solubility and permit its straightforward purification and biotinylation. A priori, it was possible that the peptide identified by phage display would not be active when fused to GST, due to different interactions between the peptide and the pVIII or GST protein. However, to date we have successfully transferred four of five mimotopes identified from phage display libraries from pVIII to GST. The reasons for our success are unclear but may reflect the expression of the peptide libraries on the tip of pVIII, as opposed to other phage surface proteins. F-specific filamentous phage are composed of five different phage proteins, of which pVIII is the most abundant, with thousands of copies per phage. The pVIII protein forms a highly ordered, tightly packed coat while the remaining four phage proteins are present at small copy number on the ends of the phage particle (60). The large number of pVIII protein copies per phage reduces the likelihood of peptides interacting among themselves. Moreover, the highly ordered packing of pVIII may reduce the number (diversity) of interactions between peptides and phage surface molecules and thus, increase the chance of successfully fusing the peptide to an unrelated protein, such as GST. The ease with which mimotopes identified by screening phage libraries of pVIII fusion peptides can be transferred to GST enables the surrogate antigen strategy to potentially be applied for the engineering of other mAbs.

Previously, peptide mimotopes have been identified for mAbs that bind epitopes of diverse chemical nature and furthermore, mimotopes have been used as antigen substitutes in multiple applications including immunoassays, affinity matrices, elicit structural information, and as immunogens. All these applications provide evidence that peptides can in fact mimic epitope structure effectively and support the notion that mimotope-guided humanization is potentially applicable to a wide range of mAbs, even when the target antigen is unknown or difficult to isolate. Mimotope-guided humanization of MRK-16 is one example of antibody engineering in the absence of antigen. It is also possible that peptides can be used as reagents for in vitro affinity maturation of mAbs. However, amino acid substitutions in the CDRs that improve mAb affinity for the peptide may be directed toward portions of the peptide that are not structurally related to the antigen. Consequently, although the resulting mAb may bind peptide with higher affinity, binding to antigen may be unaltered or diminished. Therefore, for affinity maturation purposes it might be beneficial to optimize the mAb by screening against multiple mimotopes in parallel.

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REFERENCES

Mimotope-guided Humanization of Monoclonal Antibodies

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