Protein Engineering, Design & Selection vol. 19 no. 6 pp. 255–264, 2006
Published online March 20, 2006 doi:10.1093/protein/gzl008

Development of a novel strategy for engineering high-affinity proteins by yeast display

S.A.Richman[1], S.J.Healan[1], K.S.Weber[1], D.L.Donermeyer[2], M.L.Dossett[3,4], P.D.Greenberg[3,4], P.M.Allen[2] and D.M.Kranz[1,5]

[1]Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Avenue, Urbana, IL 61801, [2]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO 63110, [3]Department of Immunology, University of Washington, and [4]The Division of Clinical Research, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, USA

[5]Author to whom correspondence should be addressed.
E-mail: d-kranz@uiuc.edu

Yeast display provides a system for engineering high-affinity proteins using a fluorescent-labeled ligand and fluorescence-activated cell sorting (FACS). In cases where it is difficult to obtain purified ligands, or to access FACS instrumentation, an alternative selection strategy would be useful. Here we show that yeast expressing high-affinity proteins against a mammalian cell surface ligand could be rapidly selected by density centrifugation. Yeast cell–mammalian cell conjugates were retained at the density interface, separated from unbound yeast. High-affinity T cell receptors (TCRs) displayed on yeast were isolated using antigen presenting cells that expressed TCR ligands, peptides bound to products of the major histocompatibility complex (MHC). The procedure yielded 1000-fold enrichments, in a single centrifugation, of yeast displaying high-affinity TCRs. We defined the affinity limits of the method and isolated high-affinity TCR mutants against peptide variants that differed by only a single residue. The approach was applied to TCRs specific for class I or class II MHC, an important finding since peptide-class II MHC ligands have been particularly difficult to purify. As yeast display has also been used previously to identify antigen-specific antibodies, the method should be applicable to the selection of antibodies, as well as TCRs, with high-affinity for tumor cell-surface antigens.

Keywords: directed evolution/major histocompatibility complex/T cell receptor/yeast display

Introduction

Cell surface receptors perform critical functions in the communication of extracellular information to the inside of the cell. Receptors for soluble molecules, including hormones, growth factors, or cytokines, control properties that range from cellular proliferation to cell migration. Other receptors function in cell-to-cell communication by interacting with a cognate ligand that is expressed on the surface of an opposing cell. Frequently, such receptor–ligand pairs exhibit very low affinities that can make biochemical and structural analyses difficult (Maenaka *et al*., 1999). By engineering the affinities or kinetics of such interactions it is possible to explore the mechanisms that dictate biological effects (Rao *et al*., 2005). Furthermore, engineering high-affinity soluble receptors or monoclonal antibodies that bind to cell surface ligands can provide potential therapeutic agents that antagonize the receptor–ligand interactions or that target a cell for destruction.

To engineer peptides or proteins for improved binding properties, various methods of directed evolution have been developed. These methods include systems such as yeast display (Boder and Wittrup, 1997), phage display (Smith, 1985; Bradbury and Marks, 2004; Marks and Bradbury, 2004), *Escherichia coli* display (Francisco *et al*., 1993), baculovirus/insect cell display (Boublik *et al*., 1995) and ribosome display (Hanes *et al*., 2000). While purified ligands have been used most often in these approaches, there has been considerable effort to develop strategies that involve ligands present in whole cell preparations. For example, in a process called biopanning, phage display libraries are either incubated with target cells or introduced *in vivo* into animals (Kupsch *et al*., 1999; Giordano *et al*., 2001; Roovers *et al*., 2001; Trepel *et al*., 2002). Phage that display a peptide or antibody against cell- or tissue-specific surface molecules are then isolated, and the process is repeated for further enrichment.

Recently, yeast display has been used as a system to isolate human scFv fragments that have specificity for various antigens, with the goal of identifying lead candidates for further directed evolution (Feldhaus *et al*., 2003). In several cases, yeast display has been used to isolate scFv (Boder *et al*., 2000; Rajpal *et al*., 2005; Razai *et al*., 2005) or other cell surface receptors (Buonpane *et al*., 2005) that exhibit picomolar affinity constants for their ligands. Furthermore, yeast display has been used to engineer T cell receptors (TCRs) (Holler *et al*., 2000; Kieke *et al*., 2001; Holler *et al*., 2003; Chlewicki *et al*., 2005), natural killer cell receptors (Dam *et al*., 2003) and proteins of the major histocompatibility complex (MHC) (Brophy *et al*., 2003; Starwalt *et al*., 2003; Esteban and Zhao, 2004) with improvements in stability and/or affinity. Each of these cell surface receptors is involved in cell-to-cell interactions through its specific binding to cognate ligands on the surface of an opposing cell. As interactions such as these are typically very low affinity (Maenaka *et al*., 1999; Davis *et al*., 2003), there has been considerable interest in the development of engineering methods for these classes of proteins.

TCRs are excellent examples of proteins recognizing cell surface ligands that in many cases are not amenable to isolation and purification with retention of native

© The Author 2006. Published by Oxford University Press. All rights reserved

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oxfordjournals.org

conformation. The TCR recognizes an antigenic peptide, derived from a foreign protein, presented on the host cell surface bound to a protein of the MHC (Davis *et al.*, 1998; Rudolph and Wilson, 2002). The binding of a TCR to its cognate peptide-MHC (pMHC) ligand on the target cell stimulates T cell effector function (e.g. cytokine release, target cell lysis). Like that of an antibody, the antigen binding site of a TCR is made up of hypervariable loops called complementarity determining regions (CDRs) that make contact with ligand (Garcia *et al.*, 1996). While the expression and purification of soluble pMHC ligands has been an area of intense effort, many pMHC protein complexes are unstable and/or difficult to manipulate. The variable success with this class of proteins arises from both the diverse nature of the antigenic peptides and the extensive polymorphisms in the MHC. We present here a strategy that allows the isolation of high-affinity TCRs against different pMHC ligands, without the need to express and purify soluble forms of pMHC.

In the yeast display system, the TCR has been cloned as a V_β-linker-V_α single chain (scTCR) fused to the gene for the yeast cell surface protein AGA2 (Kieke *et al.*, 1999). This fusion construct is displayed on the surface of yeast, where it is amenable to *in vitro* engineering for higher affinity binding to the pMHC ligand. The 2C TCR, derived from a murine cytotoxic T lymphocyte (CTL) clone, has been subjected to affinity maturation in this yeast display system (Holler *et al.*, 2000; Holler *et al.*, 2003). This TCR recognizes a peptide from a mitochondrial protein, QL9 peptide, presented by the allogeneic MHC molecule L^d (Udaka *et al.*, 1992), as well as the self peptide dEV8 and the foreign peptide SIYR presented by the syngeneic MHC molecule K^b (Tallquist and Pease, 1995; Udaka *et al.*, 1996). Previously, a library of degenerate $CDR3_\alpha$ mutants was screened using fluorescent-labeled, soluble forms of the pMHC ligands and fluorescence-activated cell sorting (FACS) (Holler *et al.*, 2000; Holler *et al.*, 2003). While this approach was effective for isolating higher affinity TCRs against the three different pMHC, many T cell systems do not have available purified and well-characterized soluble pMHC that can be used for selections by FACS. Furthermore, access to expensive FACS instrumentation may limit wider application of the approach.

Here, we present a strategy to circumvent this requirement by using intact antigen presenting cells (APCs) as the selecting platform. Yeast cells that displayed a library of scTCR on their cell surface were incubated with cells that expressed the selecting pMHC ligand in its native form on the cell surface. To separate rare yeast cells that bear the high affinity scTCR mutants from other yeast cells in the library, the approach takes advantage of the density differential between lymphoid-derived cells and yeast cells. Yeast bearing scTCR that bind with high affinity to the pMHC ligand on the APC can be separated from nonbinding or low affinity yeast by centrifugation through a discontinuous density gradient of a commercial media (Ficoll-Paque). In this single-step selection, yeast that formed stable conjugates with the pMHC-bearing lymphoid cells were retained at the interface, whereas unbound yeast sediment to the bottom. The strategy was validated using yeast that express TCRs with different affinities, spiked at various frequencies into a population of yeast cells that express non-binding TCRs. We show that this procedure can effectively enrich 1000-fold scTCR mutants with affinities in the nanomolar range. Also, novel high-affinity TCR mutants were isolated against peptide variants, thereby obviating the need to purify each peptide variant bound to the MHC ligand. Finally, the procedure was shown to be effective using TCRs that recognize either class I or class II MHC ligands, which should prove particularly useful since class II MHC ligands have been more difficult to purify (Ferlin *et al.*, 2000; Hackett and Sharma, 2002; Starwalt *et al.*, 2003). The general procedure should be readily applicable not only to the isolation of TCRs but also to antibodies that recognize cell surface tumor antigens now that a human scFv library in yeast is available (Feldhaus *et al.*, 2003).

