November 2005

Yeast Surface Display of a Noncovalent MHC Class II Heterodimer Complexed with Antigenic Peptide

Eric T. Boder  
*University of Pennsylvania, boder@seas.upenn.edu*

Jerome R. Bill  
*University of Colorado Health Sciences Center*

Andrew W. Nields  
*University of Pennsylvania*

Philippa C. Marrack  
*University of Colorado Health Sciences Center*

John W. Kappler  
*University of Colorado Health Sciences Center*

Follow this and additional works at: [http://repository.upenn.edu/cbe_papers](http://repository.upenn.edu/cbe_papers)

Recommended Citation


Postprint version. "This is a preprint of an article published in Biotechnology and Bioengineering, Volume 92, Issue 4, November 20, 2005, pages 485-491."

Publisher URL: [http://dx.doi.org/10.1002/bit.20616](http://dx.doi.org/10.1002/bit.20616)

This paper is posted at ScholarlyCommons. [http://repositoryupenn.edu/cbe_papers/61](http://repositoryupenn.edu/cbe_papers/61)

For more information, please contact repository@pobox.upenn.edu.
Yeast Surface Display of a Noncovalent MHC Class II Heterodimer Complexed with Antigenic Peptide

Abstract
Microbial protein display technologies have enabled directed molecular evolution of binding and stability properties in numerous protein systems. In particular, dramatic improvements to antibody binding affinity and kinetics have been accomplished using these tools in recent years. Examples of successful application of display technologies to other immunological proteins have been limited to date. Herein, we describe the expression of human class II major histocompatibility complex allele (MHCII) HLA-DR4 on the surface of Saccharomyces cerevisiae as a noncovalently associated heterodimer. The yeast-displayed MHCII is fully native as assessed by binding of conformationally specific monoclonal antibodies; failure of antibodies specific for empty HLA-DR4 to bind yeast-displayed protein indicates antigenic peptide is bound. This report represents the first example of a noncovalent protein dimer displayed on yeast and of successful display of wildtype MHCII. Results further point to the potential for using yeast surface display for engineering and analyzing the antigen binding properties of MHCII.

Keywords
yeast display, class II MHC, peptide loading, heterodimer expression in yeast, protein engineering

Comments
Postprint version. “This is a preprint of an article published in Biotechnology and Bioengineering, Volume 92, Issue 4, November 20, 2005, pages 485-491.”
Publisher URL: http://dx.doi.org/10.1002/bit.20616

This journal article is available at ScholarlyCommons: http://repository.upenn.edu/cbe_papers/61
Yeast Surface Display of a Noncovalent MHC Class II Heterodimer Complexed with Antigenic Peptide

Eric T. Boder\textsuperscript{1,2,3*}, Jerome R. Bill\textsuperscript{4,5,8}, Andrew W. Nields\textsuperscript{1,3}, Philippa C. Marrack\textsuperscript{4,6,8,9}, and John W. Kappler\textsuperscript{4,7,8,9}

\textsuperscript{1}Department of Chemical & Biomolecular Engineering, \textsuperscript{2}Department of Bioengineering, \textsuperscript{3}Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{4}Integrated Department of Immunology, \textsuperscript{5}Department of Medicine, \textsuperscript{6}Department of Biochemistry and Molecular Genetics, \textsuperscript{7}Department of Pharmacology and Program in Biomolecular Structure, University of Colorado Health Sciences Center, Denver, CO 80262; \textsuperscript{8}Integrated Department of Immunology, \textsuperscript{9}Howard Hughes Medical Institute, National Jewish Medical and Research Center, Denver, CO 80206.

