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The common equine class I molecule Eqca-1*00101 (ELA-A3.1) is characterized by narrow peptide binding and T cell epitope repertoires

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Abstract

Here we describe a detailed quantitative peptide-binding motif for the common equine leukocyte antigen (ELA) class I allele Eqca-1*00101, present in roughly 25 % of Thoroughbred horses. We determined a preliminary binding motif by sequencing endogenously bound ligands. Subsequently, a positional scanning combinatorial library (PSCL) was used to further characterize binding specificity and derive a quantitative motif involving aspartic acid in position 2 and hydrophobic residues at the C-terminus. Using this motif, we selected and tested 9- and 10-mer peptides derived from the equine herpesvirus type 1 (EHV-1) proteome for their capacity to bind Eqca-1*00101. PSCL predictions were very efficient, with an receiver operating characteristic (ROC) curve performance of 0.877, and 87 peptides derived from 40 different EHV-1 proteins were identified with affinities of 500 nM or higher. Quantitative analysis revealed that Eqca-1*00101 has a narrow peptide-binding repertoire, in comparison to those of most human, non-human primate, and mouse class I alleles. Peripheral blood mononuclear cells from six EHV-1-infected, or vaccinated but uninfected, Eqca-1*00101-positive horses were used in IFN- γ enzyme-linked immunospot (ELISPOT) assays. When we screened the 87 Eqca-1*00101-binding peptides for T cell reactivity, only one Eqca-1*00101 epitope, derived from the intermediate-early protein ICP4, was identified. Thus, despite its common occurrence in several horse breeds,

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Eqca-1*00101 is associated with a narrow binding repertoire and a similarly narrow T cell response to an important equine viral pathogen. Intriguingly, these features are shared with other human and macaque major histocompatibility complex (MHC) molecules with a similar specificity for D in position 2 or 3 in their main anchor motif.

Keywords

MHC class I; Peptide binding; MHC binding motif; Tcell epitope; *Equus caballus* (Eqca)

Introduction

As in other species, intracellular pathogens are of major health importance in domestic and wild horse (*Equus caballus*) populations. Viruses including equine herpesvirus type 1 and 4 (EHV-1 and EHV-4), equine arteritis virus (EAV), and equine influenza virus (EIV) have plagued horses worldwide (Ostlund 1993). Particularly in the case of EHV-1 and EHV-4, vaccines are only partially effective (Patel and Heldens 2005; Rosas et al. 2006). EHV-1, an α -herpesvirus, which primarily infects horses and wild equids, is the causative agent of rhinopneumonitis, abortions, and myeloencephalopathy (Ma et al. 2013; Van de Walle et al. 2009).

Protective immunity is observed only for few months after primary EHV-1 infection (Doll et al. 1955). Inactivated vaccines stimulate virus-neutralizing antibodies and reduce the amount and duration of virus shedding from the nasopharynx. However, although circulating and mucosal antibodies play a role in protection, they do not protect against infection (Hannant et al. 1993; Heldens et al. 2001), as spread via cell-to-cell contacts renders the virus inaccessible for antibodies. These findings suggest that antiviral immunity might mainly be conferred by virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) (Allen et al. 1995). Indeed, memory CTL frequencies clearly correlate with protection against EHV-1 infection (Kydd et al. 2003; O'Neill et al. 1999).

T cell immunity is critical for protection against intracellular pathogens, but robust assays for measuring antigen-specific T cell responses for the horse or other livestock species are either not available or poorly defined (Gaddum et al. 1996; Guzman et al. 2010). CTLs use the polymorphic T cell receptor (TCR) to recognize viral peptides produced in infected cells and presented in a complex with major histocompatibility complex (MHC) class I molecules, which play a critical role in adaptive immunity. The MHC-I-peptide assembly presents non-self structures to CD8⁺ CTLs and also provides an inhibitory signal to NK cell-mediated lysis (Germain and Margulies 1993; Lanier 1997). MHC-I heterodimers are comprised of a membrane-bound heavy chain that forms the peptide binding groove and the β -2-microglobulin light chain. In the case of intracellular infection, pathogen-derived peptides are transported into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP) where they specifically associate with the binding groove of an MHC-I molecule and are then transported to the cell surface and presented for immunological recognition (reviewed in Neefjes et al. 2011).

MHC-I molecules display a high degree of diversity among a population, defined by polymorphic residues in the peptide binding groove (Spurgin and Richardson 2010). The equine MHC (equine leukocyte antigen. ELA) is organized in haplotypes on chromosome 20 comprising up to 15 classical and nonclassical loci as well as pseudogenes (Gustafson et al. 2003; Tallmadge et al. 2010; Vaiman et al. 1986). So far, 19 distinct ELA-A haplotypes have been identified and differentiated serologically (Bailey et al. 2000; Lazary et al. 1988), with similar levels of polymorphism estimated using intra-MHC microsatellites (Tseng et al. 2010) and sequencing (Tallmadge et al. 2010). The total number of MHC class I loci expressed on horse cells may vary, but in horses homozygous for the ELA-A3 haplotype at least seven class I molecules can be detected (Azab et al. 2014; Tallmadge et al. 2005).

In general, individual MHC-I molecules each bind somewhat unique peptide repertoires, characterized by specific features, or motifs, conferring the capacity to bind the corresponding MHC. A minimalistic approach is to summarize those binding specificities in more simple terms of primary and secondary amino acid anchor residues in the peptide, which interact with specific pockets in the MHC binding groove (Rotzschke and Falk 1991; Ruppert et al. 1993; Saper et al. 1991).

Despite the demonstrable activity and high frequency of EHV-1-specific CTLs associated with reduced clinical signs, peptide ligands for equine MHC class I molecules are grossly underexplored (Allen 2008; Allen et al. 2004). To date, only five peptides derived from equine infectious anemia virus and restricted to loci of the ELA-A1, ELA-A5, and ELA-A9 haplotypes have been described (McGuire et al. 2003; Mealey et al. 2006; Zhang et al. 1998). In 2003, it was demonstrated that peptides within the EHV-1 immediate early (equine infected cell protein 4, ICP4) protein encoded by gene 64 are presented by MHC class I molecules of the ELA-A3 allele Eqca-1*00101 (Kydd et al. 2006; Soboll et al. 2003; Tallmadge et al. 2005). The authors concentrated on this haplotype because it is found commonly in Thoroughbreds and in horses of other important breeds (Antczak et al. 1986). Furthermore, horses homozygous for this MHC haplotype, including the DNA donor for the horse genome sequence, are maintained in an experimental herd at Cornell University and available for detailed study (Gustafson et al. 2003; Tallmadge et al. 2005).