Materials and methods

Peptides

Peptides (SIYR, SIYRYYGL; QL9, QLSPFPFDL) were synthesized by standard F-moc chemistry at the Macromolecular Core Facility at Pennsylvania State University. Position 5 variants of the QL9 peptide [Y5 (QLSP**Y**PFDL), R5, H5, and M5] were synthesized at the Protein Sciences Facility at the University of Illinois. QL9 and SIYR were purified by C-18 reverse-phase HPLC. The Hb peptide (GKKVITAFNEGLK) was synthesized by standard F-moc chemistry and purified by C-18 reverse-phase HPLC at Washington University.

Cell lines

The endogenous peptide transport-deficient human T-lymphoblastoid cell lines T2-K^b and T2-L^d, which have been transfected with the K^b or L^d heavy chain, respectively, were provided by P. Cresswell (Alexander *et al.*, 1989). The I-A^k, I-E^k –expressing B cell hybridoma, CH27, was used for studies with the class II 3.L2 TCR (Evavold *et al.*, 1992). Cells were propagated in RPMI 1640 media /10% fetal bovine serum, supplemented with L-glutamine and 2-mercaptoethanol at 37°C and 5% CO_2. G418 (0.5 mg/ml) was added to T2-K^b and T2-L^d cultures.

scTCR yeast display constructs and libraries

Several mutants of the 2C TCR used here were isolated previously. The 2C-T7 scTCR was engineered for increased yeast surface expression (Kieke *et al.*, 1999) and it has been shown to have an affinity (K_D value) for QL9/L^d of ~3 μM (unpublished data). The 2C-m80 TCR mutant was engineered using yeast display and FACS and the full length TCR has been reported previously to have an affinity for SIYR/K^b of 790 nM (Holler *et al.*, 2003). More recent surface plasmon resonance (SPR) measurements of the *E. coli* expressed, single-chain form of the 2C-m80 TCR have shown it to have an affinity for SIYR/K^b of 150 nM (unpublished data). As the single-chain form was expressed on yeast in the present studies, the K_D value of 150 nM will be referred to here as the affinity of the 2C-m80 scTCR. The 2C-m6 TCR was engineered using yeast display and FACS and it has been shown to have an affinity for QL9/L^d of 6 nM (Holler *et al.*, 2000; Holler *et al.*, 2003). The 3.L2 mouse TCR recognizes a peptide from an allelic variant of hemoglobin, presented by the I-E^k Class II MHC molecule (Evavold *et al.*, 1992). The mutant 3.L2-M15 was engineered using yeast display to have high affinity for its ligand, hemoglobin peptide Hb/I-E^k (K_D = 25 nM) (Weber *et al.*, 2005). Two scTCRs were used

as controls that do not bind to the cognate ligands recognized by the 2C or 3.L2 TCRs. These included (i) C18-1, a surface-stabilized scTCR mutant (unpublished data) of the murine C18 TCR that recognizes a peptide derived from a mutated MAP kinase presented by K^d MHC (Ikeda *et al*., 1997) and (ii) mWT1-B7, a mouse scTCR raised against Wilms tumor antigen-1 and engineered previously for increased yeast surface expression (unpublished data). The 2C CDR3$_\alpha$ library has been described previously (Holler *et al*., 2000). This yeast library was cultured in selective SD-CAA liquid media [2% dextrose (w/v), 0.67 % yeast nitrogen base (w/v), and 1% casamino acids (w/v)] with kanamycin (50 μg/ml) at 30°C.

Induction of scTCR expression on the yeast cell surface

scTCR expression on the surface of the *Saccharomyces cerevisiae* strain EBY100 was induced as described previously (Boder and Wittrup, 1997). Briefly, the yeast display plasmid pCT302, which encodes a gene fusion linking the scTCR to the yeast cell surface gene AGA2, controlled by a galactose-driven promoter, was introduced into EBY100 yeast cells. Expression of the AGA2:scTCR fusion gene was induced by transferring cells growing in SD-CAA to galactose-containing media and shaking at 20°C for at least 24 h.

Flow cytometric analysis of scTCR expression and pMHC binding

Yeast that express 2C scTCR mutants (V_β8.2-positive) were detected with the anti-V_β8.2 antibody F23.2. Yeast that express 3.L2 or C18 scTCR mutants (V_β8.3-positive) were detected with the anti-V_β8.3 antibodies KT-8C1 (Cedarlane Laboratories) or 1B3.3-PE (BD Pharmingen). Yeast that express mWT1-B7 scTCR (V_β11-positive) were detected with a PE-conjugated anti-V_β11 antibody, RR3-15-PE (BD Pharmingen). F23.2 or KT-8C1 binding was detected by incubating with biotinylated goat-anti-mouse IgG (Rockland Inc.), followed by streptavidin:PE (SA:PE) (BD Pharmingen) or by incubation with PE-conjugated goat F(ab')2 anti-mouse Ig (Southern Biotech). All antibodies and secondary reagents were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin (PBS/BSA). Samples were analyzed on a Coulter Epics-XL flow cytometer, gating on yeast cells based on light scattering properties. Flow cytometry was also used to detect binding of yeast-displayed scTCR to soluble L^d-Ig fusion protein folded with specific peptide (either QL9 or Y5). The QL9/L^d-Ig and Y5/L^d-Ig were produced and purified as described previously (Chlewicki *et al*., 2005). To detect pMHC binding by yeast-displayed scTCR, ~400 μg/ml QL9/L^d-Ig or ~200 μg/ml Y5/L^d-Ig was incubated with yeast cells on ice for 1 h. Cells were washed with PBS/BSA and resuspended in PE-labeled goat F(ab'2) anti-mouse Ig.

Peptide loading of cells

APCs (T2-K^b, T2-L^d, or CH27) were pelleted by centrifugation and resuspended in fresh media to a concentration of 10^6 cells/ml. 1 ml aliquots were then dispensed into 1.5 ml microfuge tubes. Peptides were incubated with cells at the following final concentrations: 1 μM SIYR, 30 μM QL9, and 10 μM Hb. Single amino acid variants of QL9 at position 5 were incubated at concentrations previously determined to be in excess for maximum stabilization of L^d on the surface of T2-L^d cells (Schlueter *et al*., 1996). Peptide/cell mixtures were rocked at room temperature for 60–90 min to allow loading of the peptides.

Density differential centrifugation

Induced yeast cells were counted on a hemacytometer and aliquoted into tubes that contained the peptide-loaded APCs (~10^6 yeast cells per tube). Another aliquot of induced yeast served as 'pre-centrifugation' control. The yeast/APC mixtures were allowed to rock at room temperature for 1 h. Following the incubation, the yeast/APC mixtures were layered onto three mls of Ficoll-Paque PLUS (Pharmacia) in 15 ml conical tubes. The cells were allowed to settle for 10 min at room temperature, and the tubes were then centrifuged at 1500 r.p.m. (~400× *g*) at 4°C for 30 min. After centrifugation, the visible layer of cells above the Ficoll-Paque (the 'interface') was removed from each tube in a 1 ml volume. In some cases, this interface was returned to the rocker for another 30-min incubation, and the procedure was repeated (centrifugation for 15 min in this 'second centrifugation').

Plasmid rescue and sequencing

Surface display plasmids were rescued from selected yeast clones using a Zymoprep I Yeast Plasmid Minipreparation Kit (Zymo Research). Rescued plasmids were introduced into the *E. coli* strain DH10B (Invitrogen) by electroporation. Plasmids were purified from *E. coli* using a QIAprep Spin Mini-Prep Kit (Qiagen) and sequenced at the DNA Core Sequencing Facility at the University of Illinois.

Results

Isolation of yeast:APC conjugates

In a previous study, we showed that it was possible to detect conjugates of a yeast cell bearing a TCR and an APC in which the MHC had been loaded with the antigenic peptide (Shusta *et al*., 2000). Using microscopy, these conjugates were shown to be more prevalent for yeast that expressed a higher affinity TCR. To exploit this observation as a rapid method for selecting high-affinity TCRs, a procedure was developed for separating yeast that are present in conjugates from unbound yeast. Lymphoid cells are known to form a discrete layer above the density medium Ficoll-Paque upon centrifugation, whereas yeast cells sediment through this solution (data not shown). Thus, we reasoned that yeast cells present as conjugates may be retained at this interface, separating them from unbound yeast.

