A random peptide library was expressed on the surface of a mammalian cell by applying retroviral vectors. The random sequence was fused to the CCR5 chemokine receptor, which served as a scaffold to present the library at the cell surface. We used this library to isolate an epitope mimetope in a proof of principle system. This approach can become a tool for rapid creation of peptide expression domains in a eukaryotic environment. Applications include the creation of decoys for receptors in cell-cell interactions, screening for molecules that drive ligand expression on target cells in two-cell interaction screens, among other utilities.

Functional genomics can be based on the use of genomic libraries and used as a means for gene or function discovery. These libraries include the now classical cDNA libraries, small interfering RNA libraries, or random peptide libraries. cDNA libraries are based on the natural genetic information of the cell, as it is the genome of the cell that serves as template for library production (Ref. 1 and references therein). Small interfering RNA libraries are composed of short double-stranded RNA molecules for the knocking down of genes based on RNA interference (2–4).

Random peptide libraries, in contrast, are composed of a pool of shapes resulting from random, or partially random, DNA sequences that can ultimately result in the translation of specific three-dimensional structures. These peptides may bind to the surfaces of biomolecules and by doing so interfere or alter signaling pathways. The change in biological outcome or phenotype is often the basis for biological screens. The peptide responsible for that outcome becomes the subject of a study aimed at discovering the binding partner in the cell (5–8).

Examples of the usage of these libraries include cDNA libraries, as in the search for transactivators (9), degenerate random libraries such as those based on the major histocompatibility complex class I anchor residues (10), and fully random peptide libraries (11). Examples also include random peptides fused to the green fluorescent protein at both termini or protruding internal loops (12). A library of cyclic peptides based on inteins has also been successfully screened in B cells (13) and used to screen for useful functionalities.

Correct protein folding is needed for biological activity and requires endoplasmic reticulum-specific posttranslational modifications when the protein is to be secreted or embedded within the cell membrane. In this regard, certain eukaryotic viruses have been engineered for eukaryotic protein display. An example is the multiple nuclear polyhedrosis baculovirus Autographa californica. Peptides inserted into, or foreign proteins fused to, the native virus coat protein results in their incorporation onto the surface of the viral particle (14). Yeast have also been exploited for surface display as they can enable correct folding of many mammalian proteins. Single-chain Fv antibody fragments referred to as intrabodies have been used for library expression in yeast (15). In some cases, still, the scaffold protein has to be engineered for proper display as the case of the T cell receptor illustrates. The T cell receptor was displayed on the surface only after mutation of specific variable region residues (16). Others have fused single-chain Fv antibody fragment-based combinatorial libraries to the Aga2p mating adhesion receptor (17, 18).

The biochemical characteristics and nature of proteins depend obviously on the environment in which they are expressed. The milieu heavily influences the three-dimensional structure, the activation status in the case of a catalytic protein, and the posttranslational modifications that the protein will undergo; this is especially relevant in the case of certain glycosylation residues that differ between human and mouse cells. Therefore, the localization and cell-specific expression of the library are critical and will influence the structure of what is displayed.

Here, we describe the construction of a peptide library expressed on the surface of mammalian cells. We fused the peptide library to the N terminus of the chemokine receptor CCR5. The CCR5 receptor, as a naturally membrane-localized molecule, is readily expressed in that compartment unless disrupted. A retroviral Moloney murine leukemia virus-based vector was used as a vehicle to transfer the library into mammalian cells and integrate it within the genome. The peptide library was constitutively expressed on the cell membrane. A FLAG epitope, purposely incorporated into the library, was readily rescued after the standard process of sorting and amplification as proof of principle. In a further test, a search for a peptide mimicking the FLAG epitope was undertaken, and we demonstrate the rescue of such an epitope from the library following subsequent rounds of sorting and amplification. This form of library could become a useful tool in the search for antibody mimetopes, cancer antigens, autoimmune decoys, adhesion molecules, and blocking agents. By expression of the library on the mammalian cell surface, it can become an at-
Random Peptide Library Expressed on Mammalian Cell Surface

