Engineering and Characterization of a Stabilized α1/α2 Module of the Class I Major Histocompatibility Complex Product Ld*

Received for publication, May 8, 2006 Published, JBC Papers in Press, June 30, 2006, DOI 10.1074/jbc.M604343200

Lindsay L. Jones†, Susan E. Brophy†,‡, Alexander J. Bankovich†, Jeremy A. Colf†, Nicole A. Hanick†, K. Christopher Garcia‡, and David M. Kranz†

From the †Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 and the ‡Department of Microbiology and Immunology, Stanford University, Stanford, California 94305

The major histocompatibility complex (MHC) is the most polymorphic locus known, with thousands of allelic variants. There is considerable interest in understanding the diversity of structures and peptide-binding features represented by this class of proteins. Although many MHC proteins have been crystallized, others have not been amenable to structural or biochemical studies due to problems with expression or stability. In the present study, yeast display was used to engineer stabilizing mutations into the class I MHC molecule, Ld. The approach was based on previous studies that showed surface levels of yeast-displayed fusion proteins are directly correlated with protein stability. To engineer a more stable Ld, we selected Ld mutants with increased surface expression from randomly mutated yeast display libraries using anti-Ld antibodies or high affinity, soluble T-cell receptors (TCRs). The most stable Ld mutant, Ld-m31, consisted of a single-chain MHC module containing only the α1 and α2 domains. The enhanced stability was in part due to a single mutation (Trp-97→Arg), shown previously to be present in the allele Ld. Mutant Ld-m31 could bind to Ld peptides, and the specific peptide-Ld-m31 complex (QL9-Ld-m31) was recognized by alloreactive TCR 2C. A soluble form of the Ld-m31 protein was expressed in Escherichia coli and refolded from inclusion bodies at high yields. Surface plasmon resonance showed that TCRs bound to peptide-Ld-m31 complexes with affinities similar to those of native full-length Ld. The TCR and QL9-Ld-m31 formed complexes that could be resolved by native gel electrophoresis, suggesting that stabilized α1/α2 class I platforms may enable various structural studies.

The class I major histocompatibility complex (MHC) encodes a cell surface glycoprotein that presents antigenic pep-

† This work was supported by National Institutes of Health (NIH) Grant GM55767 (to D. M. K.), by NIH Grant AI048540 (to K. C. G.), by a Stanford Immunology Program Training Grant (to A. J. B. and N. A. H.), and by the National Science Foundation (to L. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Two authors contributed equally to this work.

§ Current address: Abbott Laboratories, 100 Abbott Park Rd., Abbott Park, IL 60064.

1 To whom correspondence should be addressed: Dept. of Biochemistry, University of Illinois, 600 S. Mathews Ave., Urbana, IL 61801. Tel.: 217-244-2821; Fax: 217-244-5858; E-mail: d-kranz@uiuc.edu.

2 The abbreviations used are: MHC, major histocompatibility complex; TCR, T-cell receptor; scTCR, single-chain TCR; MPI, mean fluorescence unit(s); SA-PE, streptavidin-phycoerythrin.
less stable than many other MHC complexes. For example, Ld is expressed on the surface of splenocytes at 3- to 4-fold lower levels than the class I products Kd and Dd (17, 18). Consistent with this difference, Beck et al. (19) showed that Ld complexes exhibited slower intracellular processing and weaker association with β2m compared with Kd and Dd. A chimera combining the N-terminal Dd domain (α1/α2) and the C-terminal Ld domain (α3) exhibited the same rate of intracellular processing and level of association with β2m as Dd. This result suggests that the α1 and/or α2 domains influence the stability of the Ld complex (19). More recently, using multiple chimeras of Ld and Lα, a single amino acid at residue 97 of the Ld complex was shown to be responsible for decreased cell surface expression of Ld and its lower affinity for β2m (20).

Two crystal structures of Ld, complexed with an endogenous peptide p29 (YPNVIHNF) (21) or with a mixture of peptides (22) have been solved, providing insight into the weaker association of β2m and the generally low affinity of peptides for Ld. Balendirian et al. (21) suggested that the structural instability of the Ld-β2m complex is due to the unique orientation of the α1/α2 domains relative to β2m and that this orientation causes a loss of productive contacts between α1/α2 and β2m. For example, a comparison of the intermolecular interactions, van der Waals, and hydrogen bonds between α1/α2 and β2m for three different MHC complexes, Kb, Db, and Ld revealed 17, 21, and 6 contacts, respectively. Structural features of the peptide binding cleft influence peptide selection and peptide binding affinity, and these properties can also influence the stability of the peptide-MHC complex. The Ld-β2m protein binds octamer or nonamer peptides with anchor residues of proline at peptide position 2 (P2) and leucine, methionine, or phenylalanine at the C-terminal position (23). However, peptides that lack proline at P2 have also been identified as Ld-binding peptides (e.g. tum-, TQNHRALDL and p2CA, LSPFPFDL, a peptide recognized by the 2C TCR used in the present studies) (24, 25). The peptide binding pocket for proline exhibits less shape complementarity than other MHC alleles with a proline anchor residue at P2 (26).