In the present study, we have utilized various established approaches to determine a detailed, quantitative, peptide-binding motif for Eqca-1*00101. The results unexpectedly revealed a narrow peptide-binding repertoire, and most of the peptides that did bind were not recognized by T cell responses.

Materials and methods

Isolation of peripheral blood mononuclear cells from horses

Horses sampled for this study were maintained in AAALAC-approved facilities at the Equine Genetics Center at Cornell University and the Veterinary Medical Center at Michigan State University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committees. The horses at Cornell had been vaccinated at regular intervals with killed vaccines against EHV-1 and equine influenza virus. The horse at Michigan State University had been infected experimentally with wild-

type EHV-1 on at least three occasions. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized samples of venous jugular blood using methods previously described (Antczak et al. 1982). Briefly, cells were pelleted by centrifugation at $350\times g$ for 10 min at RT. Supernatant was removed and cells were resuspended in 35 mL of RPMI 1640 media supplemented with 10 % fetal calf serum (FCS). Fifteen milliliters of Ficoll (1.077 g/mL) was overlaid with the suspension and tubes were centrifuged at $350\times g$ for 15 min at RT. The interface was collected and washed three times with RPMI media. Cells were frozen in freezing media containing FCS with 10 % dimethyl sulfoxide (DMSO) in liquid nitrogen at a density of 30×10^6 /mL and thawed as required. Viability in all samples was $>90\%$ as determined by Trypan blue exclusion.

Affinity purification of Eqca-1*00101 from cell cultures

The P815 clone expressing the Eqca-1*00101 gene (Azab et al. 2014) was used to purify MHC class I molecules by immunoaffinity columns as described previously (Sidney et al. 2013). Briefly, cells were grown in DMEM HiGlucose supplemented with 10% FCS, 2 mM L-glutamine, 0.5 μ g/mL vitamin C, 0.4 μ g/mL insulin, 10 μ M β -mercaptoethanol, and 1 % penicillin/streptomycin. Eqca-1*00101 expression was confirmed by FACS analysis according to standard protocols. One hundred fifty million cells/milliliter in 10 L was harvested and lysed in 120 mL lysis buffer containing 20 mM Tris, 150 mM NaCl, 1 % NP40, and 200 mM PMSF. The lysate was precleared by repeated runs over two columns containing only sepharose beads and one column containing Protein A-coupled sepharose beads. Subsequently, the lysate was applied twice to a Protein A-sepharose column coated with an equine MHC-I-specific antibody (CZ3) (Rappocciolo et al. 2003) and bound MHC-I finally eluted in 15 mL elution buffer with 150 mM NaCl, 50 mM diethylamine, and 1 % octylglucoside. The volume was reduced and possibly co-purified murine β 2-microglobulin was size-excluded by several centrifugation steps in CentriprepUltracel YM-30 tubes at 1,200 rpm to 500 μ L. Protein purity, concentration, and the effectiveness of depletion steps were monitored by SDS-PAGE and BCA assay.

Acid elution of endogenous MHC class I ligands

P815 cells expressing Eqca-1*00101 were grown and harvested, and MHC class I molecules purified, as described above. Briefly, 3.5×10^9 cells were pelleted and lysed in 35 mL lysis buffer containing 10 mg/mL CHAPS, 5 μ g/mL aprotinin, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin A, 1 mM PMSF, phosphatase inhibitor cocktail I and II, and 10 nM calyculin A. The lysate was ultracentrifuged for 1 h at $100,000\times g$ (Beckman L8-80M with SW28 rotor; 27,500 rpm= $\sim 100,000\times g$) and 4 $^{\circ}$ C to remove cellular debris and subsequently filtered through 0.2 μ m pore size filters to remove remaining lipids. Filtered lysate was passed over an anti-murine MHC class II antibody (MKD6)-coupled Protein A column for pre-clearing (i.e., negative control for non-specific binding) and the resulting flow-through was passed over an anti-horse MHC class I-specific CZ3 monoclonal antibody-coupled column. The Protein A beads were then washed with lysis buffer (without inhibitors), low salt buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt buffer (20 mM Tris-HCl, pH 8.0, 1.0 M NaCl), and no salt buffer (20 mM Tris-HCl, pH 8.0). Endogenous peptides were eluted from the columns as described previously (Slingluff et al. 1993). Briefly, beads were rinsed with 12 mL 0.2 N acetic acid to elute the antibody-MHC-peptide complex. To allow for

dissociation, the eluate was collected and 1.06 mL glacial acetic acid added immediately after to reach a final concentration of 10 % acetic acid. Peptides were separated by centrifugation through an Ultrafree-CL filter and volume was reduced to 250 μ L by vacuum centrifugation.

Peptide sequence analysis by MS/MS

Peptides were analyzed by nanoflow high-performance liquid chromatography (HPLC)/microelectrospray ionization, coupled directly to a Thermo Fisher Scientific FT-ICR mass spectrometer that is equipped with a home-built, front-end electron transfer dissociation (FETD) source (Earley et al. 2013).

Data were acquired as previously described (Udeshi et al. 2008). In brief, a precolumn loaded with 1×10^8 cell equivalents of MHC eluted peptides was connected with polytetrafluoroethylene tubing (0.06 in. o.d. and 0.012 in. i.d.; Zeus Industrial Products) to the end of an analytical HPLC column (360 mm o.d. $\times$ 50 mm i.d.) containing 6–7 cm of C18 reverse-phase packing material (5 μ m particles; YMC). Peptides were eluted through a laser-pulled electrospray tip directly into the mass spectrometer with an Agilent 1100 series binary LC pump at a flow rate of $\sim$ 60 nL/min. Elution gradients utilized were as follows: solvent A was 0.1 M acetic acid in H₂O and solvent B was 70 % acetonitrile. For the analyses, collisionally activated dissociation (CAD) and ETD fragmentation were performed sequentially on the same parent ions. The FETD reagent was azulene and the ion–ion reaction time was set to 45 ms. The instrument was operated in a data-dependent mode, where a full-scan mass spectrum was acquired with the high-resolution mass analyzer, which was then followed by sequential acquisition of CAD and ETD tandem mass spectrometry (MS/MS) spectra in the linear trap on the top 6, most abundant, non-excluded ions observed in the full-scan spectrum.