* Corresponding author: Dr. Eric T. Boder, Department of Chemical and Biomolecular Engineering, University of Pennsylvania, 220 South 33rd Street, Philadelphia, PA 19104.
Tel: (215) 898-5658, Fax: (215) 573-2093; Email: boder@seas.upenn.edu
ABSTRACT

Microbial protein display technologies have enabled directed molecular evolution of binding and stability properties in numerous protein systems. In particular, dramatic improvements to antibody binding affinity and kinetics have been accomplished using these tools in recent years. Examples of successful application of display technologies to other immunological proteins have been limited to date. Herein, we describe the expression of human class II major histocompatibility complex allele (MHCII) HLA-DR4 on the surface of Saccharomyces cerevisiae as a noncovalently associated heterodimer. The yeast-displayed MHCII is fully native as assessed by binding of conformationally specific monoclonal antibodies; failure of antibodies specific for empty HLA-DR4 to bind yeast-displayed protein indicates antigenic peptide is bound. This report represents the first example of a noncovalent protein dimer displayed on yeast and of successful display of wild-type MHCII. Results further point to the potential for using yeast surface display for engineering and analyzing the antigen binding properties of MHCII.

Keywords: yeast display, class II MHC, peptide loading, heterodimer expression in yeast, protein engineering
INTRODUCTION

Class II MHC are membrane proteins expressed primarily on the surface of professional antigen presenting cells (APC; e.g., macrophages, dendritic cells, and B cells) and consist of two noncovalently associated chains, α and β (Stern and Wiley 1994). Each of these chains contains two domains. The peptide antigen binding site is formed from the α1 and β1 domains, while the Ig-like α2 and β2 domains likely aid folding and association in the endoplasmic reticulum. CD4+ αβ T lymphocytes respond to antigen peptides only when bound to MHCII presented on the surface of APC.

MHCII bound to defined antigenic peptides have been produced using a polypeptide linker attaching the peptide to the β chain N-terminus (Kozono et al. 1994). Tetramerization of these molecules by site-specific biotinylation (Cull and Schatz 2000) and avidin binding has enabled dramatic progress in the study of T cell-mediated immune phenomena (Altman et al. 1996; Kotzin et al. 2000). Specifically, tracking of antigen-specific T cells during active immune responses in vivo has become possible (Reijonen and Kwok 2003; Skinner et al. 2000), as well as screening of putative peptide antigens against T cells to identify reactive peptides (Novak et al. 2001). Engineered, MHC-based reagents have also shown promise in inhibition of T cell-mediated autoimmune disease (Huan et al. 2004). Consequently, methods to engineer molecular properties of these important molecules would be of great value.

The utility of directed evolution and display technologies for protein engineering [reviewed by (Chen and Georgiou 2002; Hoogenboom 2002; Pluckthun et al. 2000)] has been well established in a number of systems including antibodies, enzymes, protease inhibitors, novel binding domains, cytokines, and mammalian cell surface receptor proteins. However,
application of this methodology to T cell receptors (TCR) and major histocompatibility complex proteins (MHC), critical surface proteins involved in T cell mediated immune responses, have been few (Chlewicki et al. 2005; Esteban and Zhao 2004; Holler et al. 2003; Holler et al. 2000; Kieke et al. 1999; Shusta et al. 2000; Weidanz et al. 1998). Phage display of MHC has proved to be particularly challenging, and only three examples of successful display of class I MHC have been demonstrated to date (Kurokawa et al. 2002; Le Doussal et al. 2000; Vest Hansen et al. 2001). The lack of MHC phage display data is unsurprising as these proteins are natively heterodimeric with intrachain disulfide bonds; furthermore, at least some MHC proteins demonstrate poor stability or nonnative conformations in the absence of bound antigen peptide (Arimilli et al. 1999; Germain and Rinker 1993). These issues complicate heterologous expression in prokaryotic hosts, which is a prerequisite for phage display.