Also, the peptide binding pocket of Ld is composed of more hydrophobic residues than other MHC complexes, limiting the possible number of hydrogen bonds that can form between the peptide and α1/α2 residues. Finally, a central bulge from the floor of the peptide binding cleft in Ld prevents peptides from having a central, stabilizing anchor residue as has been observed in Kb and other class I molecules (22). These characteristics most likely contribute to the lower affinities of peptides for Ld. It has been suggested that these unique features of the Ld-β2m complex provide an alternative mechanism to the classic antigen presentation pathway (26, 27). Notwithstanding the physiological function, the instability of peptide-Ld-β2m complexes has been problematic in attempts to examine structural features of this system.5

In the present study, Ld mutants were engineered to overcome stability problems by a process of directed evolution. Randomly mutated Ld-β2m products were displayed as single-chain fusions on the surface of yeast. Stabilized mutants of Ld, complexed with the Ld-binding peptide QL9 (QLSPFPFDL) (28), were selected by flow cytometric sorting using anti-Ld antibodies and a soluble QL9-Ld-specific high affinity TCR (29). This approach yielded several mutants, including a stabilized, single-chain MHC module consisting of only the α1/α2 peptide binding domains. The α1/α2 module was capable of binding QL9, and QL9-Ld-specific TCRs bound to the QL9-α1/α2 complex with the same affinities as wild-type QL9-Ld complex on the cell surface. Bacterial expression and characterization of soluble complexes of the QL9-α1/α2-Ld module indicated that they could be bound by soluble TCRs with affinities similar to full-length peptide-Ld complexes. The results demonstrate that class I MHC can be engineered to generate more stable complexes and that individual peptide-binding domains can be produced for biochemical and structural studies.

### EXPERIMENTAL PROCEDURES

**Peptides and Antibodies**—Peptides that bind to Ld (QL9, QLSPFPFDL; and MCMV, YPHFMrPTNL) were synthesized by standard Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry at the Protein Science Facility at the University of Illinois (Urbana, IL) or the Protein Chemistry Laboratory at Texas A&M University (College Station, TX) or the Macromolecular Core Facility at Penn State University College of Medicine (Hershey, PA). Peptides were purified by C-18 reversed-phase high-performance liquid chromatography with a linear acetonitrile gradient. The following monoclonal antibodies were used: anti-Ld (α2) antibody 30-5-7 (purified from ascites), anti-Ld (α3) antibody 28.14.8 (BD Pharrmingen), anti-His antibody (Covance, Cumberland, VA), and anti-TCR(β8.1–8.3) antibody F23.1 (purified from ascites) and anti-TCR(Cβ) antibody H57–597 (BD Pharrmingen). Polyclonal antibodies used included fluorescein isothiocyanate F(ab’)2 goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and biotinylated goat anti-mouse IgG antibodies (Rockland, Gilbertsville, PA).

**Yeast Display of the Single-chain Ld-β2m**—The mouse MHC Ld α chain gene was fused to the mouse β2m gene using PCR overlap extension (30). The Ld-β2m PCR product consisted of the Ld heavy-chain gene (α1, α2, and α3) covalently linked to the mouse β2m gene with a 45-bp Gly-Ser (Gly3Ser) linker followed by a c-myc epitope tag (Fig. 1A). The Ld-β2m PCR product and the yeast display vector pCT302 were digested with restriction enzymes Nhel/Xhol, ligated, and transformed into Escherichia coli strain DH10B by electroporation. E. coli colonies were grown on LB/ampicillin (50 μg/ml) plates and then inoculated into LB/ampicillin for 24 h at 37°C. Plasmids were isolated using Qiagen mini-prep kits (Valencia, CA), diagnostically analyzed with restriction enzymes, and sequenced. Ld-β2m-encoded plasmids were transformed by the lithium acetate transformation method (31) into Saccharomyces cerevisiae strain EBY100 (32). Transformed yeast colonies were grown on nutrient-selective media that lacked tryptophan (SD-CAA, glucose 2 wt%, yeast nitrogen base 0.67 wt%, casamino acids 0.5 wt%).

**Soluble T-cell Receptors**—Selection of yeast displayed Ld libraries was performed with a soluble high affinity derivative of the 2C TCR called m6 (2C-m6), which was expressed as an αβ heterodimer in insect cells as previously described (33, 34). Culture supernatants from these transfectants were used directly.

---

5 L. L. Jones, S. E. Brophy, A. J. Bankovich, L. A. Colf, N. A. Hanick, K. C. Garcia, and D. M. Kranz, unpublished observations.
Engineering MHC Proteins

**FIGURE 1. Yeast display of L^d^-β2m.** A diagram of the L^d^-β2m construct expressed on the surface of yeast cells as an Aga-2 fusion protein; HA (hemaglutinin) and c-myc refer to epitope tags. B and C, expression of L^d^-β2m constructs (C) was induced in the presence of exogenous QL9 peptide. For flow cytometric analysis, cells were stained with anti-L^d^ antibodies against α2 or α3, followed by biotinylated goat anti-mouse IgG and an SA-PE conjugate (left and middle), or 2C-m6 TCR, biotinylated anti-TCR Cβ and an SA-PE conjugate (right) (shaded histograms). Unshaded histograms represent background staining from cells incubated with secondary antibodies and SA-PE only. Negative populations of yeast cells that do not express the Aga-2 fusion proteins appear in each histogram; these are observed for all yeast-displayed fusions. Cells were analyzed on a Coulter XL flow cytometer.

as a source of soluble high affinity TCR for staining cells (34). For some experiments, TCR was purified from culture supernatants by affinity chromatography with nickel-nitritoltriacetic acid-agarose (Qiagen) and size exclusion chromatography (Superdex 75, Amersham Biosciences).

**Detection of L^d^-β2m on Yeast Cell Surface**—Transformed yeast colonies were inoculated into SD-CAA medium and grown at 30 °C. After 18–24 h, cells were harvested by centrifugation and incubated in SG-CAA (2 wt% galactose replacing glucose in SD-CAA) at 20 °C. Where indicated, L^d^-binding peptide QL9 (QLSPFPFFDL) was added in excess (1 μM) to SG-CAA medium to stabilize L^d^-β2m complexes expressed on the yeast surface. After 48 h, yeast cultures were harvested by centrifugation, washed with PBS (10 mM NaPO_4, 150 mM NaCl, pH 7.3) containing 0.5% bovine serum albumin (BSA), and incubated on ice with anti-L^d^ antibodies (28.14.8 or B3-34), followed by biotinylated goat anti-mouse IgG followed by streptavidin-phycoerythrin (SA-PE) or fluorescein isothiocyanate (FITC) goat anti-mouse IgG. Cells incubated with 2C-m6 TCR were washed with PBS/BSA and incubated on ice with either biotinylated goat anti-mouse IgG followed by streptavidin-phycoerythrin (SA-PE) or fluorescein isothiocyanate (FITC) goat anti-mouse IgG. Cells incubated with 2C-m6 TCR were washed with PBS/BSA and incubated on ice with either biotinylated goat anti-mouse IgG followed by streptavidin-phycoerythrin (SA-PE) or fluorescein isothiocyanate (FITC) goat anti-mouse IgG.

