The $\alpha3$ Domain of the Qa-2 Molecule Is Defective for CD8 Binding and Cytotoxic T Lymphocyte Activation

By Michael Teitell,* Hilde Holcombe,*‡ Hilde Cheroutre,*
Carla J. Aldrich,§ Iwona Stroynowski,§ James Forman,§ and
Mitchell Kronenberg*

*From the *Department of Microbiology and Immunology and the Jonsson Comprehensive Cancer Center, University of California at Los Angeles School of Medicine, Los Angeles, California 90024-1747; the ‡Department of Microbiology, Pathology, and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606; and the §Department of Microbiology, University of Texas-Southwestern Medical Center, Dallas, Texas 75235-9048*

Summary

Qa-2 is a nonclassical class I molecule encoded by the Q7 gene within the mouse major histocompatibility complex (MHC). Results from previous experiments on Qa-2, and on a chimeric L^d molecule (L^{Q3}) in which the $\alpha3$ domain is encoded by $Q7^b$, suggested that the $\alpha3$ domain of Qa-2 does not carry out the functions typical of the $\alpha3$ domains in other classical and nonclassical class I antigens. Class I molecules that contain the Qa-2 $\alpha3$ domain are poorly recognized by primary cytotoxic T lymphocytes (CTLs), and do not function normally in either positive or negative selection in vivo. By employing a cell–cell adhesion assay we demonstrate directly that the Qa-2 $\alpha3$ domain in the context of the L^{Q3} hybrid molecule cannot bind to human CD8, although other mouse class I $\alpha3$ domains bind efficiently. In addition, CD8-dependent CTL-mediated lysis of target cells, in a system which requires mouse CD8-class I $\alpha3$ domain interactions, is deficient in cells that express the Qa-2 $\alpha3$ domain. When combined with our earlier work on L^{Q3} transgenic mice, these results provide additional molecular support for the hypothesis that interaction with CD8 is required for both positive and negative selection of class I restricted T cells in the thymus. As the Qa-2 $\alpha3$ domain sequence does not differ from the previously defined minimal CD8 binding sequence of other class I molecules, these results also suggest that additional amino acids in the $\alpha3$ domain must be critical for CD8 binding and CTL activation.

The mouse genome contains a number of expressed nonclassical class I, class Ib, or medial class I genes encoded within the *Qa*, *Tla*, and *Hmt* subregions of the MHC (reviewed in reference 1). Although their function(s) remain speculative, increasing evidence suggests that some of these molecules are capable of presenting peptide antigens to CTLs. Nonclassical class I molecules have a significant degree of sequence similarity with classical class I molecules such as H-2K and H-2D, and they are also coexpressed with β_2-microglobulin (1). Recent evidence indicates that the thymus leukemia antigen (TLA),[1] a mouse class Ib molecule, and HLA-G, a human nonclassical class I molecule, can bind effectively to CD8 (2, 3). Furthermore, the Mta and Qa-1 class Ib molecules have been shown to require a functional peptide transporter for their surface expression and recognition by T cells (4), and T lymphocytes that recognize defined peptide antigens in the context of these two molecules have been described (5, 6).

The $Q7^b$ gene encodes a Qa-2 molecule in C57BL/10 mice (7). The results from several experiments suggest that the $\alpha3$ domain of this molecule may be functionally different from other class I $\alpha3$ domains. Alloreactive, Qa-2 specific CTLs can only be detected in mice that have been previously primed in vivo (8, 9). Similar results were obtained using a chimeric L^d molecule, L^{Q3}, in which the $\alpha3$ domain is encoded by $Q7^b$ and the remainder by the L^d gene (10). Primary, CD8-dependent, alloreactive or virus-specific CTLs could lyse L^d transfectant cell lines but not L^{Q3} transfected targets. Despite this, secondary CD8-independent CTLs could lyse L^{Q3} transfected targets (10). The chimeric L^{Q3} molecule was shown to bind a viral peptide that also binds to L^d and, as is seen with L^d, surface expression of L^{Q3} was increased

---

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; L^{Q3}, a chimeric class I molecule in which the $\alpha3$ domain is encoded by the $Q7^b$ gene and the remainder by the L^d gene; TLA, thymus leukemia antigen.

by viral peptide binding (10). These results strongly suggest that the L^d peptide binding site in L^{Q3} is unaffected, localizing the defect in this chimeric molecule to the $\alpha 3$ domain.

Class I molecules rely on their $\alpha 3$ domains for intercellular interaction with the T cell coreceptor molecule CD8. This interaction can be demonstrated directly in binding assays using lymphoid cell lines that overexpress transfected class I genes (11). Previous assay results have shown that all mouse class I and class Ib MHC antigens so far examined are capable of binding human CD8α/α homodimers (2, 11, 12). Using this assay, a minimal CD8 binding site has been located on an external facing loop of the human class I $\alpha 3$ domain (12). Given the CD8-independent nature of both Qa-2-specific and L^{Q3}-specific CTLs, it is reasonable to hypothesize that the Qa-2 $\alpha 3$ domain does not interact with CD8. Although most of the earlier data cited above is consistent with this interpretation, there are two pieces of evidence that are inconsistent. First, it was possible in the previous studies to obtain secondary, L^d-specific T cell clones that were not inhibitable by anti-CD8 mAbs, but which also could not lyse L^{Q3}-transfected targets (10). This suggests that there could be some other defect in the L^{Q3}, or Qa-2 $\alpha 3$ domain besides the inability to bind CD8, although the mAb blocking studies may not detect all CD8-dependent T lymphocytes. Second, there are no sequence differences in the defined CD8 binding site between the Qa-2 $\alpha 3$ domain and the $\alpha 3$ domains of class I molecules that are known to interact with CD8. Therefore, to determine directly if the Qa-2 $\alpha 3$ domain can mediate binding to human CD8, a cell–cell adhesion assay was employed. In addition, the Qa-2 $\alpha 3$ domain was tested in a functional assay: CTL-mediated cytolysis of cell targets that relies only on the ability of mouse CD8α/β heterodimers to interact with class I $\alpha 3$ domain was assessed.