In contrast to phage display, yeast surface display relies upon a eukaryotic host for expression of the gene of interest while maintaining the genotype-phenotype linkage requisite for directed molecular evolution. The method has been validated by successful studies applied to numerous single-chain antibodies (Wittrup 2001), antibody Fab fragments (Lin et al. 2003; Weaver-Feldhaus et al. 2004), and a single-chain T cell receptor (Holler et al. 2000; Kieke et al. 1999; Shusta et al. 2000). Yeast’s ability to perform many of the post-translational modifications typical of secreted mammalian proteins represents a decided advantage of this technology. Three recent studies have demonstrated the ability of yeast to surface display both class I and class II MHC (Brophy et al. 2003; Esteban and Zhao 2004; Starwalt et al. 2003). A single-chain derivative of an MHCI was successfully displayed, and putatively functional as judged by direct, antigen-specific T cell activation using yeast as the antigen presenting particle (Brophy et al. 2003). However, the yeast-displayed MHCI in this study (murine H-2Kb) was also expressed by
the T cells assayed, and the experiments performed were unable to rule out peptide transfer and T cell autostimulation as the activation mechanism (Ge et al. 2002). In an elegant study, Kranz and coworkers isolated thermodynamically stabilized mutants of single-chain murine MHCII I-A\(^\beta\) via screening of combinatorial libraries and demonstrated the importance of two polymorphic \(\beta\) chain positions in this stabilization (Starwalt et al. 2003). These yeast-displayed MHCII failed to demonstrate peptide binding or T cell activation. Likewise, directed evolution was recently applied to engineer yeast-displayable mutants of a single-chain derivative of HLA-DR1, although data regarding T cell receptor reactivity was not presented (Esteban and Zhao 2004). Most significantly, protein mutations were required to achieve expression of DR1 in this system; thus, structure-function studies using this displayed protein must be approached cautiously as the mutant protein properties may not be representative of wild-type HLA-DR1.

Recently, surface display of Fab antibody fragments on yeast was demonstrated. An anti-streptavidin antibody heavy chain was expressed as a fusion to the C-terminus of the yeast Aga2p mating agglutinin (van den Beucken et al. 2003); the light chain was expressed from a separate promoter and the heterodimer was stabilized through the Fab interchain disulfide bond. A similar expression system for Fab libraries with free N-termini has also recently been demonstrated (Weaver-Feldhaus et al. 2004), as has yeast display of a catalytic Fab (Lin et al. 2003). These studies represent the first examples of assembly and display of multimeric proteins on yeast without the aid of a single-chain linker. In this work, we further establish the ability of the yeast surface display system to correctly assemble and display a heterodimer in which interchain interactions are entirely noncovalent. Significantly, our results represent the first example of yeast display of wild-type MHCII and therefore represent an important advance toward the ability to analyze and engineer MHCII-peptide interactions in the absence of
potentially confounding MHCII mutations. Yeast displayed HLA-DR4 demonstrates peptide binding activity; however, our data indicate that yeast are incapable of stimulating antigen-specific T cells and thus suggest that engineering and analysis of MHCII by yeast display is limited to peptide-binding and structural stability applications.

MATERIALS AND METHODS

Yeast expression vectors

Expression vectors for yeast display of DR4 were based upon previously described plasmid pCT302 (Boder et al. 2000). A PCR fragment of the extracellular domain of HLA-DRB1*0401 (amino acids 30 – 223) with N-terminal HA (307 – 319) peptide and thrombin-cleavable linker (Kozono et al. 1994) was cloned into pCT302 via primer-encoded Nhe I and Xho I restriction sites. This created an in-frame fusion C-terminal to the AGA2 gene (plasmid pCT-DR4β). A dual cassette expression vector was created by cloning the yeast mating factor α1 transcriptional terminator sequence 5’ of the yeast GAL1-10 bidirectional promoter, enabling galactose-inducible co-expression of two open reading frames from one plasmid. A PCR fragment of the extracellular domain of HLA-DRA1*0101 with a synthetic PRE-PRO signal sequence (Arnold et al. 1998) was cloned into plasmid pCT-DR4β 5’ of the GAL1-10 promoter (i.e., downstream of the GAL10 promoter) to create plasmid Z27.

Plasmid Z47 (Figure 1) was constructed similarly by cloning a FLU-DR4β extracellular domain PCR product pCT4-scFv. The resulting FLU-DR4β-AGA2 cassette (AGA2 beginning with residue 20) was subcloned to place it behind the GAL10 promoter and in frame with a synthetic PRE-PRO leader sequence.