**Construction of Random Mutagenesis Libraries**—Error-prone PCR and homologous recombination were used to generate libraries of randomly mutated L^d^-β2m genes (14). Error-prone PCR products of the L^d^-β2m gene were created using a modified PCR reaction that generates ~4 to 7 nucleotide mutations per 1000 bp. Error-prone PCR reactions included Tag polymerase (Invitrogen), polymerase buffer (without MgCl_2), dNTPs (0.2 mM each), 2 mM MgCl_2, 0.3 mM MnCl_2, template L^d^-β2m in pCT302 (50 ng), and upstream and downstream primers (5 μM). To facilitate homologous recombination, upstream and downstream primers were designed within the pCT302 vector backbone ~100 bp upstream and downstream of the L^d^-β2m gene insert (upstream primer, 5'-GGC AGC CCC ATC AAC ACA CAG TAT-3'; downstream primer, 5'-CGA GCT AAA AGT ACA GTC CC-3'). Error-prone PCR products (150 ng)

---

*L. L. Jones, S. E. Brophy, A. J. Bankovich, L. A. Coff, N. A. Hanick, K. C. Garcia, and D. M. Kranz, unpublished results.*
Engineering MHC Proteins

FIGURE 2. Influence of QL9 peptide on antibody and TCR binding to the mutant Ld-m8. Yeast cells were induced to express the mutant Ld-m8 protein in the absence (A) or presence (B) of exogenous QL9 peptide. Cells were stained with anti-Ld antibodies (left and middle) or 2C-m6 TCR (right) as described in Fig. 1 (shaded histograms). Unshaded histograms represent background staining from cells incubated with secondary antibodies and SA-PE only.

and pCT302 vector (150 ng) were digested with Nhel/XhoI, combined, and transformed into EBY100 yeast by electroporation in multiple reactions (20–40 electroporations). Library sizes, determined by plating an aliquot of the pooled transformations, averaged ~10^6 independent variants. Error-prone PCR and homologous recombination were used to generate a second random error-prone library, using a template from a clone identified in the first error-prone Ld-β2m library called Ld-m8.

Flow Cytometric Sorting of Yeast Libraries—The first generation Ld-β2m error-prone yeast library was sorted in four sequential rounds with anti-Ld antibodies. Briefly, the yeast library was grown in SD-CAA media for 24 h at 30 °C followed by induction of protein expression in SG-CAA media containing 1 μM QL9 peptide (QLSPFPFDL) for ~48 h at 20 °C. Cells were harvested, washed with PBS/BSA, and incubated on ice with anti-Ld (α2) antibody (28.14.8) followed by incubation with fluorescein isothiocyanate F(ab')2 goat anti-mouse IgG. The Cytomation MoFlo cell sorter (UIUC Biotechnology Center) analyzed cells at a rate of 30,000 cells/s, and the 1% of yeast cells exhibiting the highest fluorescence was collected. These cells were immediately cultured in SD-CAA for 3 h at 30 °C and induced for ~48 h at 20 °C. The second, third, and fourth sorts used anti-Ld (α3) antibody (30-5-7), but in these sorts the 0.25–0.5% of yeast cells that exhibited the highest fluorescence was collected. Following four rounds of sorting, cells were plated on SD-CAA agar and individual clones were analyzed.

Site-directed Mutagenesis—Single-site mutations were generated using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) with overlapping forward and reverse primers that contained the mutations. Reactions included Pfu polymerase (2.5 units/μl), reaction buffer, Ld-β2m pCT302 template (10 ng), dNTPs (25 μM), and primers (125 ng). PCR cycles were as follows: 1 cycle of 95 °C for 30 s; 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 16 min. The restriction enzyme DpnI (Invitrogen) was added to PCR reactions for 60 min at 37 °C to degrade methylated template Ld-β2m pCT302 plasmid. Plasmid DNA was precipitated with ethanol and transformed into electrocompetent DH10B E. coli cells.

Ld-m31 Protein Expression and Purification—For recombinant Ld-m31 expression, the platform MHC (Ld-m31) was shortened by seven additional amino acids, truncating it between the α2 and α3 domains. The resulting construct was refolded as efficiently as the original yeast mutant and was used for all further studies. Ld-m31 was cloned into pET28a as two different constructs, one containing a GGS spacer and a C-terminal biotin ligation domain (GLNDIFEAQKIEWHE) (41) and one without this domain into NcoI/XhoI sites. Expression of Ld-m31 was done in BL21 (DE3) Codon Plus E. coli (Stratagene). Cultures were grown in LB/kanamycin (30 μg/ml) to an A600 of 1.0 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3.5 h. Cells were passed through a microfluid-
izer, and inclusion bodies were solubilized in 8 M urea, 50 mM MES, pH 6.5 at room temperature overnight. Biotinylated L\textsuperscript{d}-m31 was expressed using an \textit{in vivo} biotinylation method in which L\textsuperscript{d}-m31-biotin pET28a was co-transformed into BL21(DE3) \textit{E. coli} (Stratagene) with a plasmid containing an arabinose-inducible gene for \textit{E. coli} BirA ligase (provided by John Cronan at UIUC) (42). Expression was carried out as described above, with the exception that 10 \mu g/ml chloramphenicol was added to all media for maintenance of the BirA plasmid, and BirA ligase expression was induced with 2 mM L-arabinose in the presence of 50 \mu M d-biotin when A\textsubscript{600} \sim 0.6.

