The Thymus Leukemia Antigen Binds Human and Mouse CD8

By Michael Teitell,* Matthew F. Mescher,‡ Clifford A. Olson,§
Dan R. Littman,‖ 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; the ‡Division of Membrane Biology, Medical Biology Institute, La Jolla,
California 92037; the §Department of Microbiology and Molecular Genetics, University of
California at Los Angeles, Los Angeles, California 90024; and the ‖Department of
Microbiology and Immunology and Howard Hughes Medical Institute, University of California
at San Francisco, San Francisco, California 94143

Summary

The thymus leukemia antigen (TLA) is a class Ib, or 'nonclassical' class I molecule, one of several encoded within the Tla locus of the mouse major histocompatibility complex (MHC). It structurally resembles the H-2K, D, and L class I transplantation antigens, which present processed peptides to cytotoxic T lymphocytes (CTLs). Although their function(s) are unknown, there has been recent speculation concerning the possibility that class Ib molecules may present antigens to T cells that express $\gamma\delta$ T cell antigen receptors (TCRs). In this report, using both a cell-cell adhesion assay and adhesion of T lymphocyte clones to purified plate-bound TLA, we provide evidence that TLA can bind to both human and mouse CD8. We also show that a chimeric class I molecule containing the peptide antigen binding site of L^d and the $\alpha 3$ domain, transmembrane, and cytoplasmic segments of TLA, can support a CD8-dependent immune response by CTLs. These results demonstrate for the first time binding of a class Ib molecule to CD8 with a functional outcome, as is observed for the class I transplantation antigens. The capacity to interact with CD8 has been conserved despite the extensive sequence divergence of TLA in the peptide antigen binding site, suggesting this interaction is highly significant. TLA is expressed by epithelial cells in the mouse small intestine. As these epithelial cells are in close contact with intestinal intraepithelial lymphocytes that are nearly all CD8+, and many of which express the $\gamma\delta$ TCR, the data are consistent with the hypothesis that TLA is involved in antigen presentation, perhaps to $\gamma\delta$-positive lymphocytes in this site.

Genes encoding both class I and II cell surface proteins are located within the mouse MHC (reviewed in reference 1). Functional class I sequences encode the transplantation antigens that interact with both the TCR and CD8 in presenting peptide antigens to CTLs (2, 3). In binding class I transplantation antigens on target cells, the CD8 molecule act as a co-receptor with the TCR for antigen recognition, thus forming a multimolecular, intercellular complex (3). Formation of this complex causes the delivery of specific signals to the T cell with resultant cytolysis of the target cell (reviewed in reference 4).

Most of the MHC class I genes in mice do not lie within the K or D subregions where the transplantation antigens are encoded; rather, the majority are located within the Qa, Tla, and Hmt subregions. Although not all of the class Ib genes within these subregions are capable of encoding mature polypeptides, products of at least five of these genes have

been identified either by immunochemical techniques or by measuring T cell responses to them (reference 5, reviewed in reference 6). The functions(s) of the class Ib proteins are unknown, although several have been proposed including a role in lymphocyte differentiation, antigen presentation, and in the death signal involved in negative selection in the thymus (reviewed in references 7 and 8). It has also been suggested that the class Ib genes serve as a reservoir of sequences used to diversify the transplantation antigens, and finally, that these genes have no function at all (9). Regarding their proposed function as antigen presenting elements, several groups of investigators have demonstrated alloreactive, autoreactive, and peptide antigen specific responses by $\gamma\delta$ TCR-positive lymphocytes that recognize class Ib surface molecules (5, 10, 11). Based largely on these observations, it has been proposed that T lymphocytes expressing the $\gamma\delta$ TCR utilize class Ib molecules as antigen presenting elements (12, 13). However, it

is not yet clear whether these results, obtained from very few T-cell clones and hybridomas, are physiologically relevant for immune responses by populations of γδ T cells.

