Combinatorial surrobody libraries

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A unique type of combinatorial protein libraries has been constructed. These libraries are based on the pre-B cell receptor (pre-BCR). The pre-BCR is a protein that is produced during normal development of the antibody repertoire. Unlike that of canonical antibodies, the pre-BCR subunit is a trimer that is composed of an antibody heavy chain paired with two surrogate light chain (SLC) components. Combinatorial libraries based on these pre-BCR proteins in which diverse heavy chains are paired with a fixed SLC were expressed in mammalian, *Escherichia coli*, and phagemid systems. These libraries contain members that have nanomolar affinity for antigen. We term this type of antigen-binding protein a "surrobody" to distinguish it from the canonical antibody molecule.

antibody engineering | combinatorial antibody libraries | surrogate light chain

Combinatorial antibody libraries allow the synthesis and selection of very large numbers of dimeric antibodies *in vitro* (1–6). Such libraries, whose size can exceed the natural repertoire by many orders of magnitude, offer several advantages over conventional methods for finding antibodies, in that they allow access to rare specificities, can be fully human, and are not limited by systemic constraints such as immunological tolerance (1). The latter aspect is of particular importance, because many therapeutic antibodies, such as the combinatorial antibody library-derived anti-TNF antibody Humira (in clinical use for the treatment of rheumatoid arthritis), are directed against self antigens, where production in humans would normally be forbidden because of self tolerance (1).

The success of such combinatorial antibody libraries and the attendant thinking that the production of therapeutic antibodies is now simply an engineering problem have naturally led scientists to ponder how one might improve on the antibodies themselves, rather than simply increasing their numbers in a library (7–9). Recently, there has been a particular emphasis on some intriguing alternative protein scaffolds that might be used to generate reagents equal to or better than antibodies for specific purposes such as access to the central nervous system or to intracellular compartments where conventional antibodies, for the most part, have not been successful. However, given that the immune system has evolved to generate selective and high-affinity binding, we reasoned that its potential should continue to be explored, because one starts with a system of binding proteins whose sophistication and breadth might be difficult to duplicate. Toward this end, it is remarkable that the immunological proteins that are the developmental precursors to mature antibodies have not yet been put into service for improving or expanding antibody libraries. To understand this potential, one must consider the developmental challenge in shaping the immunological repertoire and the nature of the protein molecules that are used to solve the problem of selective high-affinity antigen recognition.

The overall problem for the development of the mature B cell repertoire is to recombine the large number of germ-line antibody genes for expression of mature antibodies so that each B cell expresses a unique antibody on its surface. During this process, imperfect heavy chains (HCs), nonfunctional V_H-V_L pairings, and cells that express antibodies to self antigens must be eliminated at the pre-B cell stage of development. Over the last 20 years, the mechanism by which this is accomplished has largely been elucidated (10). The central feature of this mechanism involves the assembly of a pre-B cell receptor (pre-BCR) at the pro-B–pre-B cell junction of the developmental B cell cascade (10). The pre-BCR has a structure different from that of mature Ig. When the signal-transducing Iga/Igb dimeric complex is excluded, the pre-BCR structure can be said to be composed of two μHCs and two surrogate light chains (SLC) (11–20). The SLC is a nondiversified heterodimer composed of the noncovalently associated Vpre-B and λ5 proteins. The VpreB chain is homologous to a Vλ Ig domain, and the λ5 chain is homologous to the Cλ domain of canonical antibodies, respectively. The heterodimeric SLC is covalently associated with the HC in the pre-BCR complex by disulfide bonds between the Cλ domain and the first constant domain of the pre-BCR HC.

A unique feature of the SLC is that the VpreB1 and the λ5 domains each have noncanonical peptide extensions. VpreB1 has an additional 21 residues on its C terminus, and λ5 has a 50-aa-long tail on its N terminus (10). Although not completely understood, these non-Ig peptide extensions are thought to play a key role in the cell biology of the pre-B cell checkpoint with particular reference to trafficking through cellular compartments, signaling, and quality control of the many Ig molecules that ultimately will be added to the repertoire (14, 16, 17, 19).