For the refolding reaction, 8 mg of purified QL9 peptide was added to 400 ml of refolding buffer (100 mM Tris-HCl, 400 mM L-arginine, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM oxidized/5 mM reduced glutathione, pH 8.0). Over a 24-h period, 200 mg of urea-solubilized L\textsuperscript{d}-m31 inclusion bodies were added in three equal additions. The refold mixture was filtered, dialyzed against 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, concentrated in a stirred cell concentrator (Amicon), and subjected to size-exclusion chromatography (Superdex 75, Amersham Biosciences). Purity of select fractions was determined by SDS-PAGE, and fractions were pooled and concentrated in a YM10 Centriprep (Amicon). The yield of refolded protein from a 400-ml refold was 2–4 mg. Excess QL9 peptide was added to the purified protein to prevent unfolding of L\textsuperscript{d}-m31 due to dissociation of peptide.

**TCR-QL9-L\textsuperscript{d} Affinity Measurements**—To examine if L\textsuperscript{d} mutants displayed on the surface of yeast exhibited structures similar to normal L\textsuperscript{d} on T2-Ld cells, the affinity of QL9-L\textsuperscript{d} complexes for the 2C-m6 TCR was measured. Purified 2C-m6 TCR was used in an equilibrium binding flow cytometry assay. Yeast cells or human T2 cells transfected with Ld (44), loaded with exogenous QL9 peptide, were washed with PBS/0.5% BSA, and incubated with varying concentrations of 2C-m6 TCR at 4 °C for 60 min. Flow cytometry experiments for detection of bound 2C-m6 TCR were carried out as described above.

Binding of 2C scTCRs to immobilized biotinylated QL9-L\textsuperscript{d}-m31 was analyzed by surface plasmon resonance (SPR) on a BIAcore 3000 instrument. Streptavidin sensor chips (BIAcore) were conditioned with 1 M NaCl, 50 mM NaOH. Biotinylated QL9-L\textsuperscript{d}-m31 was immobilized to a level of 450–500 response units, and the remaining surface was blocked with 1 M d-biotin. 2C scTCRs were diluted to the desired concentrations (0.0008–11.4 \mu M, depending on the scTCR being tested) in HBS buffer containing 0.005% Tween-20 and 1 \mu M QL9 peptide and injected at 25 °C at a flow rate of 30 \mu l/min. Binding to a blank biotin-blocked control surface was subtracted from all measurements. Kinetic data were analyzed using simultaneous k\textsubscript{a}/k\textsubscript{d} determination with a 1:1 Langmuir binding model on BIAevaluation 3.2 software (BIAcore). Equilibrium affinities were determined by linear regression of response units at equilibrium versus response units/concentration (40).

**Structural Models**—Models were generated using Swiss-Pdb Viewer (GlaxoSmithKline) and are based on the p29-L\textsuperscript{d}/\beta2m structure (21). Swiss-Pdb Viewer was used to insert mutations and to energy minimize the model using the GROMAS96 algorithm with 100–200 steps of steepest decent and 100–200 steps of conjugate gradient.

**RESULTS**

Expression of Single-chain L\textsuperscript{d}-\beta2m on Yeast—To engineer more stable L\textsuperscript{d}-\beta2m complexes, a single-chain gene was fused
initial error-prone PCR derived library of mutants, generated using the Ld-β2mPro-126 fusion as a template, yielded mutants that all contained the leucine at position 126 (see below). Thus, the selection process was capable of identifying single-site mutations that confer increased yeast cell surface levels.

Selection of Ld-β2m Variants from Error-prone PCR Libraries—Previous studies have shown that it is possible to identify mutations that increase stability of a fusion protein by selection for increased yeast display (36, 37, 40, 44). As described in the methods, an error-prone PCR library of \(-10^6\) independent transformants was generated from the Ld-β2mPro-126 mutant. The yeast library was incubated with Ld-binding peptide QLSPFPFDL (QL9) and selected through four rounds of sorting, growth, and induction. Fluorescence-activated cell sorting was performed with the anti-Ld (α3) antibody in the first round and the anti-Ld (α2) antibody in subsequent rounds. Following the fourth round of sorting, yeast clones were screened for binding to the anti-Ld (α3) antibody. Multiple clones with the same sequence were identified.

Flow cytometry results for one of these clones, Ld-m8, incubated with and without QL9 peptide are shown in Fig. 2 (A and B). Addition of QL9 to the yeast cells enabled binding of the soluble 2C-m6 TCR. This suggests that cell surface Ld was capable of binding the QL9 peptide and presenting it in a conformation recognized by the 2C TCR. Sequencing revealed that Ld-m8 had three mutations: Pro-126 → Leu (CCG to CTG), Lys-196 → Asn (AAA to AAT), and a deletion in the c-myc tag at the C terminus that extended the open reading frame another 23 residues to a stop codon, resulting in a hydrophilic tail (CNSSRSDNNSVDVTKSTLKL). Expression of the Lys-196 → Asn mutation alone in Ld-β2mPro-126 did not yield increased display as measured by anti-Ld (α3) antibody binding, suggesting that the Lys-196 → Asn mutation does not contribute to the stability of this protein (data not shown). Thus, it is very likely that the Pro-126 → Leu reversion accounted for the higher surface levels of the Ld-m8 protein.