A class Ib molecule named the thymus leukemia antigen (TLA)[1] is encoded by the *T18^d* gene located within the *Tla* subregion (nomenclature according to reference 14). Historically, TLA was first identified as a leukemia-specific cell surface antigen in X-irradiated A strain mice (15), although its expression was soon detected in immature cortical thymocytes as well as leukemias of particular mouse strains. By inspection of its gene organization and amino acid sequence, TLA appears similar to the transplantation antigens and contains separate exons encoding for external α1, α2, and α3, as well as transmembrane, and cytoplasmic domains (16). Comparison of the TLA amino acid sequence with an H-2 consensus sequence shows a 60% identity in the α1 domain, a 66% identity in the α2 domain, and an 86% identity in the α3 domain (6). TLA contains three potential sites for N-linked glycosylation at positions 86, 90, and 283 compared with H-2 consensus glycosylation sites at positions 86, 176, and 256 (6). Four conserved cysteines are present in TLA at positions 101, 164, 203, and 259, as is also seen in all the transplantation antigens (17, 18). These cysteine residues are required for disulphide bond formation and proper folding in all class I molecules. TLA also associates noncovalently with β2-microglobulin, as do K, D, and L class I antigens (6). Despite all these similarities to the transplantation antigens, a cell-mediated immune response to TLA has never been reported, possibly due to its low levels of expression outside of the thymus and intestine (19, 20). If TLA were to function similarly to a transplantation antigen, perhaps it would also be bound simultaneously by both the TCR and CD8 as in the co-receptor model for antigen recognition. To determine if this is the case, the ability of TLA to interact with CD8 was assessed.

Materials and Methods

Gene Cloning and Transfections. A *T18^d* cDNA clone was obtained from Dr. F.-W. Shen, Tampa Bay Research Institute (St. Petersburg, FL) Constructs pDT18^{d}hGH and pHβAprT18^d were generated by standard molecular biology methods using pBluescript KS+ (Stratagene, La Jolla, CA) or pBR322-based cloning vectors. The H-2D^d promoter, human β-actin promoter-intervening sequence 1 (IVS 1), and human growth hormone coding sequence DNA fragments are described elsewhere (21–23). The plasmid pL^{d}neo, containing the H-2L^d promoter and genomic coding sequences colinear with pSV2neo, was a gift of Dr. Martha Zuniga, UC Santa Cruz, Santa Cruz, CA. A chimeric L^d/TLA MHC class I gene construct, designated pHβAprLT18^d, was generated from a 1.8-kb XbaI genomic fragment encoding the L^d leader, α1, and α2 domains and a 4.5-kb XbaI genomic fragment of pTla.1 encoding the *T18^d* α3, transmembrane, cytoplasmic, and 3' UTR (16, 24). The human β-actin promoter was inserted 5' of this chimeric gene in the proper orientation for expression in the cloning vector pBluescript KS+.

20 μg of the appropriate construct was coelectroporated (Gene Transfector 300; BTX Corp., San Diego, CA) with 10 μg of pSV2neo (25) into either 10^7 CIR B cells or L cell fibroblasts. G418 (Gibco BRL, Gaithersburg, MD) selection at 600 μg/ml active drug for CIR transfectants and 400 μg/ml active drug for L cell transfectants began 48 h postelectroporation in 96 well flat-bottom microtiter plates. Stable transfectants were chosen at 3 to 4 wk, grown in drug-free RPMI 1640 supplemented with 10% FCS, and stained for cell surface expression of TLA or L^d. Staining was done as previously described (20) with TL.m4 primary mAb (a kind gift of Drs. S. Kimura and E. Boyse, Sloan-Kettering Memorial Cancer Institute, NY) followed by a FITC-conjugated goat anti–mouse Ig second reagent (CalTag, South San Francisco, CA) for detection of TLA. L^d expression was assessed using mAbs 30-5-7 (specific for the α2 domain of L^d), which stains both L^d and L^d/TLA positive transfectants, and 28-14-8 (specific for the α3 domain of L^d), which detects only L^d transfectants (26, 27). Cells were fixed in 1% paraformaldehyde and analyzed on a Coulter EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL) CIR TL.J, a high expressing CIR TLA (+) transfectant, using the pHβAprT18^d construct, was used in both the cell-cell adhesion assays and to purify TLA glycoprotein. L^d and L^d/TLA L cell transfectants that express approximately equal levels of surface protein, as measured by staining and flow cytometry with mAb 30-5-7, were used as targets for primary CTLs.