Many aspects of the pre-BCR-like constructs then make them an attractive candidate for the construction of combinatorial libraries. First, although it is not an antibody, its components are derived from classical Ig domains, and thus one starts with structures that are homologous to nature's most highly evolved antigen recognition system, the antibody. Although one might be initially dissuaded because the endogenous SLC is not inherently diverse, this is nowadays not a problem, because unlimited diversity can be incorporated into the SLC protein loops by genetic engineering in much the same way as affinity maturation is accomplished for antibodies derived from combinatorial libraries (1). Second, that these pre-BCR-like constructs have three components rather than the two of classical antibodies should lend them to the construction of very large combinatorial trimeric protein libraries. Such libraries, therefore, will exceed the diversity of antibody libraries by a factor that equals the number of components. Thus, a combinatorial antibody library of 1.0×10^6 HCs and 1.0×10^6 light chains will yield a library of 1.0×10^{12} members, whereas a surrobody library of 1.0×10^6 μ HCs, 1.0×10^6 VpreB chains, and 1.0×10^6 λ5 chains will yield a library of 1.0×10^{18} members. The advantage of a larger system is not so much related to the absolute library size that can be achieved but rather concerns the achievable size relative to the transfor-

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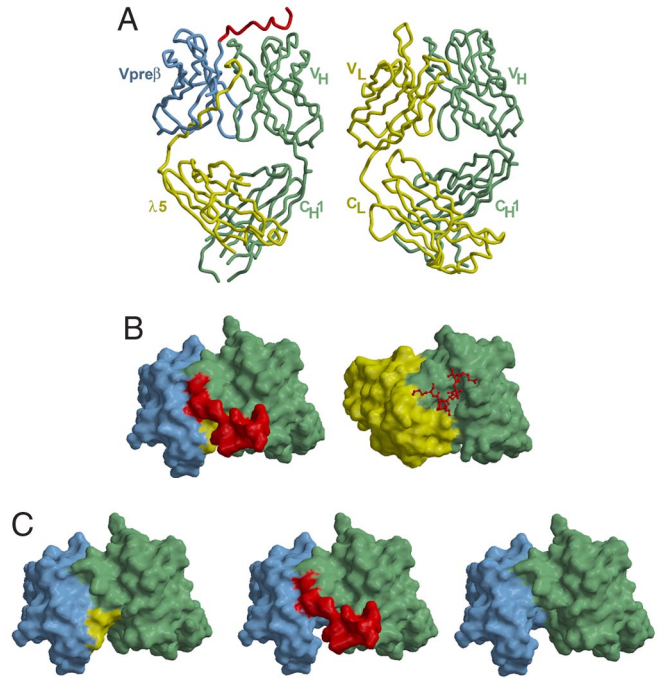

Fig. 1. Structural comparison of antibodies and surrobodies. The antibody used for comparison is a myohemerythrin antipeptide antibody. The base structure used for the surrobody is the pre-BCR structure (21). (*A*) The arrangements of the Ig domains of the pre-BCR (*Left*) and antibodies (*Right*) are compared. The VpreB1 domain (blue) and the λ5 domain (yellow), and HC (green) are indicated. The peptide extension of VpreB1 is shown in red (*Right*). Antibody domains (*Right*) light chain (yellow), and HC (green) are indicated. (*B*) View of the antigen-binding surface of a surrobody (*Left*) compares favorably with that of an antibody (*Right*). Surrobody domains: VpreB1 domain (blue), the λ5 domain (yellow), and HC (green) are indicated. The peptide antigen bound to the antibody is shown in red. (*C*) Surrobody variants with peptide extensions removed: [minus VpreB1 peptide extension (*Left*), minus λ5 peptide extension (*Middle*), minus both VpreB1 and λ5 peptide extensions (*Right*)]. The VpreB1 peptide domain (red), the λ5 domain (yellow), and HC (green) are indicated.

mation frequency of the organism in which the library will be expressed.

Here, we report the construction of pre-BCR like proteins that bind a selected antigen with high affinity from the bone marrow of patients immunized by infection. These affinities were achieved without maturation of the VpreB1 or λ5 chains. Combinatorial pre-BCR like libraries were successfully expressed in *Escherichia coli* and on the surface of M13 phage, where antigen-binding proteins were selected. We term these libraries "surrobody libraries" in recognition of their unique SLC component and to distinguish them from antibody libraries.

Structural Comparison of B Cell and pre-BCRs. Over the years, there has been much discussion and experimentation surrounding several aspects of the relationship between B cell and pre-BCRs (10, 11, 14, 16). These discussions have centered on the structural relationships between the molecules, the role of the peptide extensions on the (SLC components, and whether antigen binding by the pre-BCR was important to its function as a "quality control" element in B cell development. Although many biological questions remain, the recent elegant crystallographic structure determination of the pre-BCR has allowed it to be compared in detail to conventional antibodies (Fig. 1) (21). This x-ray structure provides a road map for the initial construction of surrobody libraries and how they might be further elaborated to provide unique functions that go beyond the capabilities of ordinary antibody molecules (Fig. 1). Overall, many structural similarities exist between the pre-BCR and conventional antibodies (Fig. 1). There should be no *a priori* reason why antigen binding by the pre-BCR should be excluded, if diversity is engineered into the SLC.