Expression and purification of soluble HLA-DR4
A baculovirus shuttle vector containing p10 and polyhedrin promoters driving expression of HLA-DR4 β with covalent FLU peptide and HLA-DRα extracellular domains was used to cotransfect Sf9 cells along with BaculoGold baculovirus DNA (Pharmingen) according to the manufacturer’s instructions. Viral stocks were isolated after 10 days and amplified once by infection of Sf9 cells and culturing for 7 days. The resulting high titer stocks were used to infect suspension-adapted High Five (Invitrogen) cells at 2 x 10⁶ cells/mL. Protein was produced by spinner flask culture for 6 days at 27°C in TMN/FH medium plus 10% Pluronic F-68. Assembled HLA-DR4-FLU heterodimers were purified from culture supernatants by affinity chromatography. Anti-DRα mAb LB3.1 was produced from hybridoma culture, purified by protein A-Sepharose affinity chromatography (Pharmacia), and coupled to activated CNBr-Sepharose.

Following affinity chromatography, monomeric DRα chain was removed by size exclusion chromatography (Pharmacia FPLC, Superdex 10/200). Heterodimer identity was confirmed by reducing and non-reducing SDS-PAGE electrophoresis and Coomassie staining. DR4-FLU protein was concentrated to ~4 mg/mL and enzymatically biotinylated as described (Crawford et al. 1998). Free biotin was removed by FPLC, and biotin incorporation was confirmed by avidin-agarose depletion and quantitative ELISA using anti-DR4β mAb L227 and biotinylated anti-DRα mAb LB3.1.

**Immunofluorescent staining and flow cytometry**

3 mL of SD-CAA (Boder et al. 2000) were inoculated with a single yeast colony and grown overnight at 30° to an OD₆₀₀ of 2.0 – 5.0. 3 x 10⁷ cells were collected, switched to 3 mL SG-CAA (galactose replaces glucose) + 10% glycerol, grown 24 hrs at 30°C, then stored at 4°C. 1.6 x 10⁷ cells were collected, spun, resuspended in 1 mL PBSB (137 mM NaCl, 2.7 mM KCl,
10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1% (w/v) BSA, pH 7.4, spun again, resuspended in 0.4 mL PBSB, and incubated at 80°C for 1 hr. A control group was kept at 4°C. After the 80°C incubation, both sets of cells were washed twice with 1 mL PBSB and resuspended in 0.8 mL PBSB. 100 µL aliquots of these suspensions (~ 2 x 10⁶ cells) were individually centrifuged and resuspended in 100 µL PBSB containing 1° Ab: 9E10 (Covance, 1:100), bio-12CA5 (Roche, 1:20), L243 (BD Pharmingen, 2:5), LB3.1 (22 µg/mL), L227 (15 µg/mL), KL295 (15 µg/mL), or KL304 (15 µg/mL). The samples were then added to a 96-well plate and incubated on ice for 45 min. The plate was washed three times by centrifuging at 3000g for 5 min and adding 200 µL PBSB to each well. After washing, 100 µL of PBSB containing 2° Ab (goat anti-mouse-PE, Sigma, 1:20) was added to each well, and the plate was incubated on ice for 45 min. After washing, the cells were analyzed on a BD FACSscan flow cytometer.

**T cell activation assays**

75IP fibroblasts transfected with HLA-DR4 (Boen et al. 2000) were cultured in D-MEM (LTI) + 10% FCS. FLU-specific and HLA-DR4-restricted T cell hybridoma HA1.7 (Lamb et al. 1982) were cultured in complete tumor media (CTM) (Kappler et al. 1981). To test for T cell activation, stimulating particles (10⁵ for fibroblasts, or 2 x 10⁵ – 1 x 10⁶ for yeast or antigen-coated beads) were added to a 96-well plate to a final volume of 250 µL. 75IP-DR4-FLU positive controls elicited >1200 U/mL IL-2 in all experiments. In some experiments, yeast were lethally UV irradiated prior to the assay using a Stratalinker UV crosslinking apparatus (Stratagene) at ~1 J/cm² for 3 mins. This prevented overgrowth of the culture during the overnight stimulation assay. Following incubation for 24 hrs at 37°C in 5% CO₂, medium supernatants were isolated and IL-2 levels were assayed by the HT-2 bioassay (Kappler et al.
1981). Wells containing live HT-2 cells were identified by light microscopy following overnight incubation in serially diluted activation supernatants.