Materials and Methods

Gene Constructs and Transfectants. The TLA expression construct pHβAprT18^d and the chimeric L^d/TLA molecule expression construct pHβAprLT18^d have been previously described (2). pHβAprT18^d encodes a full-length *T18*^{d} cDNA while pHβAprLT18^d encodes a genomic sequence containing the *H-2L*^{d} $\alpha 1$ and $\alpha 2$ domains coupled to a *T18*^{d} genomic sequence encoding the $\alpha 3$, transmembrane, and cytoplasmic domains. Both class I gene constructs are expressed under the control of the human β-actin gene promoter (13). This promoter gives higher levels of expression of mouse class I genes in CIR B cells than do either the *H-2D*^{d} or *H-2L*^{d} promoters (2; data not shown). A chimeric gene (*L*^{Q3}) encoding the *H-2L*^{d} $\alpha 1$, $\alpha 2$, transmembrane, and cytoplasmic domains along with an $\alpha 3$ domain supplied by *Q7*^{b}, expressed under the control of the β-actin gene promoter, was created as follows: A 2.9-kb BamHI fragment containing the *Q7*^{b} $\alpha 3$ domain fused to *L*^{d} transmembrane and cytoplasmic domains was excised from the previously reported expression vector LLQL-23 (10). This fragment was cloned into the BamHI polylinker sites of the cloning vector p34E (14). The fragment was then excised from the resultant construct with XbaI digestion and ligated into place in XbaI digested pHβAprLT18^d. XbaI digestion removes all *T18*^{d} gene sequences. Proper orientation was determined by appropriate restriction enzyme digestion and agarose gel electrophoresis of the resultant plasmid clones. This new construct was designated pHβAprLLQL.

Gene transfer of 20 μg of each expression vector along with 10 μg of the drug selection plasmid pSV2neo (15) into human CIR B lymphoma cells was accomplished by standard coelectroporation procedures (Gene Transfector 300; BTX Inc., San Diego, CA; [2]). G418 (GIBCO BRL, Gaithersburg, MD) selection at 600 μg/ml active drug began 48 h after electroporation, and stable transfectants were chosen 3–4 wk later as previously described (2). A CIR B cell transfectant expressing the human class I transplantation antigen HLA-A2.1 was obtained as a kind gift from Dr. D. Littman, University of California at San Francisco, San Francisco, CA (11).

Detection of Class Ib MHC Antigen Expression. Cell surface mAb staining of CIR transfectants was performed as described previously (16). The primary mAbs used included the anti-TLA mAb TL.m4 (17), which was protein-A column purified and used at a 1:200 dilution. The TL.m4 mAb was kindly provided by Dr. S. Kimura and Dr. E. Boyse, Sloan-Kettering Memorial Cancer Institute, New York. The anti-L^d mAb 30-5-7 (18) was obtained as tumor ascites fluid and used at a 1:25 dilution. It was kindly provided by Dr. M. McMillan, University of Southern California School of Medicine, Los Angeles, CA. The anti-human β_2-microglobulin mAb BBM.1 (19) was used as an undiluted tissue culture supernatant and was kindly provided by Dr. L. Sherman, Scripps Research Institute, La Jolla, CA. The secondary staining reagent employed was a 1:100 dilution of phycoerythrin-conjugated goat anti-mouse IgG (CALTAG Labs, South San Francisco, CA) that detects all three mAbs. After staining and washing, cells were analyzed in propidium iodide using a FACScan® instrument (Becton Dickinson and Co., Palo Alto, CA, and University of California at Los Angeles Flow Cytometry Core Facility, Los Angeles, CA).

Cell-Cell Adhesion Assay. This method has been described in detail elsewhere (11, 12). Briefly, 3 d before the assay adherent human CD8α(+) or mock-transfected Chinese hamster ovary (CHO) cells at 5×10^3–10^4 cells per well were placed in 96-well flat-bottom microtiter plates. Optimal confluent monolayers, as judged by visual inspection, were used on the day of assay. Parent and transfectant CIR B cell lines were incubated for 2 h in [^{35}S]methionine (>12.5 mCi/10^6 cells, >600 Ci/mmol; Amersham Corporation, Arlington Heights, IL) in 1× MEM without L-methionine (Flow Laboratories Inc., McLean, VA). The labeled cells were then added to the plate wells, centrifuged to initiate contact, and incubated at 37°C for 1 h with gentle shaking. After incubation, the wells were washed 10–12 times each with assay buffer (1× PBS, pH 7.4, 10% FCS, and 0.01% NaN$_3$) and the remaining bound cells were solubilized in 1% Triton X-100 for scintillation counting.