Surrobody Constructs and Expression. We engineered a series of vectors that encoded pre-BCR-like protein variants that might bind antigen (Fig. 2). The vectors were designed for mammalian and *E. coli* expression and for phage display formats. These formats included the native trimeric pre-BCR-like functional unit of the pre-BCR, fusion of VpreB1 to λ5, and trimers that eliminated either the λ5 N-terminal 50 aa or the VpreB1 C-terminal 21 aa or both

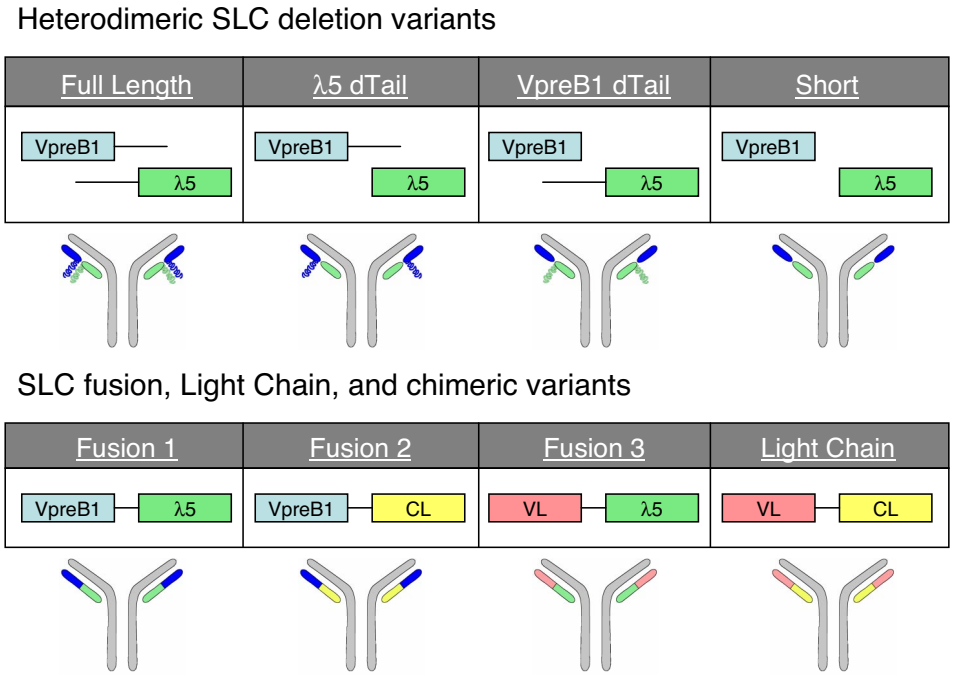

Fig. 2. Graphical depiction of surrobody variants (*Upper*) and fusions *(Lower)* produced for this study.

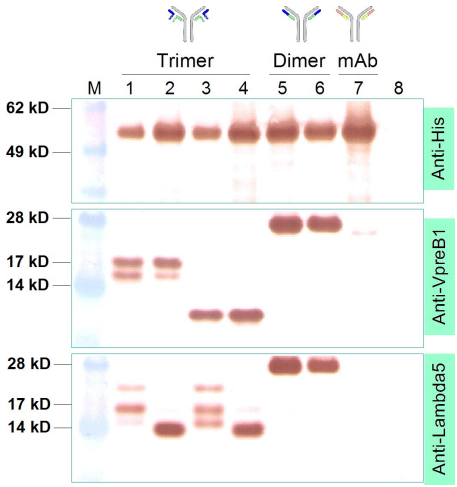

Fig. 3. Purified SLC variants and fusions form stable soluble complexes. Gel loading was as follows: molecular mass marker (lane M), full length surrobody (lane 1), λ5dT variant (lane 2), VpreB1dT variant (lane 3), short variant (lane 4), fusion 1 (lane 5), fusion 2 (lane 6), kappa-type parental antibody (lane 7), and mock transfection (lane 8). Surrobody components were analyzed by Western blot as follows: HCs (*Top*); VpreB1 variants and fusions, (*Middle*); and λ5 variants and fusions (*Bottom*). Specific detection of HCs was accomplished with anti-His-tagged full length HC antibodies, whereas VpreB1 and λ5 antibodies were detected with hyperimmunized rabbit sera raised against purified peptides conjugated to KLH. The anti-λ5 sera was raised against a 15-aa peptide near the C terminus. This differs from Cλ by only a single amino acid; thus, the antisera also efficiently recognizes Cλ fusions.