Fig. 1. Library construct. A, retroviral backbone used as transfer vector for library delivery into mammalian cells. The vector pMGIB-CCR5 carries the blastocidin resistance gene (Blast.R) for selection of infected cells. The primers used for PCR rescue are represented by arrows at their approximate position. LTR, long terminal repeat; V, packaging signal; Figure B, as shown below; IRES, internal ribosome entry site. B, construct for FLAG epitope or library insertion. Shown are the open reading frame of the prolactin signal sequence and CCR5 gene, spacers flanking cysteines upstream and downstream peptide or library insertion site, and the sites of restriction enzymes used for the ligation of insert into pMGIB-CCR5 (FseI and AscI).

tractive tool for biological screenings in more contextual and physiological environments.

MATERIALS AND METHODS

Generation of Vector-carrying Library—The insert carrying the library was produced as described previously (19). Briefly, a single-stranded DNA template of the form 5' -ATACGGGGCCGGCAGAANNNKGGGGCGGCGCTTGGGATCCACAGGA-3', where N is an equimolar mixture of all four nucleotides, K is an equimolar mixture of G and T, and n is 9 or 13, was used to prime the reaction with the reverse primer 5'-TGGTCGGCAACCAAGGCGGGC-3'. Following T4 DNA polymerase extension, the resulting double-stranded DNA was purified in a low melting agarose gel (Invitrogen). DNA was then subjected to FseI and AscI restriction overnight. DNA was run again in a low melting agarose gel and ethanol purified. One g of a mixture of pMGIB-PL-CCR5-9 and pMGIB-PL-CCR5-13 were hybridized to 5'-GAGGCAGCCAAGGCGGGC-3' for direct ligation into the linear vector.

Infection of Cells with Library-carrying Virus—The viral supernatant carrying the library was produced as described previously (20). Briefly, 5 µg of a mixture of pMIGB-PL-CCR5-9 and pMIGB-PL-CCR5-13 were used to transfect each of three 10-cm phoenix-e plates using FuGENE 6 (Roche Applied Science). A total of 30 ml of viral supernatant was used to transfect each of three 10-cm phoenix-e plates with 0.5 µg/ml final concentration were added to each well. The plates were spun 108 supt-1 cells. The cells were distributed in a total of 16 6-well plates with 106 cells from each clonal clone. 106 cells were resuspended in fluorescence-activated cell sorting (FACS) buffer and analyzed by fluorescence-activated cell sorter (FACS) calibrator, BD Biosciences.

Purification of Genomic DNA—1-5 × 106 cells from each clonal population were spun and resuspended in phosphate-buffered saline to purify genomic DNA according to the manufacturer's protocol (DNeasy Tissue Kit, Qiagen).

PCR Rescue of Peptide Sequence—Genomic DNA from individual clones was used as template. A 5'-AGAGCTTACACAGTCCTGC-3' oligonucleotide that binds to the packaging signal within the retroviral backbone was used as forward primer. A 5'-TGTCGAGCGGATCGCT-3' oligonucleotide that binds within the CCR5 open reading frame was used as reverse primer. Following PCR reaction, the DNA products were analyzed by agarose gel. Relevant DNA bands were extracted (QIAEX II gel extraction kit, Qiagen) and sequenced.

RESULTS

Choice of Scaffold for Membrane Expression—To express the peptide library on the surface of a mammalian cell, two goals had to be achieved: (1) stable expression of the peptide library in a mammalian cell line; and (2) the efficient expression of the library members on the cell surface. For stable and constitutive expression, a retroviral shuttle vector system was used. For the expression on the surface of a mammalian cell, we chose to express the library as a fusion with an adaptor or scaffold molecule that is expressed on the surface. We tested several different surface expression systems, and the CCR5 system showed the most utility and robustness (data now shown). The scaffold chosen was the CCR5 chemokine receptor of the G-coupled, seven-transmembrane domain family of proteins.