Cell Lysis Assay. Anti-K^b alloantigenic CTL clone lines C30 and C35 were obtained from Dr. M. Mescher (Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN). These clones were grown in HEPES-supplemented RPMI 1640 with 10% FCS and were stimulated once each week with irradiated C57Bl/6 splenocytes and twice each week with rIL-2. The CTL clones were activated by preincubation with either 10 μg/ml of fluid-phase mAb 2C11 (anti-CD3, both clones) or 10 μg/ml of mAb F23.1 (anti-Vβ8, clone C35 only) at room temperature for 10 min. mAb-preincubated or untreated T cell clones were then added to 96-well "V" bottom plates containing 10^4 ^{51}Cr-loaded CIR transfectant cells in various effector to target ratios. Assay plates were spun at 160 *g* for 3 min to initiate effector-to-target contact, and lysis allowed to proceed at 37°C in 5% CO$_2$ for 8 h. After incubation, 100 μl of supernatant from each assay well was removed for measurement of ^{51}Cr release and calculation of specific lysis above background.

Results and Discussion

The cell–cell adhesion assay measures the binding of nonadherent cells expressing transfected class I genes to monolayers of CHO cells that express high levels of human CD8α. The ability of mouse class I and class Ib molecules to bind human CD8α molecules in this assay is well established (2, 20). CIR, the recipient human B lymphoma cell line for the class I gene constructs, lacks HLA-A and HLA-B genes, and expresses only low levels of HLA-C (11, 21). Untransfected CIR cells therefore do not bind well to CD8α-positive CHO cells. CIR cells can, however, express high levels of transfected class I genes whose expression is controlled by their own or heterologous promoters. Transfected CIR cell lines will express the introduced mouse or human class I heavy chains along with human β_2-microglobulin. Staining with an anti–human β_2-microglobulin mAb can therefore be used to compare the level of total class I surface expression of cells transfected with the different gene constructs. Fig. 1 shows the cell surface staining profiles as analyzed in the flow cytometer of four CIR B cell lines with the anti-human β_2-microglobulin mAb BBM.1. The level of expression of endogenous class I molecules in mock transfected CIR cells is very low. In contrast, the levels of β_2-microglobulin are significantly higher than in the CIR parent, and roughly equivalent for transfected cell lines that express HLA-A2.1, TLA, or the L^{Q3} chimeric molecule. Staining with mAbs specific for the various transfected class I heavy chains confirms that the increased level of β_2-microglobulin expression is due to expression of the transfected genes (data not shown). In a separate experiment, similar results were obtained using a CIR cell transfected with a gene encoding a chimeric L^d/TLA gene (data not shown). Because the levels of expression of the different transfected genes are nearly equivalent (Fig. 1), the CD8 binding ability of each line can be compared.

Fig. 2 depicts representative data on the binding of the transfected CIR cell lines to CD8α-positive and control CHO cell monolayers. The L^{Q3} transfectant lines A and D did not bind detectably above background to the CD8α-positive monolayers. By contrast, the three cell lines expressing HLA-A2.1, TLA, or L^d/TLA class I molecules bound well. Although the CIR cells overexpress the transfected class I genes and the CHO cells overexpress human CD8α, it could be argued that a low but functional binding affinity is not detected by the method employed. However, in the studies of Salter et al. (12), there was complete concordance between results from the binding assay and the ability of transfectants that express class I mutants to be recognized by primary, alloreactive CTLs. The binding data shown are also consistent with our own prior functional studies, in that L^d/TLA can serve as a target for primary, CD8-dependent CTLs while L^{Q3} cannot (2, 10). Similar results were obtained in three separate experiments in which the binding to CD8 positive and negative monolayers were compared. The ratio of the percent of TLA and HLA-A2.1 positive control cells bound to CD8 positive monolayers to the percent binding of these cells to CD8 negative monolayers ranged from 2.0 to 5.1. In every case the difference in cells bound to CD8$^+$ vs. CD8$^-$ CHO monolayers was statistically significant, with $p < 0.001$ by the Student's t test. In contrast, in two experiments, the ratio of binding of L^{Q3} expressing CIR cells to CD8$^+$ and CD8$^-$ CHO monolayers was 1.0 and 1.1, respectively, and the difference in percent cells bound was not statistically significant. In the third experiment, the ratio of CD8$^+$

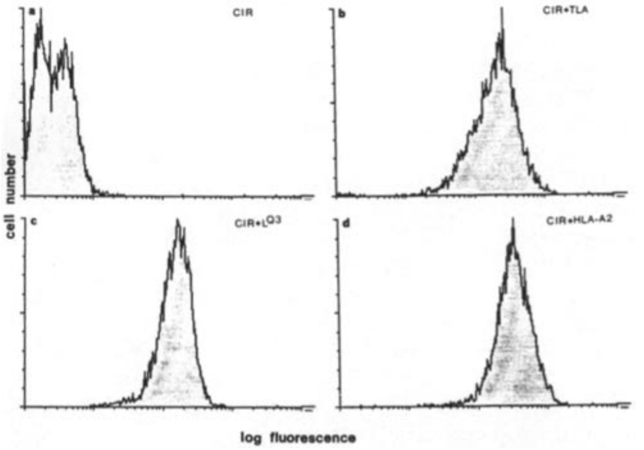

Figure 1. Flow cytometric analysis of CIR parent (*a*) and stable transfectant (*b–d*) cell lines. Staining with the anti–human β_2-microglobulin mAb BBM.1 is shown (19). The relative cell number (*ordinate*) is plotted as a function of the log of the fluorescence intensity (*abscissa*). Staining for line CIR TL.J (*b*), CIR L^{Q3} line D (*c*), and CIR-A2.1 (*d*) are shown.