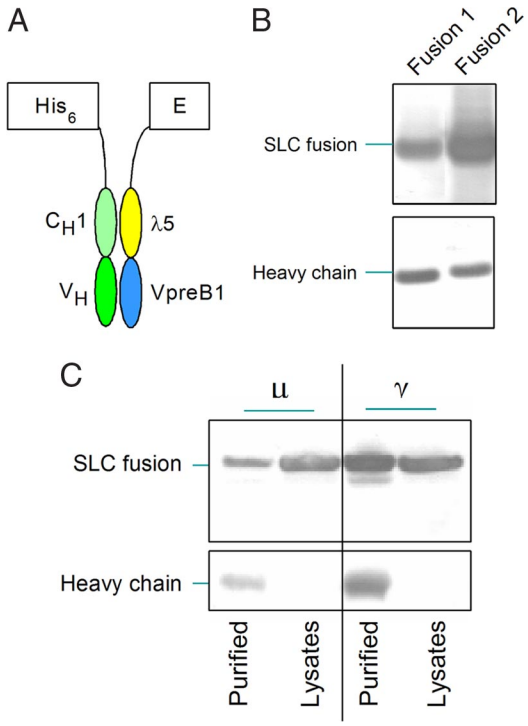

Fig. 4. Surrobody fusions are secreted in *E. coli* and associate better with HC fragments containing CH1 domains from IgG vs. IgM to form Fab-like complexes. (*A*) Graphical depiction of a surrobody fusion, with C-terminal epitope tags in a Fab-like complex. (*B*) *E. coli* periplasmic lysates were prepared and SLC fusions 1 and 2 (*Upper*) and HCs (*Lower*) were analyzed by Western blots using anti-E tag and anti-his tag antibodies, respectively for detection. (*C*) Fusion 2 surrobodies were analyzed as purified proteins and as crude lysates, before Nickel chelate chromatography. SLC fusion 2 (*Upper*) and HCs (*Lower*) were detected by Western blot analysis using anti-E tag and anti-his tag antibodies, respectively.

peptide extensions. In addition, we made chimeric constructs using the constant components of classical antibody light chains (Fig. 2).

A human HC from an antiinfluenza H5N1 hemagglutinin antibody was used for this part of the study (22). When paired with the full length HC, the complete pre-BCR trimer and its variants expressed well in mammalian cells, although the full length surrobody trimer appeared to express less well (Fig. 3).

Constructs in which VpreB1 was fused to λ5 with the requisite loss of both peptide extensions also expressed well in *E. coli*, when paired with the V_H-C_H1 fragment of the HC (Fig. 4). In general, expression was improved for all systems when the λ5 peptide extension was removed (Fig. 3, lanes 2 and 4). For the particular HC used here, it appeared that the surrobody fusions and the construct in which the peptide extensions of the VpreB1 and the λ5 proteins were both removed (short) gave the best expression. The observation of multiple bands for the expressed constructs that contained the VpreB1 and λ5 peptide extensions likely indicates that there is some proteolysis of the peptide tails (lanes 1-3, Fig. 3). Presumably, the proteolytic sites can be removed when the peptide tails are diversified for improved binding affinity and/or generation of new biological functions (see below).

To determine whether the choice of the HC C_H1 isotype was important, Fab-like dimers were constructed. HCs that used either μ or γ sequences were combined with either fusion 1 or fusion 2 SLCs and studied for their efficiency of expression in *E. coli* (Fig. 4*B*). For these constructs, chain association appeared to be more efficient when the γ C_H1 isotype was used (Fig. 4*C*). This was unexpected, because in the pre-B cell, the SLC is paired with an IgM HC to form the pre-BCR.

Assembly of Surrobodies on Phage Surfaces. A phage-capture assay was used to study whether the HC paired with the VpreB1-λ5 fusion could be expressed on phage [supporting information (SI) Fig. S1]. To provide immunological markers for the assay, the VpreB1-λ5 fusions and the HCs were tagged with an E or His epitope, respectively (Fig. 4*A*). As expected, the anti-His tag antibody whose epitope is located between the HC, and the phage gene III protein efficiently captured the phage particles. Likewise, the anti-E antibodies whose epitopes are located on VpreB1 also captured the phage, indicating a stable association of both components of the dimer. Using similar assays, it was demonstrated that the full trimeric surrobody could also be assembled on phage surfaces, although its assembly seemed to be less efficient that that of the fusions (data not shown; see below).

Antigen Binding by Surrobodies. Initially, an iterative approach using combinatorial antibody libraries was used to study whether surrobodies could bind antigen with high affinity. Several combinatorial antibody libraries prepared from the bone marrow of Turkish patients that survived H5N1 influenza virus infection were created (22). Large numbers of classical antibodies that bound the viral hemagglutinin were selected from these libraries. The HCs from the previously selected antibodies were then recombined with various SLC fusions to prepare the protein constructs illustrated in Fig. 2 *Lower*. This is a complementation strategy similar to that sometimes used to improve the binding energy of antibodies selected from patients (1). For example, Humira was generated by shuffling large numbers of heavy and light chains against each other by using a mouse antibody that bound TNFα as an antigen (23).