Data from MS/MS experiments were searched against the Swiss-Prot (Bairoch and Apweiler 2000) mouse database using the Open Mass Spectrometry Search Algorithm software (Geer et al. 2004) to generate a list of candidate peptide sequences. Instrument parameters included a precursor mass tolerance of $\pm$ 0.01 Da and a monoisotopic fragment ion mass tolerance of $\pm$ 0.35 Da. Database search parameters allowed variable modifications for phosphorylation on serine, threonine, and tyrosine residues and oxidation of methionine. Database assignments were confirmed by manual interpretation of the corresponding MS/MS spectra.

MHC class I peptide-binding assays

Performance of quantitative in vitro competitive binding assays utilizing purified MHC-I and an iodine¹²⁵-labeled standard probe peptide was performed using a monoclonal antibody capture assay platform essentially as described previously (Sidney et al. 2013). Briefly, 0.1–1 nM of radiolabeled peptide (an F6>Y analog of EHV-1 1059–1067, sequence RDGARYGEL) was co-incubated at room temperature with 1 μ M to 1 nM of purified MHC-I in the presence of a cocktail of protease inhibitors and 1 μ M human β 2-microglobulin (Scripps Laboratories) at pH 7 in PBS with 0.05 % NP40. Following a 2-day incubation, MHC-I bound radioactivity was determined by capturing MHC-I/peptide complexes on CZ3 (anti-ELA class I)-coated Lumitrac 600 plates (Greiner Bio-one,

Frickenhäusen, Germany) and measuring bound radioactivity using the TopCount (Packard Instrument Co., Meriden, CT) microscintillation counter. The concentration of peptide yielding 50 % inhibition of the binding of the radiolabeled peptide was calculated. Under the conditions utilized, where $[label] < [MHC]$ and $IC_{50} \ll [MHC]$, the measured IC_{50} values were reasonable approximations of the true K_D values (Cheng and Prusoff 1973; Gulukota et al. 1997). Each competitor peptide was tested at six different concentrations covering a 100,000-fold dose range and in three or more independent experiments. As a positive control, the unlabeled version of the radiolabeled probe was also tested in each experiment. Peptides utilized for screening studies were synthesized as crude material on a 1-mg scale by A&A (aka Synthetic Biomolecules; San Diego). For quality control, randomly selected peptides from each synthesis run were evaluated by mass spectrometry; >75 % purity is estimated in >90 % these standard crude syntheses. Peptides utilized as radiolabeled ligands were synthesized as purified (>95 %) material.

Positional scanning combinatorial libraries and bioinformatic analyses

Positional scanning combinatorial libraries (PSCL) of 9-mers were synthesized as described previously (Pinilla et al. 1992). Each library contained randomized 9-mer peptides with one fixed residue at a single position. With each of the 20 naturally occurring residues represented at each position along a 9-mer backbone, the entire library consisted of 180 peptide mixtures. A library completely randomized at each position was also tested.

PSCLs were tested in a competition assays as above, and data analyzed as described previously (Sidney et al. 2008a). Briefly, IC_{50} nM values for each mixture were standardized as a ratio to the geometric mean IC_{50} nM value of the entire set of 180 mixtures and then normalized at each position so that the value associated with optimal binding at each position corresponded to 1. For each position, an average (geometric) relative binding affinity (ARB) was calculated, and then the ratio of the ARB for the entire library to the ARB for each position was derived. We have denominated this ratio, which describes the factor by which the normalized geometric mean binding affinity associated with all 20 residues at a specified position differs from that of the average affinity of the entire library, as the specificity factor (SF). As calculated, positions with the highest specificity will have the highest SF value. Primary anchor positions were then defined as those with an SF ≥ 2.4 . This criterion identifies positions where the majority of residues are associated with significant decreases in binding capacity. Secondary anchors were defined as those positions where the standard deviation (SD) of residue specific values was >2 .

Receiver operating characteristic (ROC) curves were used to measure the quality of a prediction, which classifies objects into one of two categories (Swets 1988). Given a cutoff for the predicted value, predictions for peptides were separated into positive and negative subsets. Depending on whether a peptide was found to be a binder, a positive prediction can be either true or false. Normalizing the number of true and false positive predictions by the maximum number possible gives rates of true positive and false positive predictions. Plotting the rates of true positive predictions as a function of the rate of false positive predictions gives an ROC curve. Calculating the area under the ROC curve (AUC value) provides a highly useful measure of prediction quality, which is 0.5 for random predictions

and 1.0 for perfect predictions. The AUC value is equivalent to the probability that the predicted score for a randomly chosen antigenic peptide is lower (=better) than that of a randomly chosen peptide that is not antigenic. PSCL AUC metrics were compared with the performance of other previously evaluated MHC class I binding predictions (Peters et al. 2006; Trolle et al. 2015; Wang et al. 2008).

EHV-1 peptides were selected from a comprehensive set of 76 non-redundant EHV-1 strain Ab4 open reading frames (ORFs) retrieved from NCBI. Influenza Avirus peptides were selected from the complete sequence of equine influenza Florida clade 2 isolate H3N8/Richmond/1/2007.

Binding repertoire assessment

The peptide binding repertoires of various human (HLA), macaque (Mamu), and mouse class I alleles were determined by running the respective SMM algorithms against the entire EHV-1 sequence to determine the number of 9-mer peptides predicted to bind with an IC₅₀ of 500 nM or better, as previously described (Paul et al. 2013). Predictions were performed using the analysis tools available on the IEDB website.