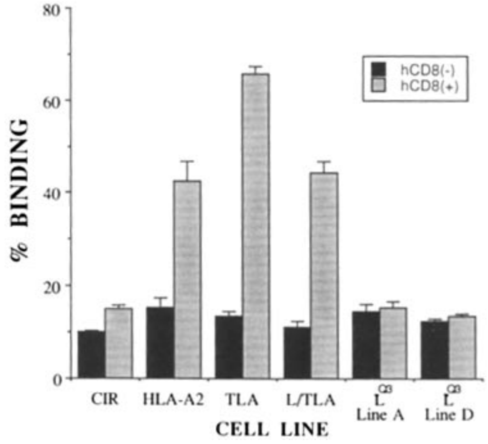

Figure 2. Cell–cell adhesion assay. Binding of radiolabeled CIR cells to CD8α(−) and to human CD8α(+) adherent CHO cell monolayers is depicted as the percentage of input CIR cells retained after extensive washing. Mock transfected CIR parent (CIR), CIR A2.1 (HLA-A2), CIR TL.J (TLA), L^d/TLA (L/TLA), and L^{Q3} lines *A* and *D* have been described previously (2, 11) or are described in Fig. 1 and Materials and Methods. CHO cell lines CHO.1 (hCD8[−]) and CHO.4 (hCD8[+]) have been previously described (11). Error bars represent the SD of triplicate samples. Representative data from one of three experiments is shown.

to CD8$^-$ binding by L^{Q3} cells was 1.9, 31.2% ± 3.0 (SE of the mean) cells bound to CD8$^+$ CHO monolayers vs. 16.8% ± 0.6 to CD8$^-$ monolayers. However, in this experiment the negative control CIR cells also bound better to the CD8$^+$ CHO monolayers, 21.8% ± 1.0 vs. 13.5% ± 0.7. The difference in binding to the CD8 monolayers for both L^{Q3} and control CIR cells is statistically significant ($p < 0.005$) in both cases. It is therefore possible that this third experiment was for some reason unusually sensitive, enabling detection of binding by the low level of HLA-C expressed by CIR cells to human CD8. Consistent with a possible enhanced sensitivity, in this same experiment the TLA and A2.1 expressing CIR cells gave the highest percent cells bound that has been obtained in these assays, 81.8% and 67.4%, respectively. In summary, the data indicate that L^{Q3} cannot bind to human CD8 because in all cases the L^{Q3} transfectant bound much less well to the CD8$^+$ CHO cells than did either the TLA or HLA-A2 transfectants, in all three experiments L^{Q3} binding was similar to that of the CIR parent negative control, and in two of three cases binding of L^{Q3} cells to CD8$^+$ and CD8$^-$ CHO cells was equivalent.

Although the Qa-2 $\alpha 3$ domain of L^{Q3} is incapable of binding human CD8α/α homodimers, it may still be capable of interacting with mouse CD8α/β heterodimers. To date, there has been complete concordance in the ability of mouse class I $\alpha 3$ domains to interact with both human and mouse CD8 (2, 11, 12, 22). However, the data are not that extensive, particularly for nonclassical class I molecules for which only one example currently exists (2). In addition, recent work by two independent groups indicates that the presence of the mouse CD8β chain appears to both broaden the range and strengthen the response of CD8-$\alpha 3$ domain interactions (23, 24). Therefore, in order to rule out the possibility that L^{Q3} could interact with mouse CD8α/β heterodimers expressed at physiologic levels, a second assay was performed.

CTLs are capable of binding and initiating a degranulation response to purified class I and class Ib MHC antigens coated on a plate when they are first suboptimally activated by preincubation with soluble anti–T cell receptor mAbs (22, 25). In the absence of mAb preincubation these functions do not occur. Furthermore, both binding and degranulation can be blocked with anti–CD8 mAbs (22). These observations suggest that, following T cell receptor activation with fluid-phase antibodies, a second signal which can be mediated through a CD8 interaction with an "irrelevant" class I molecule is required to activate the CTL. This second signal is also required for the initiation of polyphosphoinositide hydrolysis (25). In the present study, we modified this assay by using CIR target cells that express equivalent levels of transfected class I or class Ib genes (see Fig. 1) instead of purified, plate-bound MHC antigens. By using two mouse CTL clones that are K^b-specific, no influence from bound peptides or the $\alpha 1$ and $\alpha 2$ domains of the transfected class I gene is possible (26). Additionally, use of human cell line targets should reduce the possibility of other second signals, aside from those delivered through other adhesion molecules (reviewed in reference 27), from activating the CTL. Finally, the CIR transfectant A2.1 is an ideal negative control because it has been shown that human class I $\alpha 3$ domains are deficient for interactions with mouse CD8 (28, 29).