As a first step, it was critical to assess the location of library insertion within the CCR5 gene. The signal leader sequence of the chemokine receptor is not well-determined; thus any change at the N terminus of the protein may disrupt the transfer of the mature protein to the endoplasmic reticulum and finally to the outer cell membrane. To locate the peptides in the library in the extracellular compartment of the cell, several sites within the CCR5 receptor were considered. These included the N-terminal region just downstream from the signal sequence and any of the three extracellular loops of the seven-transmembrane domain molecule. We took advantage of a construct (kindly provided by Warner Greene, Gladstone Institute, San Francisco, CA) that bears an artificial prolactin signal sequence fused to the N terminus of the CCR5 molecule. This construct, based on the pcDNA-3 (Invitrogen) mammalian expression vector, also bears an epitope tag at the point of fusion between the prolactin signal sequence and the CCR5 open reading frame; the sequence is YKDDDDY and is commonly

1 The abbreviations used are: PE, phycoerythrin; FACS, fluorescence-activated cell sorting; PL, peptide library; FITC, fluorescein isothiocyanate.
referred to as the FLAG tag (21). Because this construct has been previously shown to deliver efficient expression of CCR5 on the cell surface, we chose to introduce the library insert at that same position.

Construction of a Random Library—The general scaffold of the peptide sequence was first engineered. We constructed a library with a constrained structure (22–24). For that purpose, we introduced cysteine-codon residues at both the 5' and the 3' termini of the library insert (see Fig. 1A). This would presumably enable disulfide bond formation on the outer membrane and consequently would stabilize loop structure formation in the peptides. Upstream and downstream of each cysteine we incorporated a spacer sequence consisting of glycines, prolines, and alanines; this sequence was expected to impart flexibility to the structure and appropriate separation from the CCR5 molecule. Two restriction enzyme sites were added within the inner spacers to enable the insertion of the random or other desired sequence (see Fig. 1B).

The library was constructed according to the method described by Wolkowicz and Nolan (1). A single-stranded DNA template harboring the library sequence was primed with a reverse primer at a constant region. Following polymerization with T4 polymerase and restriction digest, the double-stranded DNA insert was purified. We constructed two libraries, one consisting of a random sequence of 9 codons and a second one with 13 codons. The 9-mer and 13-mer libraries are referred to

![Fig. 2. Library expression.](image)

**A**

Jurkat-e

PL-CCR5 (no selection)

Jurkat-e-fCCR5

PL-CCR5 (after selection)

![Forward scatter plots](image)

**B**

Jurkat-e

PL-CCR5 (no selection)

Jurkat-e-fCCR5

PL-CCR5 (after selection)

![Forward scatter plots](image)
as PL-CCR5-9 and PL-CCR5-13, respectively. The vector carrying the CCR5 scaffold, pMGIB-CCR5, was ligated to the peptide library (PL) inserts. Bacteria were transformed with the ligation reaction. A titration was performed to characterize the complexity of each library. The complexities obtained from PL-CCR5-9 and PL-CCR5-13 were $6.5 \times 10^7$ and $3.0 \times 10^7$, respectively. In this study, we used a library containing both peptide lengths (referred to as the mixed library). The functional complexity of this library was around $6.5 \times 10^7$. This is due to a theoretical 34% probability of getting a stop codon, according to expectations (1). As the control, we have also introduced the FLAG epitope in the same location as that of the random sequence, and it is referred to as fCCR5.

Expression of a CCR5 Scaffold via Retroviral Vectors—To express the library in a stable manner within mammalian cells, we used pMGIB, a Moloney murine leukemia virus-based vector (see Fig. 1A). This vector enables the production of virus by transfection into the Phoenix-e packaging cell line (see “Materials and Methods”). The virus produced was collected and used to infect naive Jurkat-e cells. The human Jurkat-e cell line was chosen as it does not naturally express the CCR5 receptor, harbors the ecotropic receptor for murine leukemia virus, and is readily infected by the packaging phoenix-e produced virus that is pseudotyped with the ecotropic murine envelope.