The surrobodies generated from the six constructs (Fig. 2) were tested by ELISA for their ability to bind H5N1 influenza virus coated wells. CHOK1 cells were transfected, and the expressed surrobodies were purified from supernatants using Ni-NTA agarose. The surrobodies from all six variants were shown by ELISA to bind antigen (Fig. 5). In those constructs where the VpreB1 and

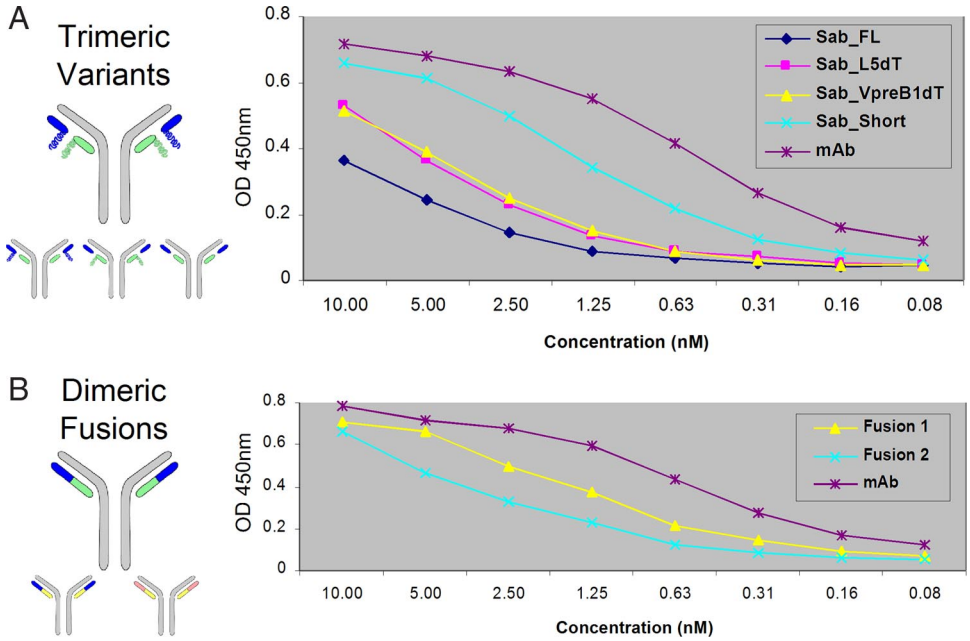

Fig. 5. Mammalian expressed surrobodies bind antigen. Purified surrobodies were titered against H5N1 Vietnam 1203/04 virus. (*A*) Trimeric surrobody variants were compared with bona fide antibodies and quantitatively detected by anti-HC Fc antibody. (*B*) Dimeric surrobody fusions were compared with bona fide antibodies selected from antibody libraries and similarly quantitatively detected by anti-HC detection, as used above.

λ5 peptide extensions are possible, binding is best when the peptide tails of both VpreB1 and λ5 are removed. However, in these constructs, the peptide extensions were not diversified. We expect that, when the extensions are diversified, the loss of binding energy will be at least returned and likely improved (see below).

To establish that surrobodies expressed in *E. coli* could also bind antigen, surrobody fusions 1 and 2 (Fig. 2 *Lower*) were studied. As with proteins expressed in mammalian cells, purified surrobodies expressed in *E. coli* also bound influenza virus hemagglutinin antigen (Fig. S2). The affinity of a variety of surrobody constructs compared with antibodies is shown in Table S1. The affinities of fusions 1 and 2 surrobodies against hemagglutinin were between 150 and 270 and 250 and 400 nM, respectively, as compared with 1 nM for the parent Fab (F5). In a second comparison with a different HC, where the affinity of the parent Fab (B11) was 13 nM, the affinities of surrobody fusions 1 and 2 were 10–21 and 15–22 nM, respectively, indicating that the binding energy of surrobodies can be comparable to that of canonical antibodies.

To show that surrobody binding was not simply attributable to the HC alone (Fig. S2), we compared the ability to bind H5N1 antigen of phage expressing only HCs to those where the HCs were paired with SLC fusions or SLC trimers (Fig. 6). We found that the phage that had HCs only did not appreciably bind antigen. The number of productive antigen-binding phage for fusions 1 and 2 was comparable to the parent Fab and better than for the full surrobody trimer, where the doubly truncated short SLC construct appreciably bound antigen. The observed differences in the numbers of productive phage produced in the case of fusions vs. noncovalently linked partners are not unlike what is generally seen when antibody single chains are compared with heterodimeric Fab constructs expressed on phage. *In toto*, these studies demonstrate that a variety of surrobody constructs can bind antigens. Importantly, surrobodies are efficiently expressed in systems that are generally used for the selection of antibodies from very large libraries (1).