To determine if yeast that express a high-affinity TCR could be isolated using this density centrifugation approach, we took advantage of a high-affinity mutant of the 2C-TCR, 2C-m80, which was engineered previously using yeast display and binds to the pMHC antigen SIYR/K^b with a K_D value of 150 nM (Holler *et al*., 2003). As a control, yeast cells that display the scTCR C18-1, which does not bind to SIYR/K^b, were used. Approximately 10^6 yeast expressing 2C-m80 or C18-1 were incubated with T2-K^b cells alone (without peptide) or with SIYR-loaded T2-K^b cells, and the cell mixture was layered onto Ficoll-Paque. Following centrifugation, the discrete interface layer was removed, allowed to incubate, and layered onto another tube of Ficoll-Paque. After

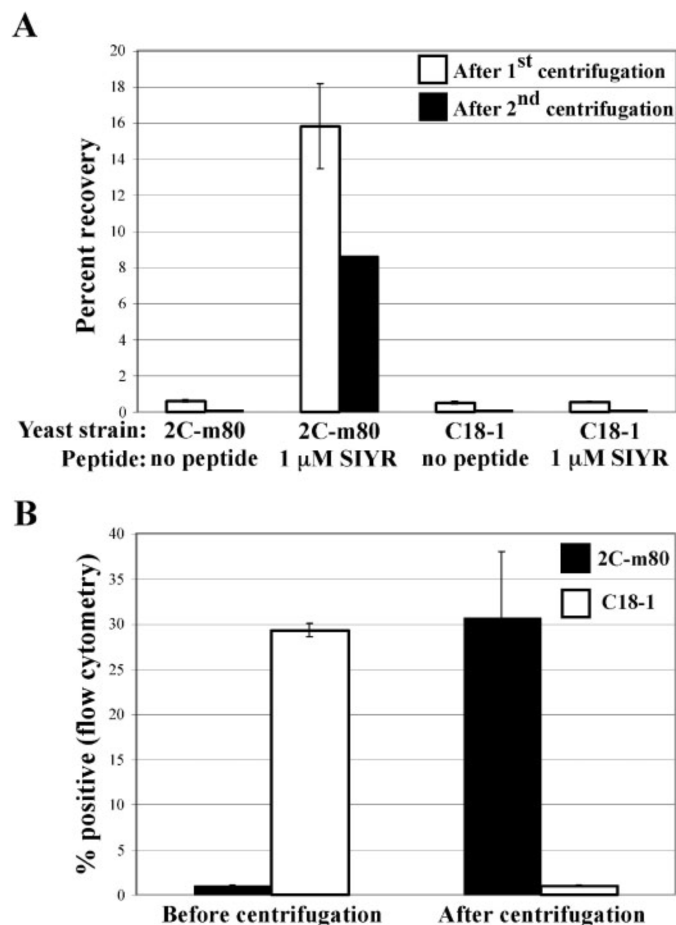

Fig. 1. Selective recovery of high affinity yeast after density centrifugation. (**A**) Percent of yeast recovered from the APC interface layer following centrifugation through Ficoll-Paque. Yeast cells expressing either the high affinity 2C-m80 scTCR or the non-binding C18-1 scTCR were incubated with T2-K^b cells that had been either loaded with exogenous SIYR peptide or used without exogenous peptide loading. The number of yeast present before centrifugation, and after the first and second centrifugation, were determined by plating aliquots of cells at each stage. The percent recovery following the first centrifugation (white bars) and second centrifugation (black bars) was taken as: (# of yeast cells present at interface layer after centrifugation)/(# of yeast cells at interface layer before centrifugation) × 100. (**B**) Isolation of high affinity 2C-m80 yeast from excess non-binding yeast. A mixture of yeast comprising a 1 to 1000 ratio of 2C-m80 to C18-1 was incubated with SIYR-loaded T2-K^b cells and subjected to two sequential rounds of density differential centrifugation. Aliquots of cells collected before centrifugation and after the second centrifugation were cultured and stained with antibodies specific for the V_β region of either C18-1 (1B3.3, white bars) or 2C-m80 (F23.2, black bars) and analyzed using flow cytometry. *Note:* The sums of the positive percentages do not equal 100 due to the negative population that is invariably observed in flow cytometric analysis in the yeast display system. See Figure 2.

this second centrifugation, the cells at the interface were again collected. Aliquots from both density selections and the original yeast cell sample were plated on sorbitol medium in order to quantitate the percentage of 2C-m80 or C18-1 yeast cells that were recovered from the APC interface layer. As shown in Figure 1a, a substantial fraction (16%) of yeast expressing the high affinity 2C-m80 TCR remained at the interface following the first centrifugation, and 8% of the yeast remained following the second centrifugation. This retention was SIYR/K^b dependent, as only 0.1% of the high-affinity 2C-m80 yeast was recovered when T2-K^b cells were used in the absence of the SIYR peptide. Furthermore, only 0.1% of yeast expressing the control C18-1 scTCR were recovered, indicating that the high affinity binding of the yeast 2C-m80 TCR to SIYR-K^b on the surface of the APCs was responsible for retaining these yeast at the APC interface layer.

Quantitative analysis of enrichment and selection potential

Given that yeast bearing the high affinity TCR, but not irrelevant yeast, were retained at the interface, we next addressed whether or not density differential centrifugation could be used for selection or enrichment methods. A library selection process was simulated by attempting to isolate high affinity mutants among an excess of non-binding yeast. In one experiment, 2C-m80 yeast were mixed with non-binding C18-1 yeast at a ratio of 1 to 1000, and this mixture was incubated with SIYR-loaded T2-K^b cells and subjected to two sequential rounds of centrifugation through Ficoll-Paque (as described above). The ratio of binding to non-binding yeast, before and after centrifugation, was monitored by flow cytometry using antibodies specific for the V_β region of either 2C or C18. As expected, yeast cells that displayed the 2C-m80 in the pre-centrifugation 1 to 1000 mixture were undetectable by flow cytometry. Remarkably, almost all yeast recovered from the interface following centrifugation expressed the 2C-m80 TCR (Figure 1b). Thus, this procedure yielded almost 1000-fold single pass enrichment.

Based on this success, we sought to determine if the procedure would work with a different high-affinity TCR, and if the procedure would be capable of selecting high-affinity binders that might be even more rare (e.g. 1 in 10 000 or 1 in 100 000). Here, we used yeast that express the high-affinity 2C TCR mutant 2C-m6, that binds to a different ligand, QL9/L^d, with a K_D value of 6 nM. Yeast cells that express 2C-m6 were mixed at different ratios with excess C18-1 yeast. These yeast cell mixtures were incubated with QL9-loaded T2-L^d APCs, and the cells were subjected to two sequential rounds of centrifugation through Ficoll-Paque as described above. The ratio of 2C-m6 yeast to C18-1 yeast was monitored as described above, before and after centrifugation, by flow cytometry. Because these ratios are potentially subject to stochastic variations in the number of 'binders' (e.g. the starting populations contained only 10 or 100 yeast cells that could bind the APC), the selection procedure was performed in triplicate for both the 1:10 000 and 1:100 000 experiments. In each case, one of the three replicates yielded detectable enrichment following the second centrifugation, as evidenced by the population of yeast that stain positive for an antibody that recognizes 2C but not C18 (Figure 2). These results indicate that it is possible to isolate very rare high affinity mutants. It was not surprising that enrichment of a starting population consisting of only 10 or 100 desired cells in 10^6 is less consistent than when 1000 high affinity cells were present in the sample. Such variation can be minimized by over-sampling libraries that might contain rare mutants.

Selection analyzed across a range of TCR affinities

In order to explore the affinity ranges that could be selected using the density centrifugation procedure, we monitored enrichment of a high affinity mutant 2C-m6 under circumstances when its affinity for different pMHC ligands ranged from >1 μM to 6 nM (including QL9/L^d, discussed in the

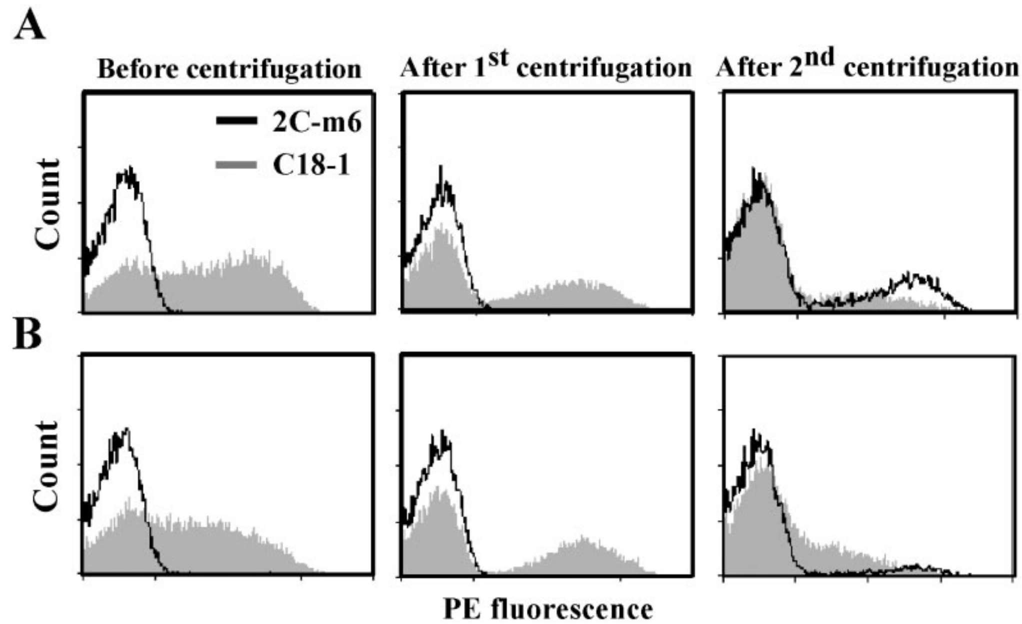

Fig. 2. Enrichment of rare high affinity yeast. Yeast expressing the high affinity 2C-m6 were mixed with non-binding yeast at a ratio of (**A**) 1 to 10 000 or (**B**) 1 to 100 000. The mixtures were incubated with QL9-loaded T2-L^d cells and subjected to two sequential rounds of density centrifugation. Aliquots of cells collected before centrifugation, after the first centrifugation and after the second centrifugation were cultured and stained with antibodies specific for the V_β region of either non-binding scTCR C18-1 (1B3.3, grey) or high affinity scTCR 2C-m6 (F23.2, black outline). *Note:* The negative population in each histogram is invariably observed in the yeast display system and serves as an internal control.