RESULTS
Yeast expression of HLA-DR4flu

Yeast display of proteins has been commonly accomplished by recombinant expression of the protein of interest fused to the Aga2p subunit of α-agglutinin, a cell wall protein involved in cell-cell adhesion during mating (Shen et al. 2001). Two constructs were developed to test surface expression of human MHCII HLA-DR4 in yeast (Figure 1). Both constructs attached the Aga2p fusion partner to a covalent peptide-MHCII β chain construct via an intervening (Gly–Ser)3 linker sequence. The influenza hemagglutinin 307 – 319 peptide (FLU) was attached to DR4β through a previously described flexible linker segment (Kozono et al. 1994). One fusion (construct A) incorporated Aga2p at the N-terminus of the FLU-DR4β protein, a format similar to that used in prior yeast display studies (Boder and Wittrup 2000). N-terminal extensions appended to covalently associated peptides have been previously shown to affect the conformation of MHCII (Rotzschke et al. 1999); thus, a second construct with the Aga2p fusion partner linked to the C-terminus of FLU-DR4β was designed. In both cases, the DRα chain was expressed as a soluble protein from a separate cassette making use of the bidirectional GAL1-10 promoter, as shown in Figure 1. Heterodimeric protein expression on the yeast surface was assessed by immunofluorescent staining. As shown in Figures 2 and 3(B), mAb specific for both the α (L243 and LB3.1) and β (L227) chains of DR4 recognize the yeast-displayed protein. Furthermore, binding of both L243 and LB3.1 is sensitive to native conformation and heterodimeric assembly of HLA-DR molecules (Horejsi et al. 1986). These data therefore indicate that yeast-displayed HLA-DR4 is assembled as a native heterodimer. Yeast expressing
construct A exhibit little reactivity with L227 and substantially lower reactivity with L243 and LB3.1 in comparison to construct B; thus, linkage of the Aga2p fusion partner at the C-terminus of MHCII β appears to be necessary for efficient expression in this context.

**Peptide loading status of yeast-displayed HLA-DR4**

To confirm that peptides were indeed loaded in the antigen-binding groove of yeast displayed HLA-DR4, additional immunofluorescence studies were performed. Monoclonal antibodies KL295 and KL304 (LaPan et al. 1992) recognize denatured HLA-DR1 and HLA-DR4 by binding to a sequence in the helical region of the β1 domain (Zarutskie et al. 1999). A significant increase in cell staining by KL295 and KL304 was observed upon heat denaturation of yeast at 80°C (Figure 3). Likewise, complete loss of L243 and LB3.1 epitopes on HLA-DRα was observed in these studies, while staining for the HA epitope tag at the C-terminus of the DR4β-Aga2p fusion remained unchanged. Taken together, these results indicate that yeast-displayed HLA-DR4 is fully native and peptide-loaded.

**Failure of T cell stimulation**

Despite displaying native HLA-DR4, yeast proved unable to stimulate IL-2 production by FLU-specific and HLA-DR4-restricted T cell hybridoma HA1.7 (data not shown). To ascertain the ability of yeast to engage in requisite intercellular interactions with T cells, yeast were coated with HLA-DR4-FLU protein produced by baculovirus-infected insect cells (bvDR4). Randomly biotinylated yeast were incubated with ExtrAvidin™ at high concentration to yield immobilized protein with unoccupied biotin binding sites. Avidin-coated yeast were then incubated with bvDR4 enzymatically biotinylated via a c-terminal biotin acceptor peptide tag (Cull and Schatz 2000; Schatz 1993). Final yeast surface density of bvDR4 was controlled by varying the
concentration of biotin in the initial yeast-labeling step, and presence and levels of the final immobilized protein on the yeast surface were assessed by flow cytometry (Figure 4). These yeast were tested for their ability to stimulate IL-2 production from HA1.7. While biotinylated bvDR4 immobilized on an avidin-coated tissue culture well yielded >1200 units/mL of IL-2, yeast samples failed to stimulate detectable IL-2 production (i.e., < 10 units/mL) by HA1.7 (data not shown). Controls using DR4-FLU-transfected fibroblasts as antigen presenting cells (Boen et al. 2000) yielded no detectable difference in IL-2 production by HA1.7 in the presence or absence of excess yeast (data not shown), confirming that yeast have no inhibitory effect on T cell function or the assay methods.