It has been shown previously that successive mutagenesis and screening for increased surface levels at higher temperatures could yield TCR variants with synergistic, stabilizing mutations (37, 40). To generate such variants in Ld, a new error-prone library was generated using the Ld-m8 gene as the template for error-prone PCR mutagenesis. A library of \(-10^6\) trans-
formants was induced in the presence of the QL9 peptide, incubated at 40 °C, and selected using two different sorting strategies. In one strategy the library was first sorted with anti-Ld (α3) antibody followed by three sorts with soluble 2C-m6 TCR. In the second strategy, the library was sorted three times with anti-Ld (α3) antibody and the fourth time with soluble 2C-m6 TCR. For both of the sorting strategies, 0.25–1% of the cells with the highest fluorescence were collected. Increased surface levels of Ld, as judged by staining with anti-Ld (α3) antibody and 2C-m6 TCR, were observed by the fourth sort using both strategies (data not shown).

**Characterization and Structural Analysis of Yeast-displayed Ld Mutants**—Clones from each sorting strategy were examined by flow cytometry for binding to anti-Ld (α2) and anti-Ld (α3) antibodies and the 2C-m6 TCR (Fig. 3 and data not shown). Most of the clones showed improved surface levels by anti-Ld (α2), staining compared with Ld-m8 (Fig. 3A and data not shown). Although the anti-Ld (α2) antibody bound all of the clones, many of the clones now lacked binding to the anti-Ld (α3) antibody (Fig. 3B and data not shown). Clones Ld-m31, Ld-m40, and Ld-m61 exhibited the highest levels of Ld as detected with the 2C-m6 TCR (Fig. 3C). Flow cytometry profiles of two clones (Ld-m31 and Ld-m37) that represent the two different classes of mutants (i.e. reactive or non-reactive with anti-Ld (α3) antibody) are shown in Fig. 4. Mutant Ld-m31 expressed surface levels that were ~10-fold higher than Ld-m8, as detected with both the anti-Ld (α2) antibody and the 2C-m6 TCR. Mutant Ld-m37 expressed surface levels that were 2–5-fold higher than Ld-m8, as detected with all three reagents.

Plasmids from several clones were rescued and sequenced. Identical sequences were found for two clones, Ld-m31 and Ld-m40, which were negative for anti-Ld (α3) antibody binding. Mutant Ld-m31 contained six mutations, Asn-30 → Asp, Ala-49 → Val, Ile-66 → Val, Trp-97 → Arg, and Lys-131 → Arg, and a deletion in codon 186. The deletion at codon 186 resulted in a frameshift generating a stop codon after three additional C-terminal residues. Thus, the lack of binding by the anti-Ld (α3) antibody is due to the complete absence of the α3 and β2m domains in this truncated protein. The sequence of the anti-Ld (α3) antibody-positive clone Ld-m37 was also determined. This mutant contained seven new mutations: two mutations in the α heavy chain (Met-5 → Leu and Trp-97 → Arg), and five mutations in β2m (Ile-7 → Thr, Glu-16 → Gly, Pro-20 → Ser, Ile-22 → Thr, and Lys-41 → Arg).

The positions of mutations for Ld-m31 and Ld-m37 in energy-minimized models are shown in Fig. 5. In the truncated α1/α2 mutant Ld-m31, two mutations (Ile-66 → Val and Trp-97 → Arg) are located near the peptide binding site, while the three other mutations are conservative changes residing on the surface of the molecule. Mutant Ld-m37 shares only the Trp-97 → Arg mutation with Ld-m31. This mutation has been shown to stabilize peptide binding and β2m association (20). Because Trp-97 → Arg is the only mutation shared between these two mutants, we examined the influence of the mutation when cloned into the Ld-β2m construct. As shown in Fig. 6, the Trp-97 → Arg mutation enhanced the surface levels of Ld, as detected with all three reagents. However, the surface levels of the Ld-m37 mutant were severalfold higher than the Ld-Trp-97 → Arg mutant, suggesting that the β2m mutations in Ld-m37 may contribute additional stability. Among the five mutations in β2m, none of them seem to be included in the interface with Ld.
To evaluate if the protein could form complexes with soluble TCRs, the purified QL9-L<sup>d</sup>-m31 protein was incubated with soluble scTCR 2C-m6 and the wild type 2C scTCR (T7). The mixtures were electrophoresed in gradient native polyacrylamide gels (Fig. 8B). A unique band, indicating complex formation, was observed in samples that contained the high affinity scTCR 2C-m6 and the QL9-L<sup>d</sup>-m31 protein, when compared with each component analyzed separately. Furthermore, this complex was only observed when QL9 and not a control peptide called MCMV, was used in the refolding of the L<sup>d</sup>-m31 protein (data not shown). The lower affinity scTCR 2C-T7 has been shown to bind to QL9-L<sup>d</sup>-m31 (see below) but does not show binding by native gel.

Surface plasmon resonance was used to measure scTCR-QL9-L<sup>d</sup>-m31 binding affinities. The L<sup>d</sup>-m31 gene was cloned with a C-terminal tag for enzymatic addition of biotin. L<sup>d</sup>-m31-biotin inclusion bodies were purified from E. coli cells that overexpressed the biotin ligase and refolded. QL9-L<sup>d</sup>-m31-biotin was immobilized on streptavidin-coated chips, and both the high affinity scTCR 2C-m6 and wild type scTCR 2C-T7 were analyzed (Fig. 8C). The

**DISCUSSION**

Various studies have sought to engineer peptide-class I/β2m complexes to facilitate expression on the mammalian cell surface or to improve yields of properly assembled soluble forms of the complexes. In many cases, efforts have focused on the generation of covalent linkages between these molecules (reviewed in Ref. 8). Early studies explored the linkage of β2m to the class I heavy chain (47) or of the peptide to class I heavy chain (48, 49). It has been argued that single-chain strategies with peptides may be of variable success, due to the need for anchoring peptide termini into class I pockets. However, Hansen and colleagues recently generated mammalian cell surface-expressed

---

**Engineering MHC Proteins**

---

**FIGURE 6. Contribution of the Trp-97 → Arg mutation to surface levels of L<sup>d</sup>-β2m.** Yeast cells were induced to express the indicated L<sup>d</sup>-β2m constructs, in the presence of exogenous QL9 peptide. Cells were stained with anti-L<sup>d</sup> antibodies (left and middle) or 2C-m6 TCR (right) as described in Fig. 1 (shaded histograms). Fluorescence of these yeast cell populations is indicated as mean fluorescence units (MFU). Unshaded histograms represent background staining from cells incubated with secondary antibodies and SA-PE only.