Ex vivo primary ELISPOT assay

Enzyme-linked immunospot (ELISPOT) assays were carried out essentially as described elsewhere for equine, mouse, and human systems (Hagen et al. 2012; Weiskopf et al. 2011), and were standardized and did not change between experiments (EHV-1 vs EIV). Briefly, Immobilon-P membrane 96-well plates (Millipore #MSIPS4510) were coated with 5 µg/mL of anti-bovine IFN-γ antibody (Mabtech #3115-3-1000) that cross-reacts with equine IFN-γ. PBMCs of ELA-A3 donor horses were thawed from liquid nitrogen stocks, and 2×10⁵ cells/well were incubated in RPMI media with 10 % FCS in the presence of 5 µg/mL of pools of peptides (DMSO, in comparable concentrations) or 5 µg/mL concanavalin A (positive control). After 20 h, the secretion of IFN-γ was detected with a biotin-conjugated secondary anti-IFN-γ antibody (Mabtech #3115-6-500) and a subsequent enzymatic/color reaction. Responses against the negative control (DMSO) were subtracted from experimental values and statistical significance was assessed using a one-tailed Student's t test. All samples were done in triplicates. A sample was considered positive if net spot-forming cells (SFCs) per one million cells exceeded 20, the stimulation index (SI) was greater than 2.0, and probability (*p* 0.05) was reached. All experiments included positive controls stimulated with concanavalin A. No adverse effects have been observed. ELISPOT screening of EHV-1 peptides against ELA-A2 typed PBMCs yielded high, reproducible numbers of spots for some peptides indicating the functionality of the assay (not shown).

Results

Elution of natural Eqca-1*00101 ligands

To derive a preliminary motif characterizing the peptide binding specificity of Eqca-1*00101, as well as to identify candidate ligands for subsequent assay development, we eluted and sequenced by mass spectroscopy a total of 24 peptides eluted from Eqca-1*00101 molecules isolated from stably transfected P815 cells (Azab et al. 2014)

(Table 1). The majority (17/24; 71 %) were 9 residues in length, conforming to the canonical ligand size for most human, non-human primate, and mouse MHC-I alleles characterized to date. Peptides of 10 ($n=4$; 16.7%) and 11 ($n=3$; 12.5%) residues were also found, albeit with lower frequencies. Peptides shorter than 9 residues were not observed.

The majority (20/24; 83.3 %) of these natural ligand peptides had aspartic acid (D) in position 2. The only other residues found in position 2 were glutamine (Q) and glutamic acid (E), which share conserved or semi-conserved chemical specificity with D. These data suggest that position 2 serves as a main anchor conferring Eqca-1*00101 binding capacity.

A similarly narrow specificity, suggesting a main anchor role, was found at the C-terminus, where most peptides (19/24; 79.2 %) had isoleucine (I). The other residues found at the C-terminus were also hydrophobic and/or aliphatic in nature (L, F, and V), although one peptide with asparagine (N) at that position was found.

Other notable, but less striking, patterns were noted in position 1 and at the C-1 residue. In position 1, 13/24 (54.2 %) peptides had a positively charged residue, arginine (R) or lysine (K). At the C-1 position, 18/24 (75 %) had a polar (N, Q, S) or charged (R, H, K, D, E) residue. Taken together, these data suggest a putative binding motif for Eqca-1*00101 associated with a predominant size of 9 residues, and recognition of the acidic residue D in position 2, and the aliphatic-hydrophobic residue I at the C-terminus. While potential contamination with co-expressed H-2 class I MHC was controlled by the use of specific monoclonal antibodies for purification, it is also notable that the motif defined here is not congruent with the well-characterized H-2d class I motifs.

The low number of eluted endogenous binders is not necessarily indicative for the size of the peptide repertoire that can bind to Eqca-1*00101, as only limited sequencing was performed for the purpose of defining a putative motif and identifying candidate ligands for assay development. The size of the MHC binding repertoire will be evaluated in in vitro binding studies with EHV-1- and EIV-derived peptides (see below). However, the distribution of Eqca-1*00101 ligands by size, showing a strong preference for 9-mers, and more limited numbers of 10- and 11-mers, is similar to the majority of class I alleles characterized to date (see, e.g., www.syfpeithi.com), suggesting that its repertoire is not likely to be subject to unusual biophysical constraints.

Development of a high throughput Eqca-1*00101 binding assay

To allow detailed functional characterization of the peptide binding specificity of Eqca-1*00101, an Eqca-1*00101 transfected P815 cell line was used to purify MHC molecules by affinity chromatography, as described in the “Materials and methods.” Next, selected peptides eluted from Eqca-1*00101 and carrying an endogenous tyrosine (Y) (necessary to allow ¹²⁵I radiolabeling) were radiolabeled and tested for activity in direct binding assays. Peptide 3600.0009 (sequence SDIYKIREI), corresponding to *Mus musculus* mCG10592 15–23, was found to bind with high efficiency, yielding a signal-to-noise ratio of 10 with as little as 0.3 nM of MHC (Supplemental Fig. 1). The specificity of binding was demonstrated by inhibition with a panel of unlabeled peptides, with affinities in the 1–50,000 nM range; 3600.0009 bound Eqca-1*00101 with an affinity of 1.4 nM.

Next, the entire panel of Eqca-1*00101 eluted and sequenced ligands was synthesized and tested for capacity to bind purified Eqca-1*00101 molecules. As shown in Table 1, 22/24 (91.7 %) eluted peptides bound with affinities of 500 nM or better, a binding threshold previously found to correspond with the majority of class I epitopes (Sette et al. 1994). Nineteen (79.2 %) of the peptides bound with very high affinity, with IC₅₀ of 50 nM or better.

Previous experiments had mapped an EHV-1-derived Eqca-1*00101-restricted T cell epitope (sequence RDGARFGEL) corresponding to residues 1059-1067 of the immediate early (IE) protein (ICP4). This peptide was also synthesized and tested for Eqca-1*00101 binding. It was found to bind with an IC₅₀ of 8.1 nM. Taken together, these data validate the binding assay developed herein, providing an assay platform amenable to high-throughput screening.

Determination of a quantitative Eqca-1*00101 peptide-binding motif using a positional scanning combinatorial peptide library

To derive a more detailed and quantitative peptide-binding motif for Eqca-1*00101, we next tested PSCL (Lauemoller et al. 2001; Sidney et al. 2008a; Stryhn et al. 1996; Udaka et al. 2000; Udaka et al. 1995) for their capacity to bind purified Eqca-1*00101 molecules. The binding capacity (IC₅₀ nM) for each mixture was normalized, and an ARB was calculated, as described in the “Materials and methods” section (Table 2).