previous section). The other ligands represented single amino acid variants of the QL9 peptide (at position 5 of the QL9 nonamer). Three position 5 variants of QL9: M5, H5, and R5, bind to L^d (Schlueter *et al*., 1996), and the affinities of 2C-m6 for these variants in the context of L^d are ~34, 78 nM, and >1 μM, respectively (Holler and Kranz, 2003). The K_D value of 2C-m6 for R5/L^d was judged to be higher than 1 μM because 2C-m6$^+$ T cells required CD8 in order to be stimulated by R5/L^d (Holler and Kranz, 2003). T2-L^d cells, loaded with one of each of the three position 5 variants, were incubated with 2C-m6 yeast mixed with non-binding C18-1 yeast at a ratio of 1 to 1000. These cells were then subjected to centrifugation through Ficoll-Paque, and the ratios of yeast cells that expressed 2C-m6 to C18-1 were monitored before and after centrifugation by flow cytometry. Not surprisingly, the single pass enrichment was correlated with the affinity of the TCR for the pMHC (Figure 3a). Density centrifugation using M5-loaded T2-L^d cells yielded 970-fold enrichment and T2-L^d loaded with H5 (for which 2C-m6 has a ~2-fold lower affinity) yielded 950-fold enrichment. Thus, as observed for selections of the 2C-m80 TCR with SIYR/K^b, affinities greater than ~100 nM yield almost 1000-fold enrichments in a single centrifugation step.

When the T2-L^d cells were loaded with the peptide for which 2C-m6 has the lowest affinity, R5, one of two replicates showed detectable enrichment. When cells recovered from the interface where enrichment was detected were re-cultured in selective yeast medium, induced to express scTCR, and then subjected to a second cycle of density centrifugation using R5-loaded T2-L^d cells, substantial enrichment for 2C-m6 was observed (Figure 3b). These results indicate that, although higher affinity mutants are more readily isolated, sequential rounds of density differential centrifugation can be used effectively to isolate mutants with lower affinities.

Notably, when T2-L^d cells were loaded with QL9 peptide, incubated with 2C-T7 yeast mixed with C18-1 yeast at a ratio of 1 to 1000, no enrichment was detected following density centrifugation (data not shown). This result was unexpected considering the affinity of 2C-T7 for QL9/L^d is believed to be equal to or higher than that of 2C-m6 for R5/L^d. One possible explanation for this observation could be related to a difference in the affinity of the peptides for the L^d MHC. The QL9/L^d complex has ~10-fold lower stability than the R5/L^d complex (Schlueter *et al*., 1996), and the density centrifugation procedure may be sensitive to such differences, favoring enrichment in cases where peptide: MHC stability is higher.

Selection of TCRs from a site-directed library of mutants

In order to examine if the density selection procedure could be used to isolate high-affinity TCRs from a mutated TCR library, we used the 2C TCR $CDR3_\alpha$ library (Holler *et al*., 2000) with the QL9 peptide and a QL9 variant called Y5. The wild-type 2C TCR binds to its allogeneic QL9/L^d pMHC ligand with a K_D value of ~3 μM (Garcia *et al*., 1997). $CDR3_\alpha$ of the 2C TCR is predicted to be positioned in close proximity to the QL9 peptide in the TCR:pMHC complex (Speir *et al*., 1998). The $CDR3_\alpha$ library was screened previously to isolate high-affinity TCR mutants, including 2C-m6, by FACS with soluble QL9/L^d (Holler *et al*., 2000). Because mutants with high affinity for QL9/L^d were shown to be present in this $CDR3_\alpha$ library, we chose to screen it for such mutants using the density centrifugation method. QL9-loaded T2-L^d cells were incubated with 600 000 2C $CDR3_\alpha$ library yeast cells (a ~6-fold over-sampling of the library), and the mixture was subjected to two sequential rounds of centrifugation through Ficoll-Paque. The interface layer was removed, and an aliquot was plated. Individual yeast colonies were isolated, cultured and assayed for high affinity.

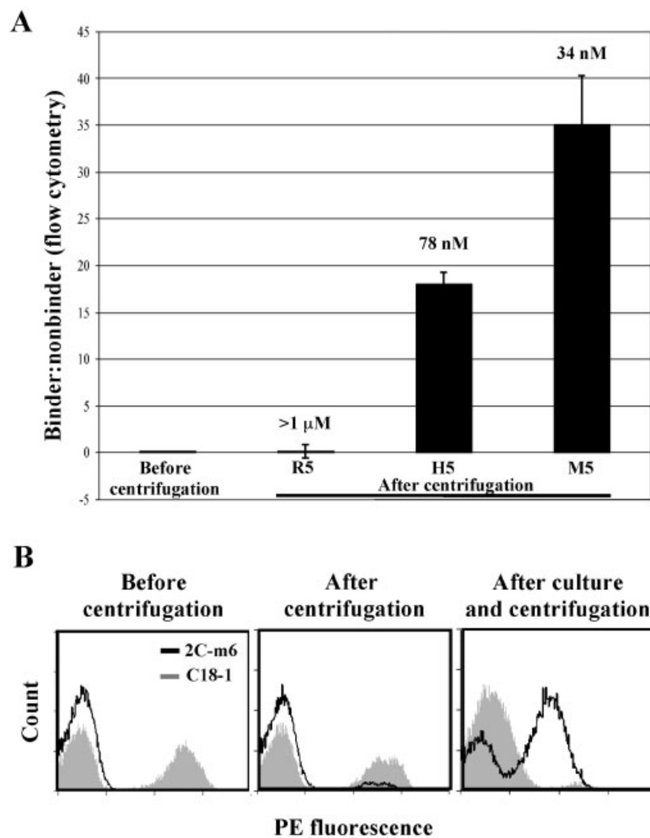

Fig. 3. Selection analyzed across a range of affinities. (**A**) T2-L^d cells were loaded exogenously with the peptide variants R5, H5, and M5. Peptide-loaded APCs were then incubated with a yeast cell mixture comprising a 1 to 1000 ratio of 2C-m6 to C18-1. Cells were then subjected to two sequential rounds of density centrifugation. Aliquots of cells before centrifugation and after the second centrifugation were cultured and stained with antibodies specific for the V_β region of either C18-1 (KT-8C1) or 2C-m80 (F23.2) and analyzed using flow cytometry. The ratios of yeast cells staining positive for F23.2 (binder) to yeast cells staining positive for KT-8C1 (nonbinder) are shown before and after centrifugation (K_D value for each 2C-m6/pep/L^d interaction is shown above bars). (**B**) R5-loaded APCs. Flow cytometry histogram overlays showing C18-1-positive yeast (grey) and 2C-m6-positive yeast (black outline) before and after centrifugation and following a second complete cycle of growth, induction, incubation with R5-loaded T2-L^d and centrifugation.