To evaluate if the protein could form complexes with soluble TCRs, the purified QL9-L<sup>d</sup>-m31 protein was incubated with soluble scTCR 2C-m6 and the wild type 2C scTCR (T7). The mixtures were electrophoresed in gradient native polyacrylamide gels (Fig. 8B). A unique band, indicating complex formation, was observed in samples that contained the high affinity scTCR 2C-m6 and the QL9-L<sup>d</sup>-m31 protein, when compared with each component analyzed separately. Furthermore, this complex was only observed when QL9 and not a control peptide called MCMV, was used in the refolding of the L<sup>d</sup>-m31 protein (data not shown). The lower affinity scTCR 2C-T7 has been shown to bind to QL9-L<sup>d</sup>-m31 (see below) but does not show binding by native gel.

Surface plasmon resonance was used to measure scTCR-QL9-L<sup>d</sup>-m31 binding affinities. The L<sup>d</sup>-m31 gene was cloned with a C-terminal tag for enzymatic addition of biotin. L<sup>d</sup>-m31-biotin inclusion bodies were purified from E. coli cells that overexpressed the biotin ligase and refolded. QL9-L<sup>d</sup>-m31-biotin was immobilized on streptavidin-coated chips, and both the high affinity scTCR 2C-m6 and wild type scTCR 2C-T7 were analyzed (Fig. 8C). The
Engineering MHC Proteins

![Graph](image)

**FIGURE 7.** Soluble 2C-m6 TCR binding titrations of Ld mutants expressed on yeast or wild-type Ld expressed on T2 cells. T2-Ld cells loaded with exogenous QL9 peptide or yeast cells induced in the presence of QL9 peptide were incubated with various concentrations of high affinity 2C-m6 TCR. Cells were washed and stained with streptavidin-PE conjugate. Cells were analyzed on a Coulter XL flow cytometer and the mean fluorescence units of bound TCR at each concentration (M) were determined from flow cytometry histograms. The maximum MFU for each titration was set as 100% binding, and the MFU value at other concentrations was calculated relative to this value.

Forms of peptide-linker-β2m-linker-heavy chain constructs (called a single-chain trimer) (9). The single-chain trimers exhibited improved stability and ability to stimulate T cells (9), and they could be expressed and refolded from E. coli in native form (50). In addition, a Tyr-84 → Ala mutation that would potentially allow the C-terminal extension of the peptide to exit the MHC groove more favorably was also shown to be properly assembled.

Although these engineering approaches have been guided to some extent by the structures of peptide-class I/β2m complexes, few studies have attempted to use a process of directed evolution to identify improvements in soluble peptide-class I/β2m complexes. We, and others, have used yeast display to select for mutated class II products that are expressed on the surface of yeast (14, 15). Based on our studies with single-chain TCRs (36, 37, 40, 44, 51), we have predicted that such displayed proteins are likely to exhibit higher levels of expression and folding as soluble constructs. Our previous study also showed that a single-chain Kβ/β2m construct could be displayed on yeast (13), but efforts to engineer the construct by directed evolution were not attempted. In this report, we used a process of random mutagenesis and selection using yeast display to identify mutants of the class I molecule Ld that were expressed at high levels on the surface of yeast. One of these mutants contained only the N-terminal α1 and α2 domains, and in the presence of peptide the protein refolded from E. coli with high yields. Such α1/α2 platforms may be of general use for studying class I MHC products. In previous studies, the structures of peptide-class II complexes were used to design a single-chain class II molecule that consisted of only the β1/α1 peptide-binding domains (52, 53), suggesting that stabilized forms of these class II platforms might also be amenable to yeast display and directed evolution.

Although the crystal structures of two Ld complexes have been reported (21, 22), crystallization of additional complexes has been problematic. For example, our efforts to crystallize QL9-Ld, the ligand for the 2C TCR used in the present studies, have been unsuccessful. These models may be associated with several features of Ld that lead to reduced stability compared with other mouse class I proteins: 1) unusual orientation
of the α heavy chain relative to β2m, 2) a reduced number of contacts between α1/α2 domains and β2m, and 3) lower affinity of the β2m for the α chain (21). Interestingly, one substitution, Trp-97 → Arg, in the structurally related Ld allele has been shown to confer additional stability on Ld (20). Remarkably, this mutation was also identified in the present study using a directed evolution approach. This observation further supports the notion that the efficiency of yeast display (i.e. cell surface levels) can be used to select for stabilizing mutations in the fused protein.

While Trp-97 → Arg was the only mutation present in both the α1/α2 mutant Ld-m31 and in the full-length mutant Ld-m37, several additional mutations were identified in each (Fig. 5). It is unclear if these mutations contribute to the enhanced surface display of the Ld mutants. In the Ld-m31 mutant, the four additional mutations are conservative substitutions. In the Ld-m37 mutant, six of the seven additional mutations are located in α3 (five mutations) and β2m (one mutation) and thus could be involved in stabilizing these two domains. While subsequent studies in the present report focused on the α1/α2 mutant, the full-length Ld-m31 mutant may be of some interest in the future.