Next, for each position, we calculated a SF (Loffredo et al. 2009; Sidney et al. 2008a; Sidney et al. 2007), which compares the normalized average binding affinity of all 20 residues at a specified position to the average of the entire library. Positions associated with the highest specificity (highest SF values) were found for position 2 and the C-terminus, with SFs of 6.35 and 16.98, respectively (Table 2). Thus, on the basis of previously established criteria for the PSCL data (Sidney et al. 2008a), and in agreement with the peptide elution data, we concluded that P2 and the C-terminus are the main anchor positions for Eqca-1*00101 binding.

Also in agreement with the pool sequencing motif, the PSCL analysis identified the negatively charged residue aspartic acid (D) as the most preferred at position 2. The polar residue glutamine was tolerated, although with an ARB approximately 20-fold lower than that of D. All other residues in position 2 were associated with ARB values <0.05. At the C-terminus, isoleucine (I) was most preferred, followed by the other aliphatic residues valine (V; ARB=0.51) and leucine (L; ARB=0.20), and the aromatic residue phenylalanine (F; ARB=0.11). All other residues at the C-terminus were associated with binding capacities more than 25-fold lower.

Secondary anchor positions were defined as those positions associated with ARB SD values >2 (Sidney et al. 2008a; Loffredo et al. 2009; Southwood et al. 2011). Accordingly, positions 1, 3, and 7 were defined as secondary anchors for Eqca-1*00101 binding. This spacing is also typically observed for HLA class I specificities (see, e.g., Sidney et al. 2008a). In position 1, as in the eluted peptide data, positively charged residues (R and K; ARB of 0.94 and 1.0, respectively) were preferred. At position 3, the aliphatic-hydrophobic

residues L, I, and M, and the aromatic residue F were preferred with ARB between 0.59 and 1.0. Finally, in position 7, the small residues S, P, T, and V were preferred.

In summary, the Eqca-1*00101 primary anchor motif is for D in position 2, and aliphatic-hydrophobic (I, L, V, and F) residues at the C-terminus, with secondary anchors in positions 1, 3, and 7. A summary cartoon depicting the Eqca-1*00101 motif is shown in Fig. 1.

Identification of equid herpes virus-derived Eqca-1*00101-binding peptides

PSCL matrices have proven to be an efficient means for identifying MHC class I binding peptides (Udaka et al. 1995; Stryhn et al. 1996; Udaka et al. 2000; Lauemoller et al. 2001; Peters et al. 2006; Sidney et al. 2007; Sidney et al. 2008a; Loffredo et al. 2009; Reed et al. 2011; Southwood et al. 2011). Accordingly, we scored all 9-mer peptides in the EHV-1 proteome (strain Ab4) using the PSCL-based matrix shown in Table 2, where the score for each peptide is the product of the values for each peptide residue-position pair. All top 0.5 % scoring 9-mer peptides ($n=194$) and a control set of 319 peptides representative of the 0.5–100 % scoring range were selected.

These 513 peptides were tested for binding to purified Eqca-1*00101. The binding affinities of these peptides (as well as a set of additional EHV-derived 10-mer peptides; see below) are provided in Supplemental Table 1, and will also be deposited in the IEDB (www.iedb.org). To define significant binding, we used a binding affinity threshold of IC_{50} 500 nM, since this affinity threshold is associated with T cell epitopes in humans, non-human primates, and mice (see, e.g., Sette et al. 1994; Kotturi et al. 2007). In total, 59 of the 513 9-mer peptides bound Eqca-1*00101 with an affinity of 500 nM or better, including 20 that bound strongly, with IC_{50} 50 nM. The set of binders represent 35 different EHV-1 ORFs.

Evaluation of PSCL prediction performance

Analysis of frequency of binding as a function of PSCL score indicated that the predictions were efficacious, as 21 of the 38 (55.3 %) peptides scoring in the top 0.1 % bound with an affinity of 500 nM or better. Lower frequencies of binding were noted for successively lower scoring ranges (Fig. 2). Specifically, 30.8 % (12/39) of the peptides scoring in the 0.1–0.2 % range, 23.1 % (9/39) in the 0.2–0.3 % range, and 12–13 % in the 0.3–2 % range (15/116, total), bound with $IC_{50}s < 500$ nM. Only 2.5 % (1/40) of the peptides scoring in the 2–5 % percentile range, and 1.6 % (1/53) in the 5–10 % range, bound. Finally, no binders (0/188) were identified in the peptide set covering the 10–100 % range.

Evaluating the predictions using ROC analysis, as described in the “Materials and methods,” yielded an AUC value of 0.877 % (Supplemental Fig. 2). By comparison, benchmarking studies of HLA class I algorithm performance, covering a wide range of alleles and prediction strategies, have found average performance to be in the 0.800–0.850 % range. Thus, the present analysis indicates that the PSCL matrix performs comparably to other validated prediction approaches for common HLA class I specificities (Peters et al. 2006; Trolle et al. 2015; Wang et al. 2008).

PCSL-based prediction of 10-mer peptides binding to Eqca-1*00101

As previously reported (Sidney et al. 2007; Sidney et al. 2008a; Loffredo et al. 2009), the 9-mer PSCL matrix can be adapted to effectively score 10-mer peptides (see also the “Materials and methods” for more details). The EHV-1 proteome was scanned accordingly and 10-mers scoring in the top 0.1% scoring peptides were selected. In this case, a smaller set of peptides was selected based on the observed approximately fivefold preponderance of 9-mers over 10-mers in the set of eluted peptides. As with the 9-mers, a set of peptides representative of various scoring ranges was also selected. We found that 34.2 % (13 of 38) of the top scoring 10-mers (percentile score 0.1) bound with an affinity of 500 nM or better. Peptides scoring in the 0.1–1 percentile range also bound at a high rate, with 41.4 % (12/29) having IC₅₀s of 500 nM or better. By contrast, only 10 % (2/20) of the peptides in the 1–5 percentile range, and 2.9% (1/35) in the 5–20 range, were binders. None of the 19 peptides with percentile scores >20 bound Eqca-1*00101. In total, 28 10-mer binders were identified, representing 21 different ORFs, including 5 not covered by the set of 9-mers identified above.

Overall, these rates of binding are comparable to those observed with the 9-mer predictions. ROC analysis of the 10-mer predictions yielded an AUC of 0.771 % (data not shown), indicating that the PSCL is reasonably effective for also predicting 10-mer binders for Eqca-1*00101, given the inherent limitations of the approach.