Anti-K^b CTL lines C30 and C35 were made competent for killing by preincubation with fluid-phase mAb 2C11 just before use. Target cells loaded with ^{51}Cr were incubated with effectors at various ratios for 8 h and the amount released, due to specific lysis above background, was measured. When either of the CTL clone lines were not made competent by mAb preincubation, or when line C30 was preincubated with the irrelevant mAb F23.1, the resultant ^{51}Cr release was very low in all repeats of the assay (maximum 0.8% at an effector to target ratio of 5:1, data not shown). However, specific lysis of CIR transfectants expressing the L^d/TLA chimeric molecule is significantly above the background seen with the negative control transfectant A2.1 (Fig. 3). Background ^{51}Cr release is consistently ∼10% in all 8-h assay repeats using effector-to-target ratios of 20:1 down to 1.25:1. Previously it was shown that the L^d/TLA chimeric molecule could support a CD8-dependent alloantigenic response and this reactivity could be blocked with anti–CD8 mAbs (2). The L^d/TLA-mediated cytolysis is blocked by 21% and 32% at effector-to-target ratios of 20:1 and 5:1, respectively, with a 10-μg preincubation of anti–CD8α mAb (M. Teitell, unpublished data, not shown). Also, the low level of background lysis due to CIR A2.1 transfectant targets was not affected by effector clone preincubation with anti–CD8α mAb (data not shown). This result further supports the proposed mechanism of CTL activation through CD8 interactions. Several other CIR transfectants that express different class I molecules are effective in this assay, including one molecule that did not function in the cell–cell adhesion assay, and this cytolysis also was partially blocked with anti–CD8α mAb preincubation (M. Teitell, manuscript in preparation). In contrast, Fig. 3 demonstrates that the chimeric L^{Q3} molecule is unable to activate a CTL response through CD8 interactions, as the amount of ^{51}Cr released from loaded targets is equivalent to that released from control CIR A2.1 targets.

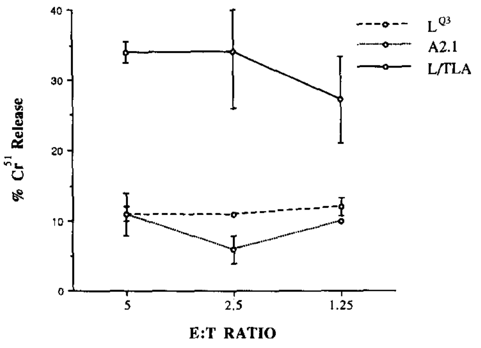

Figure 3. CD8-mediated lysis of transfected CIR target cells. The percent cytolysis is calculated as (specific release − spontaneous release)/(total release − spontaneous release) × 100 and depicted as a function of the effector-to-target ratio. The figure shows the cytolysis of cell lines expressing equivalent levels of HLA-A2.1 (A2.1), L^{Q3}, and L^d/TLA (L/TLA) transfected genes by mAb 2C11 preincubated CTL clone C30. These results are identical to those obtained with CTL clone C35 and are reproducible for effector-to-target ratios up to 20:1. Error bars represent the SD of three measurements.

The L^{Q3} molecule might not bind human CD8 or permit mouse CTL activation because the L^d $\alpha1$ and $\alpha2$ domains could potentially distort or interfere with the normal conformation or accessibility of the Qa-2 $\alpha3$ domain. We consider these possibilities unlikely because the L^d and Qa-2 $\alpha3$ domains are highly similar in sequence. Furthermore, Qa-2–specific CTLs also appear to be CD8 independent. In addition, results depicted in Fig. 3 and those of previous experiments on the chimeric L^d molecule L^d/TLA support the notion that $\alpha3$ domain functions are not disturbed in chimeric molecules by the $\alpha1$ and $\alpha2$ domains (2). It also is possible that the $\alpha3$ domain in L^{Q3} is distorted by its association with human β_2-microglobulin in the CIR transfectants and also, presumably, by bovine β_2-microglobulin obtained by exchange in the tissue culture medium. Although not formally excluded, we consider these explanations unlikely for the reasons outlined here. First, the behavior of intact Qa-2 itself is at least consistent with an inability to interact with CD8. Second, distortion of the L^{Q3} domain in the presence of heterologous β_2-microglobulin could not also apply to L^d or L^d/TLA molecules despite the high degree of sequence conservation. It should be noted that based upon the structures of both human and mouse class I molecules (30–32), all of the amino acids likely to be important for interacting with β_2-microglobulin in K^b, TLA, and Qa-2 are conserved (Fig. 4). We conclude therefore that the most likely explanation is that Qa-2 does not interact with either mouse or human CD8 and that those secondary CTLs that recognize L^d but not L^{Q3}, and which are not inhibitable with anti-CD8 mAbs (10), probably still require a CD8 interaction in order to become activated. It is still formally possible, however, that there is a second defect in the Qa-2 $\alpha3$ domain in the L^{Q3} hybrid.