We also show here that E. coli expression of the α1/α2 Ld-m31 platform yielded a stable protein that could be refolded with Ld-binding peptides. Our previous studies using single-chain TCRs or individual Vβ domains have shown that mutants with enhanced yeast surface display also showed improvements in secretion levels and thermostability (37, 40, 44, 51). Thus, we anticipated that the yeast displayed Ld mutants might also show similar improvements in E. coli expression and folding. In fact, the yields and solubility of purified peptide/Ld-m31 complexes have been excellent, allowing their use in both binding studies and crystallization trials.

Although the selection process using the high affinity TCR m6 would assure that the QL9-Ld mutant complexes were recognized by the specific TCR, it was formally possible that the affinity of the TCR-QL9-Ld interaction could be influenced by the mutations. However, two independent approaches, including SPR, showed that TCRs bound to QL9-Ld-m31 with the same affinities as reported for binding to the full-length QL9-Ld complexes. The single-chain version of both the wild-type 2C TCR and the m6 TCR recognized soluble QL9-Ld-m31 with affinities corresponding to previous measurements using full-length Ld (29, 33, 35). These results suggest that neither the Trp-97 → Arg mutation nor other mutations influence the conformation of the peptide-MHC surface that is recognized by the TCR. Furthermore, it shows that at least in the case of this TCR system, the α3/β2m domains do not appreciably influence the binding of TCRs. Interestingly, it was shown over a decade ago that a human class I MHC (HLA-Aw68), which underwent proteolytic cleavage of the α3 domain, exhibited a structure similar to the full-length peptide-HLA-Aw68-β2m complex. However, β2m was retained in the complex, and based on many studies, including early efforts to generate multimers of class I, it has generally been assumed that β2m is essential for stabilization of the entire peptide-class I-β2m complex (54, 55).

Finally, it is reasonable to predict that it will be possible to engineer other class I molecules as α1/α2 platforms, either through a process of directed evolution as described here or by rational design of the molecules (e.g. with substitutions of key hydrophobic residues that would be exposed upon removal of the α3 and β2m domains). The engineered proteins could be very useful in structural studies (e.g. NMR), in the development of tetramer reagents, or as modulating agents for T-cell responses. The elimination of the α3 domain, and thus the CD8-binding epitopes, on a class I complex could reduce problems with background binding of the class I tetramers to CD8 on T cells (56, 57). In addition, class I molecules that lack α3/β2m might be of some use in regulating the activity of T cells by either stimulating the highest avidity, CD8-independent T cells or by eliciting inhibitory signals through the TCR complex in the absence of CD8/lck signaling (58). In this regard, a recent study compared QL9-Ld monomers and multimers in the 2C system to examine activation requirements (59). It will be of interest to examine how the QL9-Ld-m31 ligand, which lacks the CD8-binding epitope, will function in eliciting T-cell activity.

Acknowledgments—We thank the University of Illinois Flow Cytometry Facility and the University of Illinois Immunological Resource Center for assistance and Phil Holler for providing some of the soluble m6 TCR preparations and for advice.

REFERENCES

1. Kulski, J. K., Shina, T., Anzai, T., Kohara, S., and Inoko, H. (2002) Immunol. Rev. 190, 95–122
2. Reche, P. A., and Reinherz, E. L. (2003) J. Mol. Biol. 331, 623–641
3. Vukmanovic, S., Neubert, T. A., and Santonti, F. R. (2003) Trends Mol. Med. 9, 139–146
4. Shina, T., Inoko, H., and Kulski, J. K. (2004) Tissue Antigens 64, 631–649
5. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) Nature 329, 506–512
6. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) Nature 329, 512–518
7. Mason, P. M., and Parham, P. (1998) Tissue Antigens 51, 417–466
8. Hansen, T. H., and Lybarger, L. (2006) Cancer Immunol. Immunother. 55, 235–236
9. Yu, Y. Y., Nethusil, N., Lybarger, L., Connolly, J. M., and Hansen, T. H. (2002) J. Immunol. 168, 3145–3149
10. Le Doussal, J., Piqueras, B., Dogan, I., Debrec, P., and Gorochov, G. (2000) J. Immunol. Methods 241, 147–158
11. Vest Hansen, N., Ostergaard Pedersen, L., Styhrn, A., and Buus, S. (2001) Eur. J. Immunol. 31, 32–38
12. Kurokawa, M. S., Ohoka, S., Matsui, T., Sekine, T., Yamamoto, K., Nishioha, K., and Kato, T. (2002) Immunol. Lett. 80, 163–168
13. Brophy, S. E., Holler, P. D., and Kranz, D. M. (2003) J. Immunol. Methods 272, 235–246
14. Starwalt, S. E., Masteller, E. L., Bluestone, J. A., and Kranz, D. M. (2003) Protein Eng. 16, 147–156
15. Esteban, O., and Zhao, H. (2004) J. Mol. Biol. 340, 81–95
16. Boder, E. T., Bill, J. R., Niedls, A. W., Marrack, P. C., and Kappler, J. W. (2005) Biotechnol. Bioeng. 92, 485–491
17. Potter, T. A., Hansen, T. H., Habbersett, R., Ozato, K., and Ahmed, A. (1981) J. Immunol. 127, 580–584
18. Dower, S. K., and Segal, D. M. (1985) J. Immunol. 134, 431–435
19. Beck, J. C., Hansen, T. H., Cullen, S. E., and Lee, D. R. (1986) J. Immunol. 137, 916–923
20. Smith, R. A., Myers, N. B., Robinson, M., Hansen, T. H., and Lee, D. R. (2002) J. Immunol. 169, 3105–3111
21. Balendir, G. K., Solheim, J. C., Young, A. C., Hansen, T. H., Nathenson, S. G., and Sacchettini, J. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 25743
Engineering MHC Proteins