Cells were analyzed for the stability of the expression of the library on the surface. We aimed for an infection efficiency of 10–20% to avoid multiple infection events per cell, which would complicate rescue of the peptide of interest in the future. 2 days after infection the cells were analyzed for the rate of infection. The cells were further incubated in the presence of blasticidin for at least 8 days to enrich the population of infected cells. An antibody against the first loop of the CCR5 receptor was used to confirm the expression of CCR5 on the surface of the cell. Fig. 2A shows that although around 13% of the cells were positive for CCR5 before blasticidin selection, upon selection the percentage increased to 59%. This is absolutely in accordance with the functional versus general complexity of the library. Around 34% of the clones infected are resistant to blasticidin but carry stop codons because of the nature of the NNK library (1/32 chance of getting a stop codon) and consequently are not expected to express the CCR5 receptor. More importantly, the pattern of CCR5 expression shows that the introduction of the cysteine-cysteine scaffold did not affect the expression of the CCR5 protein on the surface.
Rescue of a FLAG epitope. A, FACS analysis of one individual clone rescued and amplified from the third round of selection of cells expressing the FLAG-containing library. Incubation with anti-FLAG-biotin (anti-FLAG.bio) antibody was followed by incubation with streptavidin-FITC (Strp.FITC). Streptavidin-FITC staining alone is shown as a negative control for nonspecific binding. B, gel electrophoresis of the PCR products rescued from genomic DNA with the primers represented on Fig. 1A. Jkt, Jurkat. C, one of the bands was purified and sequenced. The FLAG sequence rescued is shown. FSC-H, forward scatter.

Jurkat-e cells infected with the pMGIB-fCCR5 or pMGIB-PL-CCR5-9/13 virus were compared for scaffold expression 10 days after selection with blasticidin. Naive Jurkat-e cells were used as control. Jurkat-e, Jurkat-e-fCCR5, and Jurkat-e-PL-CCR5 cells were analyzed by FACS for the presence of the receptor on the cell surface (see Fig. 2B). Anti-FLAG and anti-CCR5 antibodies coupled to FITC were used for the analysis. The cells infected with fCCR5 were positive for both FLAG and CCR5 antibodies, as expected. The library-expressing cells are positive only for CCR5 and negative for the FLAG epitope. The parental cell line was negative for both.

Screening for a Rare Epitope in a Surface-expressed Library—We then determined whether the library could be screened for a phenotype of interest and whether the peptide responsible for that phenotype could be rescued. For that purpose the construct containing the FLAG epitope was added to the mixed library at a ratio of one FLAG construct to 10^7 random clones. Because we intended to show that it was possible to rescue this specific peptide when it was present once in 10^7 peptides actually expressed on the cell surface, 0.1 pg of pMGIB-fCCR5 was mixed with 1.3 mg of the mixed library. This ratio accounted for the presence of stop codons in the library, as described previously (1). This mixture of DNA was used to transfect Phoenix-e cells, and the virus produced by them was used to infect Jurkat-e cells. Two hundred million cells were originally screened for binding capacity of the anti-FLAG antibody. To reduce false positive binders, a biotinylated form of the anti-FLAG antibody (Sigma) and a secondary staining consisting of streptavidin coupled to PE were used. Positive populations were subsequently amplified and reanalyzed. This process was repeated three times with both the parental library and that same library into which the FLAG-expressing vector had been diluted. The enrichment factor of FLAG-positive cells in the FLAG-containing library was 10-fold after just one round, 3.6% (Fig. 3, Sort 2). After two rounds (Fig. 3, Sort 3) 10% of the cells were positive. This was in striking contrast with the control library, in which only a slight enrichment factor was observed, at least within the first two rounds.