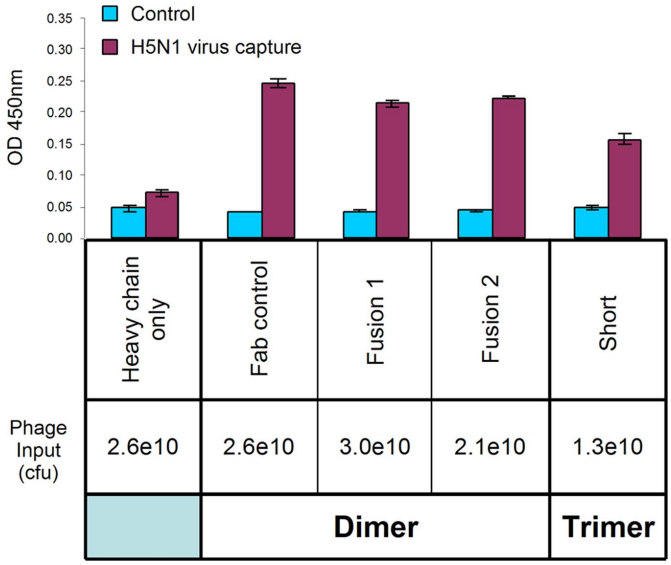

Fig. 6. Surrobodies expressed on the surface of phage bind viral antigen. Antigen binding by surrobody fusions (dimer) and trimeric surrobodies (trimer) expressed on the surface of phage were compared with Fab molecules expressed on phage by phage ELISA using antiphage antibody, as indicated. Absolute phage input was independently assessed by infectivity titrations of each phage preparation.

Selection of Surrobodies from Phage Combinatorial Libraries. Because a variety of surrobody constructs that bound antigen could be expressed in *E. coli*, it appeared they would be suitable for use in display systems. Nevertheless, it was important to establish that surrobodies that bound antigen could be directly selected from combinatorial libraries. We studied a phage system, because it is, arguably, the most powerful display system currently in use, and it lends itself to selection of binding events from the very large libraries that are anticipated for surrobodies. Also, we could directly compare the nature of surrobodies and antibodies selected from the same combinatorial libraries.

We chose to demonstrate the potential of surrobody libraries for antibody selection using combinatorial antibody libraries previously constructed from the bone marrow of patients that survived

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Table 1. The unique translated heavy chain protein sequences of a collection of similar clones isolated from surrobody library panning, related to heavy chains of known neutralizing antibodies

Group 1 heavy chains	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-56	FR3 59-92	CDR3 93-101	FR4 102-113
Vh1e	QVQLVQSGAEVKKPGSSVKVSCKASGGTF	SSYAIS	WVRQAPGQGLE	WMGGIIPIFGTAN	YAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYC	ARGSYYYESSLD	YWGQGTLVTVSS
1	----E----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---K-------
2	----H---T----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
3	----H---T----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
4	----Q----------R---T-----	----VT	----------	-----GM---T-	--------L-----EM----------	--------TT-	------M-----
5	----------S-----	--N-FT	----------	-I----GM---T-	----------ELR----------	----------	----------
6	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D------	----------	---K-------
7	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D------	----------	----------
8	----------T-----	----VT	----------	---A--GM---T-	----------EL----------D------	----------	------M-----
9	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---K-------
10	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
11	----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	------M-----
12	----------T-----	----VT	----------	-----GM---T-	----------EM----------	----------	------M-----
13	----------TT----	----VT	----------	-----GM---T-	----------EM----------	----------	----------
14	----------R---T-----	----VT	----------	------GM---T-	--------L-----EM----------	--------TT-	------M-----
15	E----------A--K---A-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
16	E----------	----VT	----------	---A--GM---T-	----------L---EL----------	----------	------M-----
17	E----------T-----	----VT	----------	---A--GM---T-	----------EL----------D------	----------	----------
18	E----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---K-------
19	E----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	----------
20	E----------T-----	----VT	----------	---A--GM---T-	----------L---EL----------	----------	---R--M-----
21	E----------T-----	----VT	----------	-----GM---T-	----------EL----------	----------	----------
22	E----------R---T-----	----VT	----------	-----GM---T-	--------L-----EM----------	--------TT-	---K-------
23	E----------RE----------T-----	----VT	----------	------GM---T-	----------EL----------	----------	------M-----

The clones containing heavy chains that are identical to those isolated from traditional antibody libraries panned against the same antigen are highlighted in grey (22). The remaining heavy chain sequences in the table were uniquely found in the surrobody libraries. Vh1e (top row) is the most closely related germline sequence to the canonical antibody heavy chains and is shown for reference.