**DISCUSSION**

To date, yeast display of MHCII has been limited to two examples: murine I-A^7^ (Starwalt et al. 2003) and human HLA-DR1 (Esteban and Zhao 2004). In both cases, display of native protein required isolation of mutants from combinatorial libraries and therefore raise questions regarding the potential impact of these mutations on peptide-binding kinetics or protein stability. Each of these examples made use of typical yeast display constructs with the Aga2p anchoring subunit N-terminal to a single-chain MHCII derivative (largely analogous to construct A in Figure 1). In this study, we have tested the ability of yeast to display an additional human MHCII, HLA-DR4, and we have assessed two fusion protein formats in which the Aga2p anchoring partner is linked to the N- and C-terminus of the DR4β chain, respectively (Figure 1). The C-terminal Aga2p fusion (construct B) more closely mimics the anchoring mechanism of MHCII expressed on APC via transmembrane domains at the C-terminus of both the α and β chains. The data indicate that HLA-DR4 expresses well with a C-terminal Aga2p (construct B) but with an N-terminal Aga2p expresses at levels sufficiently low to preclude detection of DR4β
by immunofluorescent staining (Figures 2 and 3A,B). These data are consistent with previous reports on yeast display of other MHCII (Starwalt et al. 2003; Esteban and Zhao 2004) but further indicate that successful display of wild-type MHCII on yeast is possible using the appropriate fusion protein system (construct B in Figure 1).

Staining with conformationally sensitive antibodies enables probing of DR4 conformational state and peptide loading (Figure 3). Our results indicate that yeast-displayed DR4 is native; furthermore, mAb L243 reportedly fails to react with isolated DRα chain (Horejsi et al. 1986), presumably because the MHCII α chain fails to fold natively in the absence of the β chain. Heat denaturation of the yeast quantitatively abrogates L243 binding, as expected for a noncovalently associated heterodimer. Thus, this work represents the first demonstration that yeast surface display is not limited to simple single-chain protein display or disulfide-bonded Fab display, but can also be used to successfully display complex noncovalent multimers. Monoclonal antibodies directed against a β chain epitope that is masked in native, peptide-loaded DR4 fail to react with DR4-displaying yeast, but demonstrate significant binding following heat denaturation, as shown in Figure 3C. These results strongly suggest that the yeast-displayed DR4 is functional in terms of peptide binding activity.

We were unable to demonstrate T cell receptor binding activity of our yeast-displayed protein; however, yeast coated with functional DR4 produced in insect cells likewise failed to demonstrate activity. Fluorescent tetramers of chimeric human/mouse HA1.7 TCR also failed to stain DR4-displaying yeast, but these reagents also failed to stain 75IP-DR4-FLU fibroblasts (which activate HA1.7 T cells) and thus this result is inconclusive (data not shown). BIAcore analysis of monomeric HA1.7 TCR binding to baculovirus-produced DR4-FLU suggests the affinity is low (> 50 µM; E.T. Boder, P.C. Marrack, J.W. Kappler, unpublished observations)
and thus insufficient for TCR tetramer binding, which occurs in the absence of avidity-enhancing CD4 binding.

Interestingly, 2.8 μm streptavidin-coated magnetic beads incubated with bvDR4 also failed to stimulate detectable IL-2 production from HA1.7 T cells (data not shown). Previous experiments have indicated that CD8+ cytotoxic T lymphocyte (CTL) activation by pMHCI adsorbed to latex microspheres requires “cell sized” particles ~5 μm diameter (Mescher 1992). Beads 1 - 2 μm in diameter yielded no CTL activity, while 5 μm beads stimulated a weak response. Valitutti and colleagues also showed a similar effect in a CD8 T cell system (Zaru et al. 2002). pMHC-coated polystyrene beads weakly stimulated antigen-specific T cells, but co-immobilization of anti-CD2 mAb potentiated T cell activation. Particles presenting a T cell contact surface with a small radius of curvature seem to yield nonproductive interaction despite the presence of cognate pMHC-TCR interaction.