Individual clones from Sort 3 (Fig. 3) of the FLAG-containing library were selected, amplified, and analyzed by FACS. Fig. 4A shows one of these clones. Genomic DNA was subsequently purified from several of such clones. This DNA was used as a template for PCR rescue of the insert of interest, and DNA product (Fig. 4B) was purified and sequenced, demonstrating that the sequence between the cysteines was indeed the FLAG epitope (Fig. 4C). The success of this selection process gave us confidence that this expression library would be of use in the selection of interesting biomolecules.

Rescue of a Peptide from the Original Library That Is Recognized by the Anti-FLAG Antibody—To further exploit the library and demonstrate its usefulness in biological screenings, we proceeded to rescue clones from the naive parental original library. Because an increase in enrichment was observed, although slight (Fig. 3, Sort 3), we repeated the procedure described above consisting of sort and amplification two additional times for a total of four rounds. Within the last sort; i.e. sort 5, we amplified individual clones. The clones were stained as described with biotinylated anti-FLAG antibody and streptavidin-PE and analyzed by FACS. Fig. 5A shows the clone rescued from sort 5 analyzed by FACS.

Genomic DNA from this clone was prepared, and the sequence obtained is shown in Fig. 5B. Interestingly, the rescued sequence NTAAKKDDWLGPT contains the partial FLAG motif KDD (out of the DYKDDDDY FLAG sequence). We repeatedly demonstrated that cells infected with the sequence showed reproducible increases in staining with the anti-FLAG antibody. Although the affinity of the anti-FLAG antibody to the rescued sequence referred to as “pseudo-FLAG” appeared to be slightly lower than to the consensus FLAG sequence, clear staining was observed (Fig. 5B). Thus, the pseudo-FLAG sequence that we isolated shows clearly that epitope mimics can be detected by this process at low frequency (presumably one per library), successfully enriched, and isolated.
Random peptide libraries can be thought of as libraries of shapes. The peptides expressed can affect their immediate surroundings by interacting with existing biomolecules and, as a result, influence signaling cascades or perform other useful functions. This may alter the phenotype of the cell, distinguishing it in a manner that is valuable for a variety of specific purposes. By the application of appropriate screening and selection processes one can find the peptide responsible for the phenotype of interest. Random peptides have been used in the classical approach of phage display as fusion decoys with the P-III protein of filamentous phages. This technology is essentially a refined in vitro system for the study of affinity interactions between a bait molecule and possible candidate partners within the library. Extension of this approach to mammalian cells could be beneficial for such purposes as developing ligands that work only in the context of cell-cell interactions, for instance.

Here we have expressed a random peptide library on the surface of mammalian cells. We believe this library will be useful in screens in which intracellular libraries, previously applied (12, 15, 25, 26) cannot be exploited. We have demonstrated the feasibility of this approach by fusing the random peptide sequence at the N terminus of the CCR5 chemokine receptor downstream of the signal sequence of prolactin. Other methods for expression on the cell surface could involve fusion to any natural receptor of choice, a glycosylphosphatidylinositol-anchored protein, or an artificial construct containing both a signal sequence for transfer into the endoplasmic reticulum and the membrane and a transmembrane domain for anchoring. Here we have taken a straightforward approach exploiting a naturally occurring receptor as a presentation scaffold.

Several considerations were necessary for a successful outcome in the current work. The choice of library insertion within the scaffold is as crucial as is the choice of scaffold itself. A poor insertion site could jeopardize protein expression or transport into the membrane, disrupt conformation, or all of the above. This is especially important when the scaffold protein is a receptor, at which naturally occurring signal sequences at the N terminus of the protein are often needed for transport but lost upon post translational modification. The FLAG epitope originally present at the N terminus of CCR5 in the mamma-