infection with the H5N1 influenza virus and panned against the H5N1 hemagglutinin protein (22). During panning of this library, we typically observed significant enrichment after three to four rounds of selection. For construction of the fusions 1 and 2 SLC surrobody libraries, we used a HC pool previously selected by two rounds of antibody panning. As with antibody libraries, selective enrichment of surrobodies was seen in successive rounds of panning, but because surrobody HCs had been enriched through two rounds of antibody panning, enrichment was already observed within the first two rounds of selection (Table S2). Each surrobody library produced a set of unique solutions for the binding of viral hemagglutinin. We analyzed 380 clones by ELISA against H5N1 influenza virus antigen (Fig. S3). Nucleotide sequencing was carried out on 188 of the clones, and of these, 23 unique sequences were found (Table 1). Sequence analysis of the clones showed that each library produced both a set of common and unique solutions for binding viral hemagglutinin. Not surprisingly, many sequences were identical to previously identified HCs selected for antibody binding (22). However, numerous related sequences were found, indicating that HC pairing with the SLC is not only allowable but is also capable of providing novel solutions to binding.

Discussion

The main result reported here is that pre-BCR-like molecules that bind antigen with high affinity can, in fact, be constructed. There may be several reasons why our strategy was successful. The most obvious is that we started with antibody libraries that contained HCs with high affinity for antigen. It is well known that V_H domains can bind antigen (3), but if one thinks about our strategy more carefully, the answer may be derivative of some of the central features of combinatorial antibody libraries. In this regard, there are two main points. First, one of the main advantages of combinatorial antibody libraries is that large numbers of pairing events can be simultaneously studied for the generation of functional antibody molecules. Thus, even if functional pairings are relatively rare, the process of selective enrichment can discover them. Second, if one takes a combinatorial library-against-library approach, it is possible to start with a large collection of HCs that already have been shown to partner promiscuously. Such libraries are preadapted for the rapid discovery of effective binding solutions and combine a favored set of HCs with a diverse set of SLCs. These concepts concerning promiscuous HCs are strongly supported by the fact that the same HCs were selected from combinatorial antibody and surrobody libraries. Of course, the other factor is that in these selection processes, most of the binding energy may be derived from the HC partner. As discussed below, this bodes well for increasing the binding energy by diversification of the two chains of the SLCs that are at present unitary.

The initial data reported here concerning construction of surrobody libraries are encouraging. Simply put, pairing HCs with some of the SLC variants yielded trimeric protein complexes with nanomolar affinities. Nevertheless, we see many ways to improve the affinity of surrobodies mainly by introducing diversity into the SLCs and/or by taking advantage of their unique structure. As indicated above, the most obvious way to increase the diversity of surrobodies is to randomize the otherwise invariant protein loops of the SLC as one does for conventional light chains (1). This is easily accomplished by any number of modern genetic engineering methods. Another way to increase diversity is to reconstruct the missing CDR3 region that would be present in a mature antibody light chain, but that in the pre-BCR is replaced by the VpreB1 and λ5 peptides. However, given that the HC diversity is already large, we feel that the largest opportunity resides with introducing variations into the peptide extensions of the VpreB1 and λ5 proteins. In this regard, an important result from these studies is the observation that surrobodies that contained the VpreB1 and λ5 peptides could still bind antigen. The VpreB1 and λ5 peptides could be replaced with peptides that inure to enhanced binding and/or unique effector functions. One might imagine construction of combinatorial libraries of libraries where antibody and peptide libraries selected for binding to a given target are combined. Surrobodies from such libraries could have enhanced binding energy because, in addition to that achieved by the ordinary pre-BCR complex, there would be the enthalpic advantage of having two or more selected binding peptides in place of the VpreB1 and λ5 peptide tails. There also could be entropic disadvantages to untethered peptide tails if they are unstructured in the absence of antigen. Also, they might be subject to proteolysis. Nevertheless, that the length of the VpreB1 and λ5 peptide tails is greater than most of the conventional CDR loops of antibodies, and they are not subject to the constraints imposed by the overall antibody fold, may allow them to be used to greatly increase the size of the protein surface that can be used for antigen binding. Because binding energy is inherently a function of the absolute amount of

protein surface used for binding to antigen, the potential that surrobodies have for generation of additional binding surface may allow them to break the binding affinity ceiling of antibodies for proteins; for most conventional antibodies, this ceiling is limited by the ≈700–1,000 A^2 of protein surface that can be used to interact with protein antigens. Although the affinity ceiling for naturally induced antibodies that bind to proteins is nanomolar to high picomolar, much higher affinities can be observed for engineered antibodies (24) and/or antibodies to haptenic structures, such as fluorescein (25). The even higher binding affinities close to femtomolar that may be achieved by surrobodies could permit their use in systems that are not presently accessible because the antigens of interest are present only at very low concentrations.