Taken together with our data, these results suggest that geometry of the antigen-presenting particle may be important for both CD8 and CD4 T cell stimulation, at least by artificial antigen-presenting particles lacking co-stimulatory interactions. It is noteworthy that the antigen-presenting particles in the current study (yeast) and those discussed above (synthetic beads) present spatially restricted ligands incapable of two-dimensional diffusion on the surface of the particle; thus, supramolecular activation cluster (Monks et al. 1998) or immunological synapse (Bromley et al. 2001) formation is not possible in these systems regardless of the presence of costimulatory ligands. Although these spatial patterns have been suggested as important to both thymic selection and peripheral T cell activation in vivo (Jacobelli et al. 2004; Lee et al. 2003a; Lee et al. 2002; Minter and Osborne 2003), synapse formation is clearly not required for T cell stimulation in all cases. Nonetheless, it would be interesting to investigate the
effects of antigen presenting particle size on T cell stimulation in a system allowing ligand diffusion on the presenting particle, such as a lipid vesicle. This information might further corroborate physical models of the T cell-APC interaction in which T cell membrane bending modulus and thermal fluctuations play a critical role in spatial pattern formation, with apparent effects on signaling and activation (Lee et al. 2003b; Lee et al. 2002).

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation under CAREER Award No. 0239099, the Merck Engineering Foundation, and the Howard Hughes Medical Institute. We thank the staff of the NJC Flow Cytometry Facility, the NJC Molecular Resources Facility, and the Penn Flow Cytometry and Cell Sorting Shared Resource for technical assistance.
**Figure 1.** Map of yeast display vector for expression of heterodimeric proteins. Two expression cassettes are driven by the bidirectional GAL1-10 promoter. Yeast Aga2p mediates surface linkage. Plasmid Z27 (Construct A) and plasmid Z47 (Construct B) differ with respect to Aga2p fusion at the N- and C-terminus of the peptide-DR4β chain, respectively, and with respect to the orientation of the promoter; Z27: GAL1 = AGA2-DR4β and GAL10 = DRα; Z47: GAL1 = DRα and GAL10 = DR4β-AGA2. DRα is solubly expressed and directed to the secretory pathway via a synthetic leader sequence. *HA represents hemagglutinin epitope tag sequence YPYDVPDYA recognized by mAb 12CA5.*

**Figure 2.** Flow cytometric analysis of Aga2p-FLU-DR4 expression on the yeast cell surface. Yeast transformed with Z27 (Construct A) were induced in galactose for 48 hr at 20°C and stained for expression of the HA epitope tag (mAb 12CA5), DR4β chain (L227), and DRα chain (L243), as indicated. *No 1° = control stained with secondary antibody only.*

**Figure 3.** Analysis of peptide-loading of yeast-displayed HLA-DR4-FLU. (A-C) Yeast expressing DR4 (construct B) were incubated at room temperature (filled) or 80°C (empty) and immunofluorescently stained with mAbs against the (A) HA epitope tag, (B) DRα chain, and (C) DRβ 58 - 69 epitope. (D) Control yeast expressing an unrelated protein were stained with protein specific mAb following room temperature incubation (filled) or KL295 (open, solid line) and irrelevant control mAb (open, dashed) following incubation at 80°C.

**Figure 4.** Levels of insect cell-produced HLA-DR4-FLU presented by yeast. Yeast expressing FLU-DR4-Aga2p were nonspecifically biotinylated with the indicated concentrations of NHS-
biotin prior to binding of avidin and site-specifically biotinylated HLA-DR4-FLU. Cells were stained with anti-DRα chain mAb LB3.1 and analyzed by flow cytometry.
REFERENCES


Figure 2
Boder, et al.

![Graphs showing fluorescence-activated cell sorting (FACS) analysis with different antibodies.]

- 5.4.2000.001: no 1°
- 5.4.2000.002: anti-HA
- 5.4.2000.005: anti-DR4β
- 5.4.2000.003: anti-DRα
Figure 3
Boder, et al.