The $Q7^b$ $\alpha3$ domain differs from the L^d $\alpha3$ domain, which does bind to CD8 (data not shown), by only six amino acid substitutions and a three amino acid insertion at the carboxy terminus (33, 34; Fig. 4). Although it is not possible to reach a definitive conclusion as to which of the changes are critical for interfering with CD8 binding, the carboxy-terminal three amino acid insertion at positions 275–277 is perhaps responsible for the lack of CD8 interaction. The *H-2D*^{d} $\alpha3$ domain has a carboxy-terminal insertion of similar length (35), and it is presumed not to be altered in its ability to interact with CD8. However, the $Q7^b$ $\alpha3$ domain sequence contains a bulky tryptophan group at position 277, which is not present in the *H-2D*^{d} $\alpha3$ domain, and this substitution could prevent $\alpha3$ domain–CD8 binding. Qa-2 molecules that span the plasma membrane, and which therefore are not phosphatidylinositol linked (36), could contain this tryptophan group up to 10–15 amino acids external to the plasma membrane. The K to Y and E to A substitutions, at positions 196 and 198, respectively, may also effect $\alpha3$ domain–CD8 interactions. Extrapolation of data from the crystal structure suggests that these changes are found on an external facing loop between the $\alpha3$ domain β-strands 1 and 2 (30–32), and they replace two charged amino acids with two neutral ones. However, positive, negative, and in some cases even neutral amino acids can occupy these positions in various class I molecules that are presumed capable of CD8 binding. Among the remaining substitutions that distinguish the $Q7^b$ and *L*^{d} $\alpha3$ domains, the E to D and S to P alterations at positions 183 and 184 and the R to H change at position 260 are not likely to have an important effect on CD8 binding. These changes replace L^d amino acids with those found in both TLA and the human consensus $\alpha3$ domain sequences. Finally, the Y to N substitution at position 262 is potentially important, but the human consensus $\alpha3$ domain sequence contains a glutamine at this position which is a rather conservative change from the asparagine in $Q7^b$. Whichever substitutions turn out to be critical, the data suggest that a larger than expected area of the $\alpha3$ domain may be involved directly in CD8 interactions. Alternatively, the substituted amino acids might disrupt the conformation or accessibility of the previously defined minimal CD8 binding domain.

Analysis of transgenic mice that express L^{Q3} has shown that this class I molecule does not function normally in either positive or negative selection of the T cell repertoire (37). The data presented here provide strong evidence that the defect in L^{Q3} is indeed due to the loss of binding to CD8, and therefore that effective CD8 interactions are required for positive and negative selection in the thymus. Our data on L^{Q3} is consistent with recent experiments showing that mutant class I molecules, bearing $\alpha3$ domain substitutions that disrupt CD8 binding, cannot carry out either positive or nega-

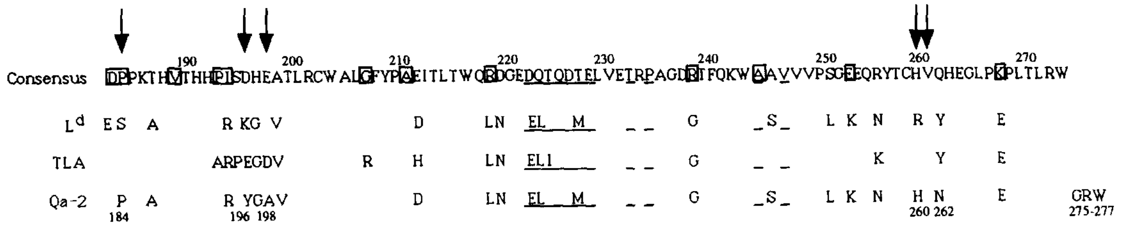

Figure 4. Consensus sequence of the human class I $\alpha3$ domain compared to L^d, TLA (encoded by *T18*^{d}), and Qa-2 (encoded by $Q7^b$) $\alpha3$ domains. Boxed areas in the consensus sequence represent human class I $\alpha3$ domain polymorphic amino acids as described in Parham et al. (45). The L^d sequence was obtained from Linsk et al. (33), the TLA sequence was obtained from Fisher et al. (46), and the Qa-2 sequence was obtained from Devlin et al. (34). Amino acids that differ between L^d, TLA, and Qa-2 and the consensus sequence are indicated. The six amino acids that differ between L^d and Qa-2 are numbered, as is the three amino acid insert near the carboxy terminus of Qa-2. Arrows above the consensus sequence indicate the position of Qa-2 variant residues, which are due to encoding of this class I molecule by different genes with the *Qa* locus (34). Underlined portions of the sequence have been implicated in binding to CD8 (12).

tive selection (38, 39). They are also consistent with studies demonstrating that human HLA class I molecules do not carry out positive selection in transgenic mice (28), probably because human class I molecules do not bind to mouse CD8 (28, 29).

The Q7-encoded Qa-2 class Ib molecule is unusual in several respects. It is attached to the plasma membrane by a phosphatidylinositol linkage (36, 40), its expression as a cell-bound molecule is controlled in part by alternate splicing mechanisms (41), and it can transmit an intracellular activation signal when cross-linked by specific cell surface antibodies (42, 43). Although, like L^{Q3}, Qa-2 is probably incapable of binding CD8, this does not imply that this molecule has no significant role in antigen presentation to T lymphocytes and it does not exclude a possibility that it participates in negative or positive selection. Some or all of the previously characterized anti–Qa-2 allogeneic CTL may have arisen as a result of positive selection of these T cells not on Qa-2, but on another class I molecule(s), most likely with CD8 binding capabilities. Recently, however, Qa-2 has been shown to bind endogenously derived nonameric peptides (44; Joyce, S., P. Tabaczewski, R. H. Angeletti, S. G. Nathenson, and I. Stroynowski. Manuscript submitted for publication). Thus, it remains possible that anti–Qa-2 CTL, which are directly selected on Qa-2/peptide complexes, originate from a novel and thus far not identified developmental pathway that does not depend on CD8 coreceptor.

We thank Dr. Valentin Isacescu of the University of California at Los Angeles flow Cytometry Core Facility for flow cytometric analysis, Dr. Matthew Mescher of the University of Minnesota for both CTL clone lines and helpful discussion, and Jose Trevejo and Katherine Williams for help with creation of the figures.