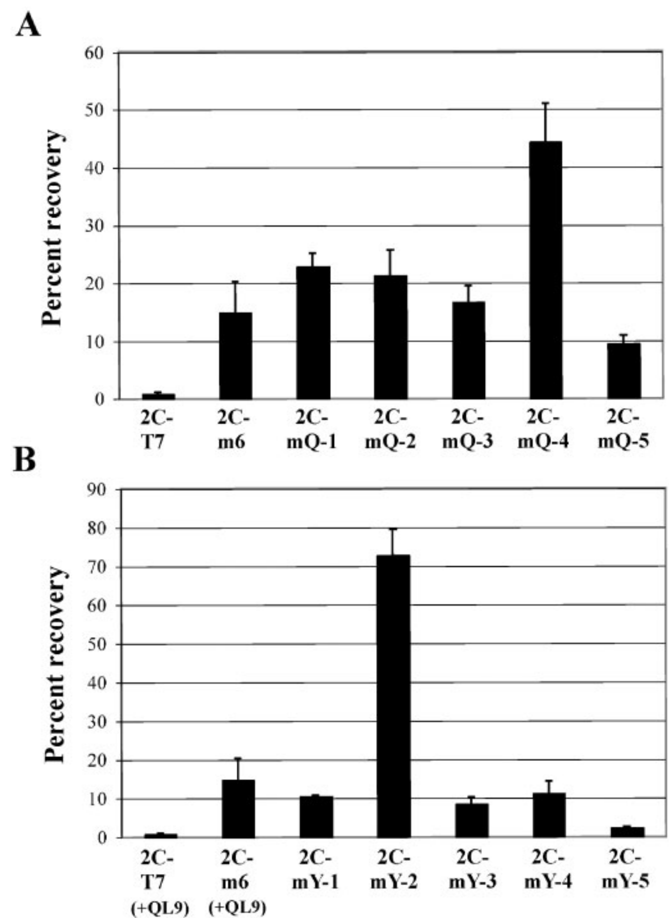

Fig. 4. Analysis of unique TCR mutants by density differential centrifugation. (**A**) QL9-loaded T2-L^d cells were incubated with yeast expressing mutant scTCRs 2C-T7, 2C-m6 or 2C-mQ-1 through 2C-mQ-5 and (**B**) Y5-loaded T2-L^d cells were incubated with yeast expressing mutant scTCRs 2C-mY-1 through 2C-mY-5. Cell mixtures were then subjected to one round of density differential centrifugation. The number of yeast present before and after centrifugation was determined by plating aliquots of cells at each stage. The percentages of yeast recovered from the interface, calculated as described for Figure 1, are shown as an average of three trials. The recovery percentages of 2C-m6 (high affinity) and 2C-T7 (wild-type affinity) when incubated with QL9-loaded T2-L^d cells and subjected to density differential centrifugation from (A) are shown in (B) for comparison.

Initial analysis to assess the relative affinities of isolated yeast clones used a qualitative density centrifugation approach. QL9-loaded T2-L^d cells were incubated with isolated yeast clones, the high affinity yeast clone 2C-m6, or the surface stabilized version of the wild-type 2C, 2C-T7 (that contains wild-type CDRs and has an affinity of $\sim$3 μM). The cell mixtures were subjected to one round of centrifugation through Ficoll-Paque, and the interface layers were removed and plated. In each case, an aliquot of yeast before centrifugation was plated to normalize for the starting number of yeast, and the percentage of yeast that remained at the interface following centrifugation was calculated (Figure 4a). Using this approach, $\sim$15% of 2C-m6-expressing yeast cells were recovered from the interface following centrifugation. All five of the new clones (2C-mQ-1, mQ-2, mQ-3, -mQ-4, and mQ-5) were retained to a similar degree as 2C-m6. In contrast, only $\sim$1% of 2C-T7-expressing yeast were retained. These results suggest that mutants 2C-mQ-1 through 2C-mQ-5 bind to QL9/L^d with higher affinity than wild-type 2C.

In an effort to isolate high affinity clones from the 2C CDR3$_\alpha$ library, using a novel peptide that had not yet been used to screen the library, the procedure described above was performed using a position 5 variant of the QL9 peptide. T2-L^d cells were loaded with Y5 peptide (which contains a Tyr substituted for Phe at peptide position 5), incubated with CDR3$_\alpha$ library cells, and subjected to centrifugation through Ficoll-Paque. Five mutants isolated from this selection, 2C-mY-1 through 2C-mY-5, were assayed qualitatively for binding to Y5-loaded T2-L^d cells by density centrifugation. Three clones, 2C-mY-1, 2C-mY-3 and 2C-mY-4, showed retention levels similar to those of the standard high-affinity pair 2C-m6:QL9/L^d (Figure 4b). One clone, 2C-mY-2, showed a remarkable recovery of $\sim$70%, whereas clone 2C-mY-5 showed reduced recovery compared to 2C-m6/QL9/L^d but greater than the lower affinity control (2C-T7/QL9/L^d).

Characterization of TCR clones isolated from a site-directed mutant library

To further characterize the TCR yeast clones isolated by density centrifugation with QL9/L^d- or Y5/L^d-bearing APCs, flow cytometry was performed using anti-TCR antibody

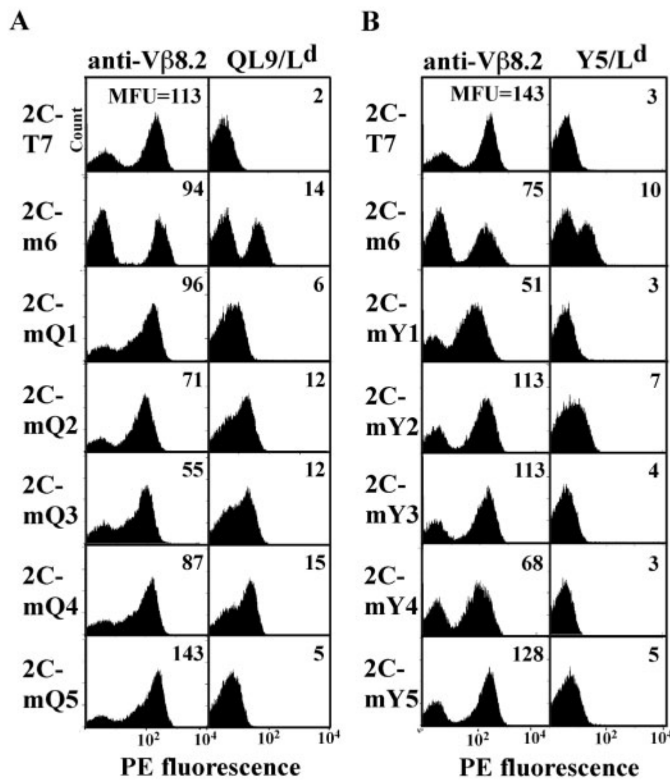

Fig. 5. Analysis of unique TCR mutants using flow cytometry. (**A**) Histograms of yeast cells expressing mutant scTCRs 2C-T7, 2C-m6, and 2C-mQ-1 through 2C-mQ-5 stained with either anti-V_{β}8.2 (F23.2) (left column) or QL9/L^{d}-Ig pMHC dimer (right column) and PE-conjugated goat F(ab')2 anti-mouse Ig. (**B**) Histograms of yeast expressing 2C-T7, 2C-m6, and 2C-mY-1 through 2C-mY-5 stained with either anti-V_{β}8.2 (F23.2) (left column) or Y5/L^{d}-Ig pMHC dimer (right column) and PE-conjugated goat F(ab')2 anti-mouse Ig. Mean fluorescence units for each histogram are indicated.

QL9-selected clones	
Clone Clone	CDR3α
Wild-type 2C	101 GFASA
2C-m6	**HQGRY**
2C-mQ-1	**RLGRY**
2C-mQ-2	**IRGTR**
2C-mQ-3	**YLGLY**
2C-mQ-4	**MHGLY**
2C-mQ-5	**YFGTR**
Y5-selected clones	
2C-m12*	**NPPPL**
2C-mY-1	**STSWY**
2C-mY-2	**APPPT**
2C-mY-3	**NPTPL**
2C-mY-4	**CPTPL**
2C-mY-5	**HGGAY**

Fig. 6. Sequences of TCR mutants. The amino acid sequences of the CDR3$_{\alpha}$ of mutants 2C-mQ-1 through 2C-mQ-5 and 2C-mY-1 through 2C-mY-5 are shown. The CDR3$_{\alpha}$ amino acid sequences of previously isolated 2C mutants 2C-m6 and 2C-m12, as well as wild-type 2C CDR3$_{\alpha}$, are shown for comparison. 2C-m12* was selected previously using QL9 peptide.

F23.2 to evaluate yeast display levels and soluble QL9/L^{d} or Y5/L^{d} to evaluate ligand binding (Figure 5). Yeast cells were stained with F23.2, dimeric QL9/L^{d} (QL9/L^{d}-Ig), or dimeric Y5/L^{d} (Y5/L^{d}-Ig), and binding was analyzed using flow cytometry. TCR clones isolated with QL9/L^{d} or Y5/L^{d}, the wild-type low affinity TCR 2C-T7 and the previously selected mutant 2C-m6 were displayed at similar levels, within 2-fold based on the mean fluorescence units of cells stained with the anti-TCR antibody. Among the mutants isolated using QL9, the mutant that exhibited the highest recovery in density centrifugation, 2C-mQ-4 (Figure 4a), also showed the highest level of QL9/L^{d}-Ig binding (Figure 5a). Similarly, the Y5/L^{d} mutant that showed the highest recovery in density centrifugation, 2C-mY-2 (Figure 4b), also showed the highest level of Y5/L^{d}-Ig binding (Figure 5b). The clone with the lowest recovery by density centrifugation, 2C-mQ-5, also showed the lowest level of staining with QL9/L^{d}-Ig. All other QL9/L^{d} clones were intermediate in both recovery and staining with QL9/L^{d}-Ig, while other Y5/L^{d} clones were very low recovery and staining in the case of Y5/L^{d}-Ig. As expected, the low affinity 2C TCR 2C-T7 did not show detectable binding to either QL9/L^{d}-Ig or Y5/L^{d}-Ig.