The peptide extensions may provide many innovative functions besides the high-affinity selective binding of the surrobody. For example, the use of cell penetrating sequences could cause the surrobodies to be internalized so that the heretofore largely inaccessible intracellular proteome of antigens can now be addressed for therapeutic purposes. The peptide extensions are long enough to form metal-binding sites, carry effector functions, or be endowed with catalytic or autocatalytic properties. They can be engineered to be cleavable by selective proteases, which are, for example, expressed only by the cell that also expresses the antigen. The generation of such surrobodies will depend on the ability to select molecules where the presence of the peptide does not significantly diminish binding to the target antigen. Finally, we expect a continuing evolution in our understanding of the biological role that the VpreB1 and λ5 proteins play in lymphocytes. It is possible that they themselves may perturb cellular proliferation or function and/or be toxic and, thus, may find therapeutic use, particularly in lymphoma or other cancers.

Materials and Methods

A description of the library construction, selective enrichment, and biophysical characterization of library members is detailed in *SI Materials and Methods*. Experimental procedures for serological analysis and antibody-binding studies are also provided.

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1. Lerner RA (2006) Manufacturing immunity to disease in a test tube: The magic bullet realized. *Angew Chem Int Ed* 45:8106–8125.
2. Huse WD, *et al.* (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246:1275–1281.
3. Ward ES, *et al.* (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* 341:544–546.
4. McCafferty J, *et al.* (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552–554.
5. Marks JD, *et al.* (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. *Bio/Technology* 10:779–783.
6. Collet TA, *et al.* (1992) A binary plasmid system for shuffling combinatorial antibody libraries. *Proc Natl Acad Sci USA* 89:10026–10030.
7. Nord K, *et al.* (1997) Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. *Nat Biotechnol* 15:772–777.
8. Nygren PA, Uhlen M (1997) Scaffolds for engineering novel binding sites in proteins. *Curr Opin Struct Biol* 7:463–469.
9. Sandstrom K, Xu Z, Forsberg G, Nygren PA (2003) Inhibition of the CD28-CD80 costimulation signal by a CD28-binding affibody ligand developed by combinatorial protein engineering. *Protein Eng* 16:691–697.
10. Vettermann C, Herrmann K, Hans-Martin J (2006) Powered by pairing: The surrogate light chain amplifies immunoglobulin heavy chain signaling and pre-selects the antibody repertoire. *Semin Immunol* 18:44–55.
11. Karasuyama H, Kudo A, Melchers F (1990) The proteins encoded by the VpreB and lambda 5 pre-B cell-specific genes can associate with each other and with mu heavy chain. *J Exp Med* 172:969–972.
12. Kudo A, Melchers F (1987) A second gene, VpreB in the lambda 5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. *EMBO J* 6:2267–2272.
13. Pillai S, Baltimore D (1988) The omega and iota surrogate immunoglobulin light chains. *Curr Top Microbiol Immunol* 137:136–139.
14. Tsubata T, Reth M (1990) The products of pre-B cell-specific genes (lambda 5 and VpreB) and the immunoglobulin mu chain form a complex that is transported onto the cell surface. *J Exp Med* 172:973–976.
15. Melchers F (1999) Fit for life in the immune system? Surrogate L chain tests H chains that test L chains. *Proc Natl Acad Sci USA* 96:2571–2573.
16. Kudo A, Sakaguchi N, Melchers F (1987) Organization of the murine Ig-related lambda 5 gene transcribed selectively in pre-B lymphocytes. *EMBO J* 6:103–107.
17. Roth PE, DeFranco AL (1996) Receptor tails unlock developmental checkpoints for B lymphocytes. *Science* 272:1752–1754.
18. Torres RM, *et al.* (1996) Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science* 272:1804–1808.
19. Gong S, Nussenzweig MC (1996) Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. *Science* 272:411–414.
20. Guo B, *et al.* (2000) Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. *Immunity* 13:243–253.
21. Bankovich AJ, *et al.* (2007) Structural insight into pre-B cell receptor function. *Science* 316:291–294.
22. Kashyap AK, *et al.* (2008) Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc Natl Acad Sci USA* 105:5986–5991.
23. Jespers LS, *et al.* (1994) Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. *Bio/Technology* 12:899–903.
24. Boder ET, Midelfort KS, Wittrup KD (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci USA* 97:10701–10705.
25. Mallender WD, Carrero J, Voss EW, Jr (1996) Comparative properties of the single chain antibody and Fv derivatives of mAb 4–4-20. Relationship between interdomain interactions and the high affinity for fluorescein ligand. *J Biol Chem* 271:5338–5346.