Cell-Cell Adhesion Assay. This assay has been described in detail previously (3, 28, 29). Briefly, 3 to 4 d before assay between 5 × 10^3 and 10^4 human CD8α(+) or CD8α(−) adherent chinese hamster ovary (CHO) 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 or transfectant CIR B cells were labeled for 2 h with [^{35}S]methionine (>12.5 μCi/10^6 cells, >600 Ci mmol^{-1}; Amersham, Arlington Heights, IL) in 1× MEM without L-methionine (Flow Laboratories, McLean, VA). The CIR cells were added to the plate wells and centrifuged for 3 min at 160 *g* to initiate contact with the CHO monolayers. The plates were then incubated at 37°C for 1 h while shaking gently. Following incubation, the wells were washed 10–12 times with assay buffer and the remaining bound cells were solubilized in 1% Triton X-100 for scintillation counting. For the antibody blocking experiments, 10^6 labeled CIR cells were preincubated with 10 μg of the appropriate mAb, or adherent CHO cells in plate wells were preincubated with 2.5 μg of the appropriate mAb. Incubations were carried out for 30 min at 4°C in 0.01% NaN$_3$, 1× PBS (pH 7.4) and 10% FCS ("assay buffer"). The mAbs used in the blocking studies were the anti-TLA mAbs TL.m4 and TL.m9 and the anti-CD8 mAbs 66.2 and 51.1 (28, 30). All mAbs were protein A-Sepharose affinity-column purified.

Purification of Class I and Class Ib Molecules. D^b was purified as described previously (31). TLA was purified from the CIR TL.J transfectant according to a slightly modified version of this protocol (31). Briefly, cells grown in vitro were lysed by nitrogen cavitation and the membrane fraction isolated by centrifugation. Membranes were extracted with 0.5% Triton X-100 in 10 mM Tris (pH 7.2) with 0.15 M NaCl, and the solubilized protein applied to a column prepared from the rat anti-TLA mAb HD168 (a kind gift of Drs. L. Old and E. Stockert, Sloan-Kettering Memorial Cancer Institute, NY) coupled to Sepharose 4B (32). Following loading and washing with extraction buffer, the column was washed with 0.5% deoxycholate (DOC), 10 mM Tris (pH 8.2) with 0.15 M NaCl (buffer 1) to remove nonspecifically bound protein, and TLA then eluted using 0.5% DOC in 15 mM sodium carbonate buffer (pH 11) with 0.15 M NaCl (buffer 2). Eluted fractions were as-

---

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; IEL, intraepithelial lymphocyte; TLA, thymus leukemia antigen.

sayed for total protein content using the BCA Protein Assay (Pierce Chemical Co., Rockford, IL), and for TLA glycoprotein by direct ELISA using mAb HD168 and HRP-goat anti-rat Ig second antibody (CalTag; reference 31).

Cytotoxic T Lymphocyte (CTL) Adhesion to Plate-Bound Class I Major Histocompatibility Complex (MHC) Glycoproteins. Purified TLA and D^b antigens were immobilized in microtiter plate wells by incubation for 1.5 h at 25°C, followed by four washes with 0.15 ml of 1× PBS as previously described (31). The amount of input protein/well is indicated in Fig. 3 B. The wells containing bound class I and class Ib molecules were then blocked by incubation with 2% FCS; control wells received only 2% FCS. $V\beta8^+$ CTL clones C35 and C11, specific for the K^b class I alloantigen (33, 34), were ^{51}Cr-labeled (sodium chromate, 100 μCi/10^7 cells, 250–500 mCi mg^{-1} Cr; Amersham) by incubation for 1 h at 37°C. Labeled cells were then washed and added to wells bearing the indicated class I or class Ib protein in the absence or presence of soluble anti-Vβ8 mAb. The anti-Vβ8 mAb F23.1 (35) was incubated with the clones at a concentration of 0.45 μg/ml for C35 and 0.9 μg/ml for C11 in 100 μl total volume. Plates were then centrifuged at 1200 rpm for 1 min to initiate contact and incubated for 2 h at 37°C, 5% CO$_2$. Cell binding was determined as previously described by adding 0.1 ml ice-cold 1× PBS to each well and placing the plates in an ice water bath for 10 min (33). Unbound cells were removed by pipeting 10 times and discarding the supernatant. Well bottoms were then cut off and the remaining radioactivity was measured.