Quantifying the Eqca-1*00101 binding repertoire

Next, we quantified the binding rates associated with various prediction percentile intervals to estimate the total number of binders in the entire set of 39,088 9-mers in the EHV-1 genome. When this was done, it was estimated that the EHV-1 genome should contain about 77 Eqca-1*00101 binding 9-mers, corresponding to a repertoire size of 0.198 % (77/39,088).

A recent study from our laboratory (Paul et al. 2013) evaluated the peptide binding repertoires of 27 well-characterized HLA class I molecules (see also Assarsson et al. 2007). By running predictions against a large set of dengue isolates, encompassing about 40,000 unique 9-mer peptides, an average repertoire of about 3 % was determined, with a range of 0.07–10.4 %. Thus, the predicted repertoire of Eqca-1*00101 binding peptides is at the lower limit of what is known for most class I specificities studied to date.

To confirm this observation, the repertoires of the same suite of alleles, as well as those of several mouse and macaque alleles, were determined by running predictions against the entire set of over 39,000 EHV-1-derived 9-mer peptides. As with the previous study, the average repertoire of the human class I alleles was about 3 % (2.8 %; range 0.11–9.7 %). The average repertoire sizes for seven macaque and five mouse alleles were 2.44 and 1.49 %, respectively. Figure 3 summarizes the repertoire sizes for the various class I alleles analyzed. As shown, the estimated Eqca-1*00101 repertoire of 0.198 % is lower than all but three alleles: HLA B*51:01 (0.11 %), H-2 Dd(0.051 %), and Mamu B*01 (0.069 %). This analysis demonstrates that Eqca-1*00101 has a smaller than normal binding repertoire.

Screening for ex vivo T cell reactivity in PBMC from vaccinated/infected horses

Several HLA class I molecules, like A*0101, which are associated with a narrow peptide-binding repertoire are also associated with a small T cell epitope repertoire. That is, these alleles bind few peptides and, in addition and independently, few of the binding peptides are recognized as immunogenic at the T cell level. For example, in the case of macaques, Mamu B*01, which shares with Eqca-1*00101 a strong preference for D in position 2 (as well as a preference for I at the C-terminus), also has a very limited peptide binding repertoire and, despite considerable effort, no T-cell epitopes have been identified to date that are Mamu B*01 restricted.

To verify whether Eqca-1*00101 was also associated with a narrow repertoire, PBMC from six EHV-1-infected, or vaccinated but uninfected, Eqca-1*00101-positive horses were used in IFN- γ ELISPOT assays to test the Eqca-1*00101 binding peptides identified above, including the previously mapped Eqca-1*00101-restricted EICP4-derived T cell epitope RDGARFGEL (positions 1059–1067 in EICP4), for T cell reactivity, as described in the “Materials and methods.” The proteins of provenance of the set of 87 peptides tested included several early, immediate early, and late viral proteins, and represented a total of 40 different proteins. Strikingly, the ICP4 epitope elicited responses in only one of the six Eqca-1*00101 horses, and no response was noted from any of the other 86 Eqca-1*00101 binding 9- and 10-mer peptides (Fig. 4).

To investigate whether this striking result was an EHV-1-specific phenomenon, or reflective of a more general characteristic of the Eqca-1*00101-specific repertoire, we next probed for responses to equine influenza virus peptides. As above, the PSCL matrix was utilized to score peptides derived from the complete sequence of equine influenza A2/H3N8/Richmond/1/2007, a virus type currently circulating in horse populations worldwide. The top 2 % scoring 9- and 10-mer peptides were selected, synthesized, and screened for responses in PBMC from the same cohort of six horses. Interestingly, none of the 174 screened influenza virus peptides elicited T cell responses in any of the six horses tested (data not shown). While these results could also be attributed to a naïve immune status towards equine influenza, as data on the vaccination and infection history of the donor horses was not available, taken together, the data presented in this section strongly suggests that Eqca-1*00101 is associated with a remarkably narrow T cell epitope repertoire.

Discussion

In the present study, we probed the peptide-binding specificity of the common horse MHC class I molecule Eqca-1*00101. A motif was derived by first sequencing endogenously bound ligands. These ligands were also utilized to develop a high-throughput binding assay using purified Eqca-1*00101 molecules. The assay was in turn used to test a panel of positional scanning combinatorial libraries, resulting in a detailed quantitative motif.

With this motif, a complete scan of the EHV-1 proteome was undertaken. In total, 87 EHV-1 peptides were identified that have the capacity to bind Eqca-1*00101 with high affinity. Binders were identified from 40 different EHV-1 proteins, including 1, 12, and 27 from immediate early, early, and late proteins, respectively.

However, in comparison with other characterized MHC class I specificities (Paul et al. 2013), the repertoire of Eqca-1*00101 appears to be very limited. The repertoire of Eqca-1*00101 extends to only about 0.2 % of all possible 9-mer ligands, compared to about 1.5–3 %, on average, for human, macaque, and mouse class I. The biological consequence of this limited binding repertoire was further magnified by an independent and similarly limited T cell epitope repertoire. When several hundred Eqca-1*00101 binding peptides derived from EHV-1 and equine influenza virus were screened for Tcell reactivity in PBMC from Eqca-1*00101 horses, only one previously known EHV-1 peptide was detected.

Interestingly, the only responsive horse was one that had been infected on several occasions. The remaining five horses had been vaccinated repeatedly with killed EHV-1 vaccines and twice in the 2 months before sampling with the live-attenuated EHV-1 strain RacH. Based on our results, we surmise that even live-attenuated vaccines do not potently activate a sufficient CD8+ CTL response for complete clinical protection.

Robust assays to reliably and reproducibly measure pathogen-specific CTL frequencies in horses are not available, although highly desirable, as they would enable us to correlate frequencies with protection and to determine vaccine efficacy. So far, there are strong hints that elevated virus-specific CTL frequencies are necessary for protection against EHV-1 infection in horses. Indeed, it has been shown that pre-infection CTL frequencies correlate with protection against abortion and other clinical symptoms (Kydd et al. 2003). Therefore, induction of high EHV-1-specific memory CTLs could help prevent infection. Establishing equine ELISPOT assays to determine antigen-specific, reactive CD8+ T cell frequencies that correlate with protection could be used to confirm protective immunity in infected and vaccinated horses.