This work is funded by National Institutes of Health grants CA-52511 (to M. Kronenberg) and AI-13111 (to J. Forman). M. Teitell is supported by Medical Scientist Training Program grant GM-08042 and H. Cheroutre is supported by a postdoctoral fellowship from the Cancer Research Coordinating Committee of California.

Address correspondence to Dr. Mitchell Kronenberg, Department of Microbiology and Immunology, UCLA Center for the Health Sciences, 10833 Le Conte Avenue, Los Angeles, CA 90024–1747. Dr. Michael Teitell's current address is the Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115.

Received for publication 1 June 1993 and in revised form 8 September 1993.

References

1. Stroynowski, I. 1990. Molecules related to class I major histocompatibility complex antigens. *Annu. Rev. Immunol.* 8:501.
2. Teitell, M., M.F. Mescher, C.A. Olson, D.R. Littman, and M. Kronenberg. 1991. The thymus leukemia antigen binds human and mouse CD8. *J. Exp. Med.* 174:1131.
3. Sanders, S.K., P.A. Giblin, and P. Kavathas. 1991. Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. *J. Exp. Med.* 174:737.
4. Attaya, M., S. Jameson, C.K. Martinez, E. Hermal, C. Aldrich, J. Forman, K. Fischer-Lindahl, M.J. Bevan, and J.J. Monaco. 1992. *Ham-2* corrects the class I antigen-processing defect in RMA-S cells. *Nature (Lond.).* 355:647.
5. Vidovic, D., M. Roglic, K. McKune, S. Guerder, C. MacKay, and Z. Dembic. 1989. Qa-1 restricted recognition of foreign antigen by a $\gamma\delta$ T-cell hybridoma. *Nature (Lond.).* 333:646.
6. Fischer-Lindahl, K., E. Hermel, B.E. Loveland, and C.R. Wang. 1991. Maternally transmitted antigen of mice: a model transplantation antigen. *Annu. Rev. Immunol.* 9:373.
7. Mellor, A.L., J. Antoniou, and P.J. Robinson. 1985. Structure and expression of genes encoding murine Qa-2 class I antigens. *Proc. Natl. Acad. Sci. USA.* 82:5920.
8. Forman, J., and L. Flaherty. 1978. Identification of a new CML target antigen controlled by a gene associated with the Qa-2 locus. *Immunogenetics.* 6:227.
9. Forman, J., J. Trial, S. Tonkonogy, and L. Flaherty. 1982. The *Qa-2* subregion controls the expression of two antigens recognized by *H-2*-unrestricted cytotoxic T cells. *J. Exp. Med.* 155:749.
10. Aldrich, C.J., L.C. Lowen, D. Mann, M. Nishimura, L. Hood, I. Stroynowski, and J. Forman. 1991. The Q7 $\alpha3$ domain alters T cell recognition of class I antigens. *J. Immunol.* 146:3082.
11. Norment, A.M., R.D. Salter, P. Parham, V.H. Engelhard, and D.R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature (Lond.).* 336:79.
12. Salter, R.D., R.J. Benjamin, P.K. Wesley, S.E. Buxton, T.P.J. Garrett, C. Clayberger, A.M. Krensky, A.M. Norment, D.R. Littman, and P. Parham. 1990. A binding site for the T-cell co-receptor CD8 on the $\alpha3$ domain of HLA-A2. *Nature (Lond.).* 345:41.
13. Leavitt, J., P. Gunning, P. Porreca, S.Y. Ng, C.S. Lin, and L. Kedes. 1984. Molecular cloning and characterization of mutant and wild-type human β-actin genes. *Mol. Cell. Biol.* 4:1961.
14. Tsang, T., V. Copeland, and G.T. Bowden. 1991. A set of cassette cloning vectors for rapid and versatile adaptation of restriction fragments. *Biotechniques.* 10:330.
15. Southern, P.J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under

the control of the SV40 early region promoter. *J. Molec. Appl. Genet.* 1:327.