Cytotoxicity Assay. Primary anti–H-2^d effector CTLs were created by stimulating $\sim$10^8 C57BL/6 splenocytes (H-2^b) with $\sim$10^8 2,000 r irradiated BALB/c splenocytes (H-2^d). Cells were incubated for 5 d in RPMI 1640 supplemented with 10% FCS at a concentration of 2 × 10^7 total splenocytes per T25 tissue culture flask. Both C57BL/6 and BALB/c mice were bred and housed in the UCLA Vivarium, UCLA School of Medicine. ^{51}Cr-loading (100 μCi/4 × 10^6 cells; Amersham) of lightly trypsinized target cells was done for 1 h at 37°C, 5% CO$_2$ with residual ^{51}Cr removed by extensive washing. The targets included L cells transfected with the L^d gene, the L^d/TLA chimeric gene, and the pSV2neo gene, along with DBA/2 strain P815 (H-2^d) mastocytoma cells. Varying amounts of effector cells were incubated with 10^4 labeled targets in 200 μl of medium containing RPMI 1640 supplemented with 10% FCS. Target cell lysis proceeded for 4 h in 96-well U-bottom microtiter plates at 37°C and 5% CO$_2$. Measurement of specific ^{51}Cr release was performed as described previously by removing 100 μl per well and assaying for ^{51}Cr in the removed supernatant (36). For blocking studies, 2 ml of effectors were preincubated with 200 μl of the anti-Lyt2.2 mAb 41-3.48 (3.2 mg/ml, ammonium sulfate precipitation of ascites fluid) for 20 min at 37°C, 5% CO$_2$ and then added to the microtiter plate assay wells (37).

Results

Cell-Cell Adhesion Assay. A full-length T18^d cDNA, encoding TLA serological determinants (38), was inserted into two different mammalian expression cassettes (Fig. 1 A). Transfectants were made using CIR cells that lack HLA-A and HLA-B genes and express only low levels of HLA-C. These nonadherent human B lymphoma cells were used in order to minimize background binding to CD8 due to endogenous human class I gene expression (28, 39, 40). Gene transfer and subsequent antibody staining of numerous clones
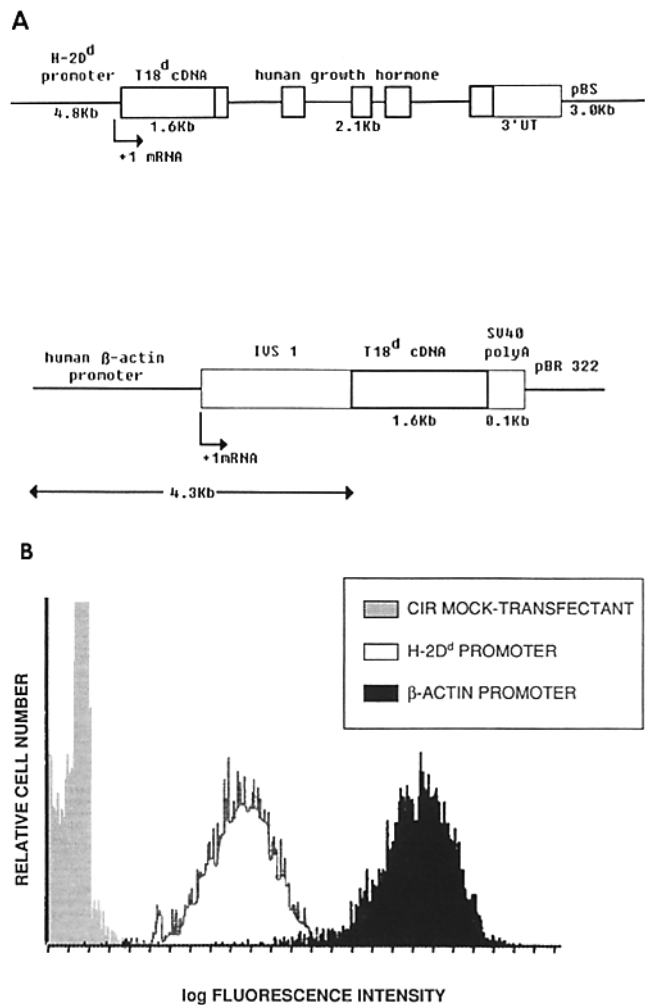
Figure 1. T18^d cDNA expression constructs and flow cytometric analysis of CIR B cell transfectants. (A) Constructs pDT18^{d}hGH (top) and pHβAprT18^d (bottom) are shown. pBS is the cloning vector pBluescript KS+. The constructs are not drawn to scale. (B) Flow cytometry of representative CIR parent and stable transfectant lines. Staining with the mAb TL.m4 is shown (30). The mAb HD168 (32) stained identically to TL.m4, while the mAb TL.m9 (30) did not stain either the parent or transfectants (data not shown).