The Eqca-1*00101 specificity for aspartic acid in position 2 is presumably driven by the unusual presence of the positively charged lysine residue in position 9, which is located in the MHC B pocket. When compared to HLA peptide-binding motifs, Eqca-1*00101 ligands are most similar to those bound by HLA alleles in the B44 supertype (Lund et al. 2004; Sidney et al. 2008b), the mouse allele K^k, and the non-human primate alleles Mamu B*01, Mamu A*11, and Patr A24. In the majority of these cases, however, the predominant specificity is for E in position 2, with D only rarely tolerated. The notable exception is Mamu B*01, which shares with Eqca-1*00101 a strong preference for D in position 2 (as well as a preference for I at the C-terminus). What is intriguing from the comparisons is that Mamu B*01 also has a very limited peptide-binding repertoire and, despite considerable effort, no T cell epitopes have been identified to date that are Mamu B*01-restricted. It is not clear whether the shared specificity for D in position 2 is a factor driving the limited repertoires of these two alleles, or whether the observation just is coincidental. Another allele with a notably limited repertoire is HLA A*01:01, which has a dominant specificity for peptides with D in position 3. While no specific mechanism implicating D as driving the repertoire limitations is apparent, it is tempting to speculate that the presence of D near the N-terminus of potential ligands somehow influences the efficiency of ligand generation by processing. Whereas nothing specific is known for horse TAPs, rat TAPs display a remarkably reduced affinity to peptides carrying an aspartate or related residues in position 2 or 3 (Gubler et al. 1998). Also, human ER-resident aminopeptidases (ERAP) have

preferences for n-terminal hydrophobic residues, which also might influence peptide repertoire for the peptide loading complex (Serwold et al. 2001).

One hypothesis to explain the identification of a limited binding repertoire in the present study pertains the various sources of β 2-microglobulin (β 2m) present in different contexts. The Eqca-1*00101 molecules analyzed with the elution studies are largely heterodimers associated with murine β 2m or, to a minor extent, with bovine β 2m. Additionally, the binding assays are performed with human β 2m, and the entire peptide library was tested in ELISPOT assays where equine β 2m is predominant. While the affinity of β 2m may affect peptide binding, especially weak binders, in general it is also accepted that the specificity of peptide selection would not change much. Indeed, given the concordance between the elution (largely mouse β 2m) and in vitro assays (human β 2m) in terms of specificity, and that our assays addressed the binding and T cell (equine β 2m) repertoires using a large set of peptides covering a broad range of predicted affinities, we do not believe the source of β 2m has a significant influence in this case.

Other motifs expressed by horses and related species are not currently available. It is therefore possible that narrow MHC binding and T cell repertoires might not be associated with only this particular Eqca-1*00101 allele, but rather reflect an as yet unappreciated feature of horse MHC in general. While we consider this an unlikely eventuality, to directly answer this question we are in the process of characterizing the peptide specificity and repertoire size of other common horse MHCs.

Finally, the hypothesis that the limited T cell epitope repertoire is an inherent feature of Eqca-1*00101, or related to immunosuppressive characteristics of EHV-1 like TAP inhibition by an early protein (Ambagala et al. 2004), was addressed by probing for T cell reactivity against candidate epitopes derived from equine influenza virus. No CTL response was observed against any of the influenza epitopes. To explain this limited CTL epitope repertoire against both viruses, in case of EHV-1, the fore mentioned TAP inhibition by the early protein pUL49.5 (Koppers-Lalic et al. 2008) could prevent any other peptide than the one derived from the immediate early protein to be shuttled into the ER and presented on Eqca-1*00101. Certain strains of EHV-1 also encode an additional early protein, pUL56, which downregulates MHC-I surface expression by dynamin-dependent endocytosis (Huang et al. 2014). Both proteins affect antigen presentation and therefore could limit the antigen repertoire presented to CD8+ CTLs. Another unlikely possibility could be thymic intolerance for precursor CTLs recognizing self-peptides that mimic viral class I-epitopes with similar binding specificities. As for influenza virus, since we did not have any data regarding the donor horses' immune status, we cannot rule out the possibility that all donor horses were influenza naïve and did not respond to antigens due to a lack of primed CD8+ CTLs. This scenario seems unlikely, however, as EIV infections are widespread and most horses have undergone vaccination. As vaccination is widely performed with inactivated preparations, priming and boosting of CTL responses may not be ideal. It is also possible that immunization of horses with current influenza vaccines results in dominant antibody responses and relatively poor T cell-mediated immunity.

In the sequences of 11 EHV-1 strains deposited in GenBank, no DNA sequence variation within the IE (ICP4) epitope RDGARFGEL could be found to implicate that escape variants have arisen. Several interaction sites have been reported for the EHV-1 sole IE protein. It physically binds to the viral proteins ICP0, ICP22, and ICP27, as well as several cellular transcription factors like TFIIB (Albrecht et al. 2003; Derbigny et al. 2000; Kim et al. 2003; Zhao et al. 1995). Although none of those interaction sites include the sequence of the identified epitope, a mutation leading to an escape variant might abolish binding or functional folding of the IE protein, which in turn might interrupt the viral replication cycle. However, the EHV-1 closely related virus EHV-4, which shares 80–90 % DNA sequence homology, encodes for a substitution of aspartic acid with glutamic acid in the identified CTL epitope (REGARFGEL). The ICP4 proteins of EHV-1 and EHV-4 share a homology of 89 % on the amino acid level, which suggests that mutations in the epitope can be tolerated provided no complementary mutations arose. On the other hand, it could imply that the Eqca-1*00101 allele, while common (allele frequency estimated at 13.4 %), does not contribute substantially to the control of EHV-1 field infections, and therefore does not exert a strong selection pressure. That actually might not be necessary as the infection could be controlled by CTL activity directed against other class I loci or the Eqca-1*00101 allele confers protection against other important equine infections.