Our previous studies have shown that high-affinity TCR mutants exhibited distinct sequence motifs in the CDR3α, which probably correlate with the structural requirements for higher affinity binding. To examine whether or not the ten mutants isolated by density centrifugation exhibited these same motifs, the plasmids from the yeast clones were sequenced. The amino acid sequences of the mutated CDR3$_{\alpha}$, wild-type CDR3$_{\alpha}$, and the CDR3$_{\alpha}$ of previously isolated mutants (2C-m6 and 2C-m12) that contain two sequence motifs are shown in Figure 6. The five QL9-isolated mutants differed from the wild-type 2C and 2C-m6. Interestingly, all of these mutants contained the Ala101$_{\alpha}$Gly substitution that characterizes one of the conserved motifs identified in the CDR3$_{\alpha}$ of high affinity 2C clones, including 2C-m6, isolated during QL9/L^{d} selection using FACS (Holler *et al*., 2000). The five mutants isolated here also contained either Ala103$_{\alpha}$Arg or Ala103$_{\alpha}$Tyr substitutions, both identified as preferential mutations in the original screening of this library.

Comparison of the CDR3$_{\alpha}$ sequences of Y5-isolated mutants and 2C mutants, isolated previously using QL9/L^{d} and FACS (e.g. 2C-m12), revealed that in this case a second conserved motif was also present (Figure 6) (Holler *et al*., 2000). This conserved motif contains three tandem prolines (Pro-Pro-Pro) and it was present in 2C-mY-2, the mutant that showed the highest level of Y5/L^{d} binding as judged by both density centrifugation and flow cytometry (Figures 4 and 5). A variation of that motif, Pro-Thr-Pro, which was also isolated previously using flow sorting (Holler *et al*., 2000), was identified in mutants 2C-mY-3 and 2C-mY-4. Mutant 2C-mY-5 contained the conserved glycine residue at 101$_{\alpha}$ that was observed in all of the QL9-isolated clones. Finally, mutant 2C-mY-1 did not contain either the proline or the glycine motif, but its unique sequence (STSWY) was somewhat similar to isolates (RWTSG and TWSPF) obtained

in previous selections (Holler *et al*., 2003). Thus, despite the fact that these two ligands (QL9 and Y5) differ by only a hydroxyl group, it appears that there are significant differences between 2C TCR recognition of QL9/L^d and Y5/L^d. QL9/L^d may preferentially interact with TCR mutants that contain Gly101$_{\alpha}$, while Y5/L^d appears to be capable of using more diverse sequences, and in particular proline-containing mutants.

Quantitation of TCR selection potential in a class II MHC system

Given the effectiveness with which density centrifugation selected high affinity mutants in the 2C TCR/class I MHC system, we extended our analysis to a TCR/class II MHC-restricted system. The class II restricted TCR 3.L2 binds to a peptide from hemoglobin (Hb) bound to the class II MHC I-E^k (Evavold *et al*., 1992). Previously, a mutant of the 3.L2 TCR, 3.L2-M15 was isolated from sequential CDR3 libraries using yeast display and FACS (Weber *et al*., 2005). Mutant 3.L2-M15 binds to the Hb/I-E^k ligand with a K_D value of ~25 nM as measured by SPR, an 800-fold increase in affinity over the wild-type 3.L2 TCR. We thus investigated whether yeast cells that express this high affinity mutant 3.L2-M15 could be isolated from a 1000-fold excess of non-binding yeast using density centrifugation. The I-E^k-positive mouse cell line CH27 was loaded exogenously with 10 μM Hb peptide. Peptide-loaded cells were incubated with yeast that express 3.L2-M15 mixed at a 1 to 1000 ratio with control yeast that express the non-binding mWT1-B7 TCR. The cell mixture was subjected to one round of centrifugation through Ficoll-Paque, and the ratio of 3.L2-M15 yeast to mWT1-B7 yeast before and after centrifugation was monitored by flow cytometry using antibodies specific for either the V_{β} region of the 3.L2 TCR or the V_{β} region of the mWT1 TCR (Figure 7a). As expected, prior to centrifugation, only non-binding yeast that express mWT1-B7, were detectable by flow cytometry. Following only a single centrifugation, the percentage of cells staining positive for 3.L2-M15 increased from undetectable to 23.6%. Furthermore, the percentage of cells that expressed mWT1-B7 decreased from 70.3 to 39%. This increase in the relative number of cells positive for the 3.L2-M15 mutant correlated with an enrichment for the high affinity scTCR of ~380-fold (from 0.1% of cells to 38% of the cells). The average enrichment for three experiments with 3.L2-M15 and mWT1-B7 was ~500-fold (Figure 7b). This experiment was repeated using the same high affinity TCR 3.L2-m15, but a different non-binding TCR, 2C-T7, and in this case an average enrichment of ~200-fold was achieved (Figure 7c).

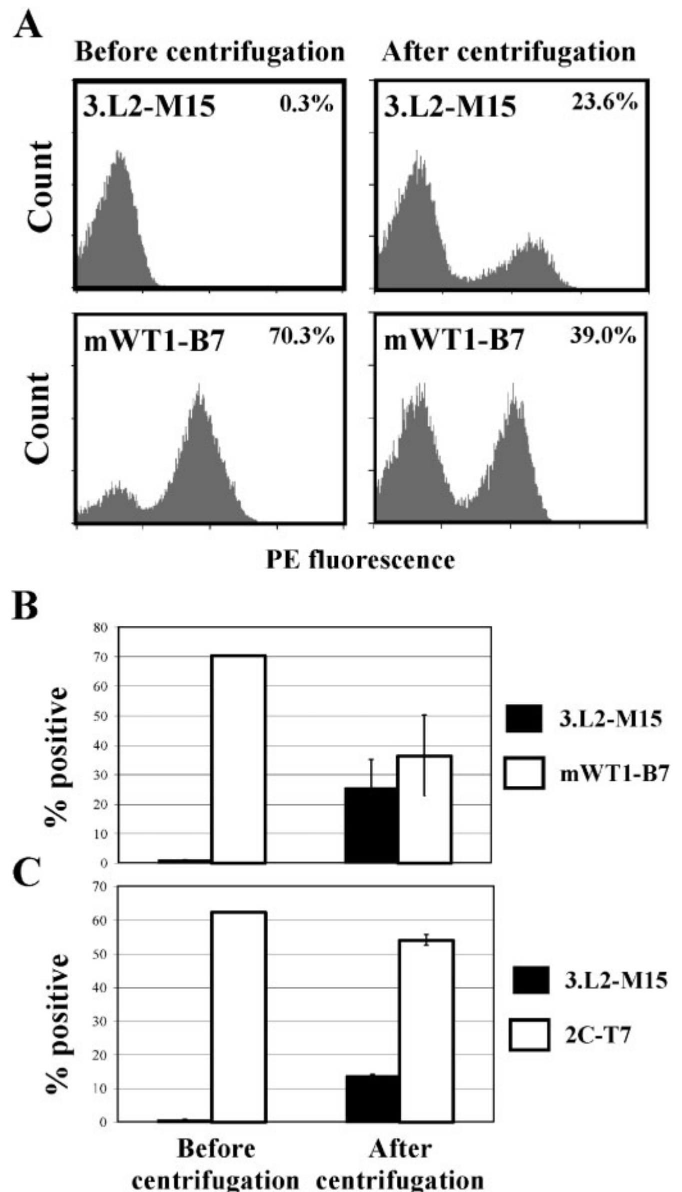

Fig. 7. Enrichment of a high affinity MHC class II-restricted scTCR mutant. Yeast expressing the high affinity mutant scTCR 3.L2-M15 were mixed at a 1 to 1000 ratio of (**A** and **B**) non-binding mWT1-B7 yeast or (**C**) non-binding 2C-T7 yeast. The yeast mixtures were incubated with Hb peptide-loaded CH27 APCs. Cells were then subjected to one round of density differential centrifugation. Aliquots of cells that were collected before and after centrifugation were cultured and stained with antibodies specific for the V_{β} region of either the non-binding scTCRs mWT1-B7 (RR3-15-PE) or 2C-T7 (F23.2) or the high affinity scTCR 3.L2-M15 (1B3.3-PE in A and B, KT-8C1 in C). Stained yeast cells were analyzed using flow cytometry. (A) Histograms of yeast before and after centrifugation stained with antibody specific for V_{β} region of the high affinity 3.L2-M15 (1B3.3-PE, top row) or mWT1-B7 (RR3-15-PE, bottom row). Percent of cells staining positive in each histogram are shown in the upper right-hand corner. (B and C) Percent of yeast before and after centrifugation staining positive for non-binding scTCR (white bars) or high affinity scTCR (black bars) (average of three trials in B or two trials in C).