![Rescue of a pseudo-FLAG epitope. A, FACS analysis of one individual clone rescued and amplified from the fifth round of selection of cells expressing the parental library. Incubation with anti-FLAG-biotin antibody was followed by incubation with streptavidin-PE. Streptavidin-PE staining alone is shown as a negative control (−) for nonspecific binding. B, the sequence of the pseudo-FLAG is depicted, with the FLAG sequence underneath for comparison.](image-url)
lian expression vector gave a potential site for insertion that others observed did not disrupt the CCR5 expression on the surface of the target cell. Moreover, the FLAG epitope was a useful control as a known peptide member within the library. The random peptide library insert engineered as a 9-mer or 13-mer was introduced within a cysteine-cysteine scaffold flanked by spacer regions to stabilize loop formation. The FLAG tag within that same constrained structure showed high levels of cell surface expression, demonstrating an appropriate choice for site library insertion. Moreover, the constrained structure sustained perfect FLAG epitope presentation, a conclusion that could be well extended to other random sequences or mimetopes.

The utility of the library is measured not by the numbers of individual clones obtained nor by absolute levels of peptide expression per cell. Success is achieved when the library can be screened for a desired phenotype, as accomplished here. An fCCR5 construct was rescued after three rounds of selection using an anti-FLAG antibody. One might expect a longer process with more rounds of selection and amplification in binding assays in which the affinity is not as strong. The fact that we did not enrich for a FLAG-positive population in the original parental library within the first three sorts, at least in a robust manner, demonstrates that (a) the perfect FLAG epitope was not present in the random library or (b) a less than perfect FLAG epitope was present but needed for more rounds of selection and amplification to be detected. To explore this latter possibility, we screened individual clones amplified from screen/sort round 5 out of the parental library. We were successful in recovering a peptide recognized by the anti-FLAG antibody. Interestingly, this peptide of sequence NTAAKDDDDWGT reasonably resembles the FLAG epitope, as it contains the KDDE motif out of the DYKDDEYY DFLAG amino acid sequence. This peptide, referred to as pseudo-FLAG, was indeed a member of the random library, as demonstrated by the feature of its DNA sequence. Although the affinity of the anti-FLAG antibody to the pseudo sequence seems to be much lower than to the original FLAG sequence, the rescue of such a peptide demonstrates the utility of the membrane-expressed peptide library for biological screenings.

A peptide library expressed on the surface of the mammalian cell could become a tool in the search for ligands or novel decoys for biological interactions including antibody mimetopes, cancer antigens, or autoimmune decoys. It would be easy to imagine the construction of such a library based on the genetic sequence of a gene or genes of interest. This kind of library could then be screened against polyclonal antibodies that recognize the protein products. This could lead to the discovery of stronger epitopes that could be used in vaccinations. Furthermore, some of the peptides may serve as potent pharmacophores, lowering the affinity binding to virus or other ligands or having physical properties that render them valuable for analysis. The library expressed on the cell surface has an important advantage. Because the library is displayed in a mammalian cell environment, it can be used for the discovery of binding partners as has been done using phage display. It can also be in biological screens for ligands that influence downstream events because of the change in binding affinities or binding partners. For example, effects on signaling pathways as a result of such binding can be evaluated in the selection scheme. The expression of the library on the mammalian cell can harbor all the posttranslational modifications that occur in the eukaryotic environment. Moreover, such a library could be used for cell-cell interaction biological assays in *ex vivo* and *in vivo* setups. One could also envision future development of a mammalian cell-based two-hybrid system for cell activation assays as well as induction of signaling pathways. Mammalian peptide display can couple both genotype and phenotype tightly, as the same cell expressing the right “hook” can be the cell under investigation with its own unique behavior that distinguishes it from its neighboring cells. We expect to apply this system in the future to create a library of peptides in search of membrane-binding inhibitors of human immunodeficiency virus 1 (HIV-1). By virtue of physical proximity between the main receptor CD4 and the CCR5 coreceptor (used here as scaffold), we believe that specific sequences at the N terminus of CCR5 will interfere with viral envelope binding to the receptor-coreceptor complex. The library, as described above, will also be used for the search of antibody mimetopes, among them human immunodeficiency virus 1-neutralizing antibodies such as IgG-b12 (27). Such mimetopes could be further used as vaccine candidates.

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