It is important to also consider these results in the context of previous data that suggested that Eqca-1*00101 had a protective effect in terms of EHV-1 infection outcomes after vaccination, where the IE gene of EHV-1 was cloned into a NYVAC vaccine that stimulated IFN- γ +T lymphocytes in PBMCs from Eqca-1*00101 horses and reduced cell-associated viremia showing an efficacious induction of cellular immunity against an IE expression construct in vivo (Paillot et al. 2006; Soboll et al. 2010). That suggests, paradoxically, that a narrow repertoire might be beneficial with respect to control of, or protection against, EHV-1 infection, perhaps allowing a more focused response, or avoiding “immune diversion” or “decoy” responses, which have been described in the case of other herpesviruses (Gillet et al. 2007). At the same time, it is notable that equine immune responses to EHV-1 are particularly short-lived, but there is very little information on whether there are differences in immune responses that would correlate with the ELA haplotype. Therefore, we consider it important to continue the characterization of horse MHC class I-binding motifs for other alleles and to try to correlate the results with the responses to the major viral diseases of equines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Summary Eqca-1*01:001-binding motif. As determined using eluted ligands and a PSCL binding analysis, the motif is summarized to show the main (*red fill*) and secondary (*blue fill*) anchor positions. The most preferred residues at the primary anchor positions are indicated by *enlarged font*. Tolerated anchor residues are also shown. The most preferred residues at secondary anchor positions are as highlighted in the text

Fig. 2.

Efficacy of PSCL predictions. The fraction of EHV-1 peptides in various PSCL percentile scoring ranges that bound Eqca-1*00101 with an IC_{50} of 500 nM or better is shown.

Peptides were tested in competition assays using purified MHC, as described in the “Materials and methods”

Fig. 3.

The peptide binding repertoire of Eqca-1*00101 is relatively limited. The fraction of EHV-1 peptides predicted to bind (i.e., repertoire size) various human (HLA, *green background*), macaque (Mamu, *yellow background*), and mouse (H-2, *white background*) class I alleles, in comparison with the predicted repertoire of Eqca-1*00101 (*red bar on gray background*). Class I repertoires were determined, as described in the “Materials and methods,” using the SMM algorithm

Fig. 4.

ELISPOT interferon gamma T cell responses to Eqca-1*00101 binding peptides.

Eqca-1*00101 binding peptides ($IC_{50} < 500$ nM) were tested for T cell responses in six Eqca-1*00101+ horses. Responses (SFC) were detected to only the previously identified ICP4 1059–1067 epitope RDGARFGEL (peptide 8), and in only one of the six horses. Peptides are sorted *left to right* from highest affinity to lowest, corresponding to the order in Supplemental Table 1

Table 1

Endogenously bound ligands eluted from purified Eqca-1*00101 molecules

Sequence	Length	Eqca-1*00101 binding ^a
SDYVKVSN	9	0.66
SDIYKIREI	9	1.4
GDYNGHVGL	9	1.5
RDLERGVEI	9	2.2
RDLPSHTKI	9	4.5
ADLKKAQEI	9	7.7
RDFKGGKILI	9	9.1
GDMKNKVVI	9	16
KDMDIITKF	9	16
KDAGLVKSL	9	20
RDIPIVHRV	9	37
VDIAAFNKI	9	48
LDKAQLLEI	9	59
KDTKQILRI	9	77
LEQEELDKN	9	90
SQSTVKVLI	9	650
ADDPSSLI	9	1073
KDYSKKVTMI	10	16
RQADFEAHNI	10	67
ADFDGVLHYI	10	92
RQMSGAIKI	10	357
KDVPNSQLRHI	11	45
RDIETFYNTSI	11	0.14
KDVEGSTSPQI	11	43

^aBinding is expressed as IC₅₀ nM

Table 2

PSCL-derived quantitative peptide-binding motif for Eqca-1*00101

Residue	Position								
	1	2	3	4	5	6	7	8	9
A	0.660	0.021	0.232	0.249	1.000	0.713	0.327	0.111	0.006
C	0.145	0.015	0.114	0.121	0.380	0.384	0.137	0.212	0.003
D	0.073	1.000	0.072	0.446	0.156	0.453	0.081	0.288	0.001
E	0.103	0.025	0.082	0.312	0.240	0.616	0.076	0.175	0.001
F	0.328	0.008	0.592	0.295	0.443	0.938	0.084	0.315	0.109
G	0.200	0.020	0.067	0.243	0.521	0.594	0.198	0.288	0.003
H	0.333	0.030	0.112	0.565	0.452	0.511	0.083	0.084	0.002
I	0.309	0.031	0.991	0.244	0.591	0.655	0.241	0.127	1.000
K	1.000	0.011	0.212	0.168	0.278	0.270	0.057	0.121	0.016
L	0.123	0.008	1.000	0.289	0.947	0.759	0.224	0.215	0.196
M	0.699	0.037	0.810	0.538	0.437	0.551	0.308	0.196	0.031
N	0.068	0.017	0.129	0.511	0.357	0.412	0.285	0.234	0.002
P	0.092	0.011	0.068	1.000	0.466	0.248	0.615	0.135	0.001
Q	0.150	0.053	0.223	0.441	0.328	0.262	0.480	0.212	0.001
R	0.937	0.016	0.097	0.587	0.354	0.343	0.194	0.175	0.001
S	0.403	0.018	0.051	0.899	0.808	0.521	1.000	0.325	0.001
T	0.251	0.034	0.053	0.495	0.663	0.758	0.567	0.328	0.010
V	0.158	0.014	0.062	0.300	0.192	1.000	0.547	0.254	0.508
W	0.038	0.011	0.054	0.525	0.752	0.181	0.158	0.248	0.038
Y	0.212	0.011	0.092	0.519	0.259	0.225	0.045	1.000	0.002
Geomean	0.22	0.02	0.15	0.39	0.43	0.47	0.20	0.22	0.01
SD	2.5	2.8	2.7	1.7	1.7	1.6	2.4	1.7	9.2
SF	0.64	6.35	0.95	0.36	0.32	0.30	0.68	0.64	16.98
10-fold	4	19	10	0	0	0	6	1	16

The PSCL was tested for binding, the data analyzed, and primary and secondary anchor positions defined, as described in the "Materials and methods." Values shown represent the average relative binding (ARB) of the corresponding library relative to other pools with the same fixed position. Values have been normalized to the optimal residue at the corresponding position. Geomean is the average ARB across all residues at the corresponding position. SD indicates the standard deviation between the ARB of pools at the same position. SF is the specificity factor, calculated as described in the "Materials and methods," representing the ratio of the average binding of the entire library to the average of pools at the indicated position. At the primary anchor positions (SF>2.4), the most preferred residues, associated

with an ARB >0.1 are highlighted by *italic font*. Tenfold indicates the number of residues at each position associated with ARB 10-fold or more lower than the optimal residue at the same position. The library average binding for Eqca-1*00101 was 8110 nM