Discussion

Antibody engineering to facilitate targeting of antigens has been used extensively in the development of antibodies as potential therapeutic agents. The most frequent uses of *in vitro* techniques seek to optimize antibody binding affinities, typically through a platform such as phage display (Hawkins *et al*., 1992; Marks *et al*., 1992). Alternatively, phage display has been used to identify lead antibodies against targets using either purified antigens (Edwards *et al*., 2003) or biopanning procedures employing whole cells or tissues (Giordano *et al*., 2001; Trepel *et al*., 2002). Like antibodies, TCRs recognize a diverse array of antigens. However, unlike the antigens recognized by antibodies, the antigens recognized by T cells represent a greater challenge from the biochemical perspective. These antigens consist of a short peptide that is non-covalently associated with a cell surface heterodimer encoded by the MHC for class II or the MHC and β2-microglobulin for class I. Ternary complexes of peptides and MHC products

exhibit widely diverse stabilities and thus the ability to express and purify these ligands varies widely. Various laboratories, including the NIH tetramer facility and commercial vendors, provide purified complexes of various well-characterized pMHC ligands. However, the variety of MHC alleles (2101 in total among human class I and II), and the large number of possible peptides makes it difficult to use purified ligands more generally. In order to develop a strategy for engineering TCRs with high-affinity for a diverse array of antigens, it would be advantageous to avoid the need for purification of the ternary antigen complexes. We show here that it is possible to combine the yeast display system with a rapid density centrifugation method to select high-affinity TCRs. The strategy required only the use of synthetic peptides that could be loaded exogenously onto an APC that expresses on its surface the proper MHC molecule.

In the examples described here, the density centrifugation method can be compared favorably to our previous experience using purified pMHC antigens and FACS (Holler *et al*., 2000; Holler *et al*., 2003; Chlewicki *et al*., 2005). In previous studies, three or four rounds of yeast cell growth, induction and sorting were required to identify TCR mutants with high affinity. This process required a period of 2–3 weeks, whereas the density centrifugation method used here to isolate QL9/L^d or Y5/L^d mutants required only a few hours for selections, through only one or two centrifugations. Thus, this method is rapid, obviates the need for a high-speed flow sorter and does not require purified ligands. While the precision and ability to perform off-rate based screens remains an inherent advantage of FACS and yeast display (Boder and Wittrup, 1998), the present strategy should prove useful for the many systems that lack purified ligands and for laboratories with limited access to FACS instrumentation. In a different selection approach that also does not require FACS instrumentation, Yeung and Wittrup used biotinylated antigens and streptavidin-coated magnetic beads to isolate yeast that express antigen-specific scFvs with an enrichment of $\sim$100-fold (Yeung and Wittrup, 2002). While the method described in the present report can be used only for ligands that are expressed on cell surfaces, our findings show that remarkable enrichments of 1000-fold can be achieved with only single-pass density centrifugations.

Recent reports have shown that high-affinity TCRs can also be engineered by phage display (Laugel *et al*., 2005; Li *et al*., 2005). It is reasonable to predict that the same type of biopanning that has been performed with phage-displayed peptides or antibodies (Kupsch *et al*., 1999; Giordano *et al*., 2001; Roovers *et al*., 2001; Trepel *et al*., 2002) could be used for the phage-displayed TCRs, using peptide-loaded APCs. It is possible that this approach may also find some use in the isolation of ‘lead’ pMHC binders from libraries of naïve TCRs, although such libraries have not yet been reported. If the density method described here were to be used with yeast-display libraries of naïve TCRs, clearly the affinities of the TCRs would need to be above a particular threshold (e.g. K_D values <1 μM). Whether TCRs can be obtained from naive libraries and, if so, whether they retain the typical diagonal docking orientation on the pMHC ligand (Garcia and Adams, 2005) remains to be determined.

In addition to providing validation that the density centrifugation method could be used to isolate high-affinity TCRs from a library of mutants, sequence analysis of the panels of high-affinity TCRs against QL9/L^d and Y5/L^d revealed CDR3α motifs that may correlate with recognition differences between these two very similar ligands. Accordingly, all mutants isolated against QL9, which contains a Phe at position 5, represented a CDR3α motif with a key glycine at residue 101α. In contrast the addition of a hydroxyl group in the Y5 peptide variant appears to allow the isolation of CDR3α mutants with more diversity in their CDR3α. The molecular explanations for these differences remain to be resolved and will require structural studies of the TCR-peptide/L^d complexes. Nevertheless, the data point to the exquisite peptide fine-specificity that is associated with the recognition of pMHC ligands by TCRs.

As indicated, many pMHC ligands of interest have not yet been successfully expressed and purified in soluble form. This limitation is particularly pronounced for class II MHC ligands (Hackett and Sharma, 2002), and, despite significant advances in class II MHC multimer technology, there are still relatively few class II MHC multimers available (Cameron *et al*., 2002). While the expression of class I MHC molecules in *E. coli* has been standardized for many alleles, it has been more difficult to develop standardized protocols for class II MHC production and only a few MHC class II molecules have be produced in *E. coli*. Some class II MHC molecules have been successfully isolated following secretion from insect cells, often as fusion products with introduced leucine zipper domains to assist in chain association (Scott *et al*., 1996). One fundamental difference between class I and class II MHC involves the chaperones that facilitate folding and peptide loading intracellularly (Cresswell and Lanzavecchia, 2001). Our method of exogenous peptide loading completely eliminates the need to develop expression and purification protocols for each peptide-class II MHC multimer. For example, such an approach may be useful in the discovery of high-affinity TCRs that bind to class II MHC antigens that are involved in autoimmunity (Lebowitz *et al*., 1999; Masteller *et al*., 2003).

Finally, it is conceivable that this methodology could be extended to other receptor–ligand interactions, as well as to the engineering of high-affinity antibodies against tumor antigens (Boder and Wittrup, 2000; Feldhaus *et al*., 2003; van den Beucken, 2003; Hoogenboom, 2005). In fact, an alternative biopanning strategy using an antibody–hapten model system was published during the preparation of this manuscript (Wang and Shusta, 2005). These studies monitored recovery of yeast expressing a high-affinity, fluorescein-specific antibody from a fluorescein-labeled, adherent endothelial cell monolayer. While this approach is limited to adherent cell lines and has yet to be applied to selections from combinatorial libraries, it provides further evidence of the potential of yeast display in cell-panning strategies. Furthermore, mammalian proteins other than antibodies or TCRs have been expressed on the cell surface via the yeast display system (Bhatia *et al*., 2003; Schweickhardt *et al*., 2003). For example, the cell adhesion molecule E-selectin, which helps initiate extravasation of leukocytes by binding sialyl-Lewis-x ligand on the leukocyte cell surface, has been expressed as a functional construct on the surface of yeast (Bhatia *et al*., 2003). As the use of yeast display expands as a tool for directed evolution, the density centrifugation strategy for selections may serve to support

broadened efforts in library screening, especially when purified soluble selecting agents are not available.

Acknowledgements

We thank Michael Lasker for helpful discussions, and the University of Illinois Flow Cytometry Facility for assistance. This work was supported by NIH grants AI24157 and AI61173 to PMA, CA33084 and CA18029 to PDG, CA111877 and GM55767 to DMK; and Leukemia and Lymphoma Society Grant 7040 to PDG. SAR was supported in part by an NIH NRSA 1 F31 ES013571-01, and MLD by a Poncin Fellowship.