16. Hershberg, R., P. Eghtesady, B. Sydora, K. Brorson, H. Cheroutre, R. Modlin, and M. Kronenberg. 1990. Expression of the thymus leukemia antigen in the intestinal epithelium. *Proc. Natl. Acad. Sci. USA.* 87:3380.
17. Shen, F.W., M.J. Chorney, and E.A. Boyse. 1982. Further polymorphism of the Tla locus defined by monoclonal TL antibodies. *Immunogenetics.* 15:573.
18. Ozato, K., T.H. Hansen, and D.H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L^d antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473.
19. Brodsky, F.M., W.F. Bodmer, and P. Parham. 1979. Characterization of a monoclonal anti-β_2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *Eur. J. Immunol.* 9:536.
20. Salter, R.D., A.M. Norment, B.P. Chen, C. Clayberger, A.M. Krensky, D.R. Littman, and P. Parham. 1989. Polymorphism in the $\alpha3$ domain of HLA-A molecules affects binding to CD8. *Nature (Lond.).* 338:345.
21. Storkus, W.J., D.N. Howell, R.D. Salter, J.R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA expression. *J. Immunol.* 138:1657.
22. O'Rourke, A.M., J. Rogers, and M.F. Mescher. 1990. Activated CD8 binding to class I protein mediated by the T-cell receptor results in signaling. *Nature (Lond.).* 346:187.
23. Wheeler, C.J., P. von Hoegen, and J.R. Parnes. 1992. An immunological role for the CD8 β-chain. *Nature (Lond.).* 357:247.
24. Karaki, S., M. Tanabe, H. Nakauchi, and M. Takiguchi. 1992. β-chain broadens range of CD8 recognition for MHC class I molecule. *J. Immunol.* 149:1613.
25. O'Rourke, A.M., and M.F. Mescher. 1992. Cytotoxic T-lymphocyte activation involves a cascade of signalling and adhesion events. *Nature (Lond.).* 358:253.
26. Sherman, L.A., S.V. Hesse, M.J. Irwin, D. LaFace, and P. Peterson. 1992. Selecting T cell receptors with high affinity for self-MHC by decreasing the contribution of CD8. *Science (Wash. DC).* 258:815.
27. O'Rourke, A.M., and M.F. Mescher. 1993. The roles of CD8 in cytotoxic T lymphocyte function. *Immunol. Today.* 14:183.
28. Kalinke, U., B. Arnold, and G.J. Hammerling. 1990. Strong xenogeneic HLA response in transgenic mice after introducing an $\alpha3$ domain into HLA B27. *Nature (Lond.).* 348:642.
29. Sanders, S.K., R.O. Fox, and P. Kavathas. 1991. Mutations in CD8 that affect interactions with HLA class I and monoclonal anti-CD8 antibodies. *J. Exp. Med.* 174:371.
30. Fremont, D.H., M. Matsumura, E.A. Stura, P.A. Peterson, and I.A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science (Wash. DC).* 257:919.
31. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (Lond.).* 329:506.
32. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (Lond.).* 329:512.
33. Linsk, R., J. Vogel, H. Stauss, J. Forman, and R.S. Goodenow. 1986. Structure and function of three novel MHC class I antigens derived from a C3H ultraviolet-induced fibrosarcoma. *J. Exp. Med.* 164:794.
34. Devlin, J.J., E.H. Weiss, M. Paulson, and R.A. Flavell. 1985. Duplicated gene pairs and alleles of class I genes in the Qa-2 region of the murine major histocompatibility complex: a comparison. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3203.
35. Sher, B.T., R. Nairn, J.E. Coligan, and L.E. Hood. 1985. DNA sequence of the mouse H-2D^d transplantation antigen gene. *Proc. Natl. Acad. Sci. USA.* 82:1175.
36. Stroynowski, I., M. Soloski, M.G. Low, and L. Hood. 1987. A single gene encodes secreted and membrane-bound forms of the major histocompatibility Qa-2 antigen: anchoring of the protein product by a phospholipid tail. *Cell.* 50:759.
37. Aldrich, C.J., R.E. Hammer, S. Jones-Youngblood, U. Koszinowski, L. Hood, I. Stroynowski, and J. Forman. 1991. Negative and positive selection of antigen-specific cytotoxic T lymphocytes affected by the $\alpha3$ domain of MHC I molecules. *Nature (Lond.).* 352:718.
38. Ingold, A.L., C. Landel, C. Knall, G.A. Evans, and T.A. Potter. 1991. Co-engagement of CD8 is required for negative selection. *Nature (Lond.).* 352:721.
39. Killeen, N., A. Moriarty, H.-S. Teh, and D.R. Littman. 1992. Requirement for CD8-major histocompatibility complex class I interaction in positive and negative selection of developing T cells. *J. Exp. Med.* 176:89.
40. Stiernberg, J., M.G. Low, L. Flaherty, and P.W. Kincade. 1987. Removal of lymphocyte surface molecules with phosphatidylinositol-specific phospholipase C: effects on mitogen responses and evidence that ThB and certain Qa antigens are membrane-anchored via phosphatidylinositol. *J. Immunol.* 38:3877.
41. Ulker, N., K.D. Lewis, L.E. Hood, and I. Stroynowski. 1990. Activated T cells transcribe an alternately spliced mRNA encoding a soluble form of Qa-2 antigen. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3839.
42. Robinson, P.J., M. Millrain, J. Antoniou, E. Simpson, and A.L. Mellor. 1989. A glycophospholipid anchor is required for Qa-2-mediated T cell activation. *Nature (Lond.).* 342:85.
43. Hahn, A.B., and M.J. Soloski. 1989. Anti Qa-2 induced T cell activation: the parameters of activation, the definition of mitogenic and non-mitogenic antibodies and the differential effects on $CD4^+$ versus $CD8^+$ T cells. *J. Immunol.* 143:407.
44. Rotzschke, O., K. Falk, S. Stevanovic, B. Grahovac, M.J. Soloski, G. Jung, and H.G. Rammensee. 1993. Qa-2 molecules are peptide receptors of higher stringency than ordinary class I molecules. *Nature (Lond.).* 361:642.
45. Parham, P., C.E. Lomen, D.A. Lawlor, J.P. Ways, N. Holmes, H.L. Coppin, R.D. Salter, A.M. Wan, and P.D. Ennis. 1988. Nature of polymorphism in HLA-A, -B, and -C molecules. *Proc. Natl. Acad. Sci. USA.* 85:4005.
46. Fisher, D.A., S.W. Hunt, III, and L. Hood. 1985. Structure of a gene encoding a murine thymus leukemia antigen, and organization of *Tla* genes in the BALB/c mouse. *J. Exp. Med.* 162:528.