6880–6885
22. Speir, J. A., Garcia, K. C., Brunmark, A., Degano, M., Peterson, P. A., Teyton, L., and Wilson, I. A. (1998) Immunity 8, 553–562
23. Corr, M., Boyd, L. F., Frankel, S. R., Kozlowski, S., Padlan, E. A., and Margulies, D. H. (1992) J. Exp. Med. 176, 1681–1692
24. Lurquin, C., Van Pel, A., Mariame, B., De Plaen, E., Szikora, J. P., Janssens, C., Reddehase, M. J., Lejeune, J., and Boon, T. (1989) Cell 58, 293–303
25. Udaka, K., Tsomides, T. J., and Eisen, H. N. (1992) Cell 69, 989–998
26. Smith, K. J., Reid, S. W., Harlos, K., McMichael, A. J., Stuart, D. I., Bell, J. I., and Jones, E. Y. (1996) Immunity 4, 215–228
27. Hansen, T., Balendiran, G., Solheim, J., Ostrov, D., and Nathenson, S. (2000) Immuno Mol. Today 21, 83–88
28. Sykulev, Y., Brunmark, A., Tsomides, T. J., Kageyama, S., Jackson, M., Sykulev, Y., and Kranz, D. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11487–11491
29. Holler, P. D., Holman, P. O., Shusta, E. V., O’Herrin, S., Wittrup, K. D., and Kranz, D. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5387–5392
30. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 61–68
31. Geitz, R. D., Schiestl, R. H., Willems, A., and Woods, R. A. (1995) Yeast 11, 355–360
32. Boder, E. T., and Wittrup, K. D. (1997) Nat. Biotech. 15, 553–557
33. 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. U. S. A. 94, 13838–13843
34. Holler, P. D., Chlewicki, L. K., and Kranz, D. M. (2003) Nat. Immunol. 4, 55–62
35. Holler, P. D., and Kranz, D. M. (2003) Immunol. 18, 255–264
36. Kieke, M. C., Shusta, E. V., Boder, E. T., Teyton, L., Wittrup, K. D., and Kranz, D. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5651–5656
37. Shusta, E. V., Holler, P. D., Kieke, M. C., Kranz, D. M., and Wittrup, K. D. (2000) Nat. Biotechnol. 18, 754–759
38. Garcia, K. C., Radu, C. G., Ho, J., Ober, R. J., and Ward, E. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6818–6823
39. Maynard, J., Adams, E. J., Krosgaard, M., Petersson, K., Liu, C. W., and Garcia, K. C. (2005) J. Immunol. Methods 306, 51–67
40. Weber, K. S., Donermeyer, D. L., Allen, P. M., and Kranz, D. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 19033–19038
41. Cull, M. G., and Schatz, P. J. (2000) Methods Enzymol. 326, 430–440
42. Cronan, J. E., Jr., and Reed, K. E. (2000) Methods Enzymol. 326, 440–458
43. Ozato, K., Hansen, T. H., and Sachs, D. H. (1980) J. Immunol. 125, 2473–2477
44. Shusta, E. V., Kieke, M. C., Parke, E., Kranz, D. M., and Wittrup, K. D. (1999) J. Mol. Biol. 292, 949–956
45. Alexander, J., Payne, J. A., Murray, R., Frelinger, J. A., and Cresswell, P. (1989) Immunogenetics 29, 380–399
46. Maynard, J., Petersson, K., Wilson, D. H., Adams, E. J., Blondelle, S. E., Boulanger, M. J., Wilson, D. B., and Garcia, K. C. (2005) Immunol. Today 22, 81–92
47. Mage, M. G., Lee, L., Ribaudo, R. K., Corr, M., Kozlowski, S., McHugh, L., and Margulies, D. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10658–10662
48. Mottez, E., Jaulin, C., Godeau, F., Choppin, J., Levy, J. P., and Kourilsky, P. (1991) Eur. J. Immunol. 21, 467–471
49. Mottez, E., Langlade-Demoyen, P., Gourmier, H., Martinon, F., Maryanski, J., Kourilsky, P., and Abastado, J. P. (1995) J. Exp. Med. 181, 493–502
50. Lybarger, L., Yu, Y. Y., Miley, M. J., Fremont, D. H., Myers, N., Primeau, T., Truscott, S. M., Connolly, J. M., and Hansen, T. H. (2003) J. Biol. Chem. 278, 27105–27111
51. Buonpane, R. A., Moza, B., Sundberg, E. J., and Kranz, D. M. (2005) J. Mol. Biol. 353, 308–321
52. Burrows, G. G., Chang, J. W., Bachinger, H. P., Bourdette, D. N., Offner, H., and Vandenbark, A. A. (1999) Protein Eng. 12, 771–778
53. Chang, J. W., Mechling, D. E., Bachinger, H. P., and Burrows, G. G. (2001) J. Biol. Chem. 276, 24170–24176
54. Dal Porto, J., Johansen, T. E., Catipovic, B., Parфи, D. J., Tuveson, D., Gether, U., Kozlowski, S., Fearon, D. T., and Schneck, J. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6671–6675
55. Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. J., McMichael, A. J., and Davis, M. M. (1996) Science 274, 94–96
56. Holman, P. O., Walsh, E. R., and Jameson, S. C. (2005) J. Immunol. 174, 3986–3991
57. Wooldridge, L., van den Berg, H. A., Glick, M., Gostick, E., Laugel, B., Hutchinson, S. L., Milicic, A., Brenchley, J. M., Doue, D. C., Price, D. A., and Sewell, A. K. (2005) J. Biol. Chem. 280, 27491–27501
58. Maile, R., Siler, C. A., Kerry, S. E., Midkiff, K. E., Collins, E. J., and Sewell, A. K. (2005) J. Immunol. 174, 619–627
59. Stone, J. D., and Stern, L. J. (2006) J. Immunol. 176, 1498–1505