References

Alexander,J., Payne,J.A., Murray,R., Frelinger,J.A. and Cresswell,P. (1989) *Immunogenetics*, **29**, 380–399.
Bhatia,S.K., Swers,J.S., Camphausen,R.T., Wittrup,K.D. and Hammer,D.A. (2003) *Biotechnol. Prog.*, **19**, 1033–1037.
Boder,E.T. and Wittrup,K.D. (1997) *Nat. Biotech.*, **15**, 553–557.
Boder,E.T. and Wittrup,K.D. (1998) *Biotechnol. Prog.*, **14**, 55–62.
Boder,E.T. and Wittrup,K.D. (2000) *Methods Enzymol*, **328**, 430–444.
Boder,E.T., Midelfort,K.S. and Wittrup,K.D. (2000) *Proc. Natl Acad. Sci. USA*, **97**, 10701–10705.
Boublik,Y., Di Bonito,P. and Jones,I.M. (1995) *Biotechnology (NY)*, **13**, 1079–1084.
Bradbury,A.R. and Marks,J.D. (2004) *J. Immunol. Methods*, **290**, 29–49.
Brophy,S.E., Holler,P.D. and Kranz,D.M. (2003) *J. Immunol. Methods*, **272**, 235–246.
Buonpane,R.A., Moza,B., Sundberg,E.J. and Kranz,D.M. (2005) *J. Mol. Biol.*, **353**, 308–321.
Cameron,T.O., Norris,P.J., Patel,A., Moulon,C., Rosenberg,E.S., Mellins,E.D., Wedderburn,L.R. and Stern,L.J. (2002) *J. Immunol. Methods*, **268**, 51–69.
Chlewicki,L.K., Holler,P.D., Monti,B.C., Clutter,M.A. and Kranz,D.M. (2005) *J. Mol. Biol.*, **346**, 223–239.
Cresswell,P. and Lanzavecchia,A. (2001) *Curr. Opin. Immunol.*, **13**, 11–12.
Dam,J., Guan,R., Natarajan,K., Dimasi,N., Chlewicki,L.K., Kranz,D.M., Schuck,P., Margulies,D.H. and Mariuzza,R.A. (2003) *Nat. Immunol.*, **4**, 1213–1222.
Davis,M.M., Boniface,J.J., Reich,Z., Lyons,D., Hampl,J., Arden,B. and Chien,Y. (1998) *Annu. Rev. Immunol.*, **16**, 523–544.
Davis,S.J., Ikemizu,S., Evans,E.J., Fugger,L., Bakker,T.R. and van der Merwe,P.A. (2003) *Nat. Immunol.*, **4**, 217–224.
Edwards,B.M. *et al*. (2003) *J. Mol. Biol.*, **334**, 103–118.
Esteban,O. and Zhao,H. (2004) *J. Mol. Biol.*, **340**, 81–95.
Evavold,B.D., Williams,S.G., Hsu,B.L., Buus,S. and Allen,P.M. (1992) *J. Immunol.*, **148**, 347.
Feldhaus,M. *et al*. (2003) *Nat. Biotechnol.*, **21**, 163–170.
Ferlin,W., Glaichenhaus,N. and Mougneau,E. (2000) *Curr. Opin. Immunol.*, **12**, 670–675.
Francisco,J.A., Campbell,R., Iverson,B.L. and Georgiou,G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10444–10448.
Garcia,K.C. and Adams,E.J. (2005) *Cell*, **122**, 333–336.
Garcia,K.C., Degano,M., Stanfield,R.L., Brunmark,A., Jackson,M.R., Peterson,P.A., Teyton,L. and Wilson,I.A. (1996) *Science*, **274**, 209–219.
Garcia,K.C., Tallquist,M.D., Pease,L.R., Brunmark,A., Scott,C.A., Degano,M., Stura,E.A., Peterson,P.A., Wilson,I.A. and Teyton,L. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 13838–13843.
Giordano,R.J., Cardo-Vila,M., Lahdenranta,J., Pasqualini,R. and Arap,W. (2001) *Nat. Med.*, **7**, 1249–1253.
Hackett,C.J. and Sharma,O.K. (2002) *Nat. Immunol.*, **3**, 887–889.
Hanes,J., Schaffitzel,C., Knappik,A. and Pluckthun,A. (2000) *Nat. Biotechnol.*, **18**, 1287–1292.
Hawkins,R.E., Russell,S.J. and Winter,G. (1992) *J. Mol. Biol.*, **226**, 889–896.
Holler,P.D. and Kranz,D.M. (2003) *Immunity*, **18**, 255–264.
Holler,P.D., Holman,P.O., Shusta,E.V., O'Herrin,S., Wittrup,K.D. and Kranz,D.M. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 5387–5392.
Holler,P.D., Chlewicki,L.K. and Kranz,D.M. (2003) *Nat. Immunol.*, **4**, 55–62.
Hoogenboom,H.R. (2005) *Nat. Biotechnol.*, **23**, 1105–1116.
Ikeda,H., Ohta,N., Furukawa,K., Miyazaki,H., Wang,L., Kuribayashi,K., Old,L.J. and Shiku,H. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 6375–6379.
Kieke,M.C., Shusta,E.V., Boder,E.T., Teyton,L., Wittrup,K.D. and Kranz,D.M. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 5651–5656.
Kieke,M.C., Sundberg,E., Shusta,E.V., Mariuzza,R.A., Wittrup,K.D. and Kranz,D.M. (2001) *J. Mol. Biol.*, **307**, 1305–1315.
Kupsch,J.M., Tidman,N.H., Kang,N.V., Truman,H., Hamilton,S., Patel,N., Newton Bishop,J.A., Leigh,I.M. and Crowe,J.S. (1999) *Clin. Cancer Res.*, **5**, 925–931.
Laugel,B. *et al*. (2005) *J. Biol. Chem.*, **280**, 1882–1892.
Lebowitz,M.S., O'Herrin,S.M., Hamad,A.R., Fahmy,T., Marguet,D., Barnes,N.C., Pardoll,D., Bieler,J.G. and Schneck,J.P. (1999) *Cell Immunol.*, **192**, 175–184.
Li,Y. *et al*. (2005) *Nat. Biotechnol.*, **23**, 349–354.
Maenaka,K. *et al*. (1999) *J. Biol. Chem.*, **274**, 28329–28334.
Marks,J.D. and Bradbury,A. (2004) *Methods Mol. Biol.*, **248**, 3: 161–76.
Marks,J.D., Hoogenboom,H.R., Griffiths,A.D. and Winter,G. (1992) *J. Biol. Chem.*, **267**, 16007–16010.
Masteller,E.L., Warner,M.R., Ferlin,W., Judkowski,V., Wilson,D., Glaichenhaus,N. and Bluestone,J.A. (2003) *J. Immunol.*, **171**, 5587–5595.
Rajpal,A., Beyaz,N., Haber,L., Cappuccilli,G., Yee,H., Bhatt,R.R., Takeuchi,T., Lerner,R.A. and Crea,R. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 8466–8471.
Rao,B.M., Lauffenburger,D.A. and Wittrup,K.D. (2005) *Nat. Biotechnol.*, **23**, 191–194.
Razai,A. *et al*. (2005) *J. Mol. Biol.*, **351**, 158–169.
Roovers,R.C., van der Linden,E., de Bruine,A.P., Arends,J.W. and Hoogenboom,H.R. (2001) *Eur. J. Cancer*, **37**, 542–549.
Rudolph,M.G. and Wilson,I.A. (2002) *Curr. Opin. Immunol.*, **14**, 52–65.
Schlueter,C.J., Manning,T.C., Schodin,B.A. and Kranz,D.M. (1996) *J. Immunol.*, **157**, 4478–4485.
Schweickhardt,R.L., Jiang,X., Garone,L.M. and Brondyk,W.H. (2003) *J. Biol. Chem.*, **278**, 28961–28967.
Scott,C.A., Garcia,K.C., Carbone,F.R., Wilson,I.A. and Teyton,L. (1996) *J. Exp. Med.*, **183**, 3.
Shusta,E.V., Holler,P.D., Kieke,M.C., Kranz,D.M. and Wittrup,K.D. (2000) *Nat. Biotechnol.*, **18**, 754–759.
Smith,G.P. (1985) *Science*, **228**, 1315–1317.
Speir,J.A., Garcia,K.C., Brunmark,A., Degano,M., Peterson,P.A., Teyton,L. and Wilson,I.A. (1998) *Immunity*, **8**, 3: 553–562.
Starwalt,S.E., Masteller,E.L., Bluestone,J.A. and Kranz,D.M. (2003) *Protein Eng.*, **16**, 147–156.
Tallquist,M.D. and Pease,L.R. (1995) *J. Immunol.*, **155**, 2419–2426.
Trepel,M., Arap,W. and Pasqualini,R. (2002) *Curr. Opin. Chem. Biol.*, **6**, 399–404.
Udaka,K., Tsomides,T.J. and Eisen,H.N. (1992) *Cell*, **69**, 989–998.
Udaka,K., Wiesmuller,K., Kienle,S., Jung,G. and Walden,P. (1996) *J. Immunol.*, **157**, 670–678.
van den Beucken,T., Pieters,H., Steukers,M., van der Vaart,M., Ladner,R.C., Hoogenboom,H.R. and Hufton,S.E. (2003) *FEBS Lett.*, **546**, 288–294.
Wang,X.X. and Shusta,E.V. (2005) *J. Immunol. Methods*, **304**, 30–42.
Weber,K.S., Donermeyer,D.L., Allen,P.M. and Kranz,D.M. (2005) *Proc. Natl Acad. Sci. USA*, **102**, 19033–19038.
Yeung,Y.A. and Wittrup,K.D. (2002) *Biotechnol. Prog.*, **18**, 3: 212–20.

Received October 30, 2005; revised February 8, 2006; accepted February 9, 2006

Edited by Hennie Hoogenboom