generated from each type of construct showed that the β-actin-promoter construct yields a higher level of surface TLA expression than does the H-2D^d-promoter construct (Fig. 1 B). Very high levels of gene expression for several other class Ib genes have been achieved using this β-actin-promoter expression cassette in CIR cells (data not shown).

The ability of TLA to bind to CD8 was measured through the adhesion of T18^d transfectants to monolayers of CHO cells that overexpress or that are negative for human CD8α (28). Due to the previously reported weak avidity of HLA-A2.1-mediated CD8 binding (28), data presented here is from a transfectant expressing high levels of TLA surface protein generated using the pHβAprT18^d (β-actin promoter) construct. However, transfectants that express lower levels of surface TLA under the control of the H-2D^d promoter also bind CD8 specifically, though not as tightly, in this assay
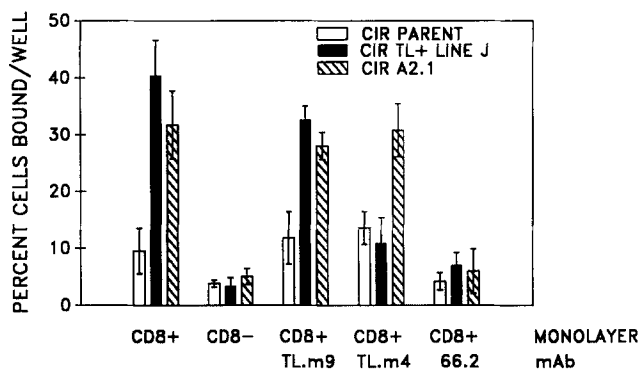
Figure 2. Cell-cell adhesion assay. Binding of radiolabeled C1R cells to CD8α(−) and to human CD8α(+) adherent CHO monolayers is measured; the percentage of input C1R cells retained after extensive washing is presented. Cell lines C1R A2.1, CHO.1 (CD8α-negative), and CHO.4 (CD8α-overexpressing) have been previously described (28, 40). Blocking mAbs TL.m4 and TL.m9 are both TLA-specific, but only TL.m4 recognizes the allele of TLA encoded by $T18^d$ (30). The anti-CD8 mAb 66.2 is characterized elsewhere (49). Error bars represent the standard deviations of triplicate samples.

(data not shown). Both C1R TL.J (TLA-positive) and C1R A2.1 (HLA-A2.1-positive) transfectants bind well to CD8α(+) but not to CD8α(−) CHO monolayers (Fig. 2). Blocking with the mouse anti-TLA mAb TL.m4 reduces the binding of C1R TL.J to a level equivalent to the mock-transfected C1R parent. TL.m9, an isotype-matched mouse mAb that does not recognize the allelic variant of TLA encoded by $T18^d$ (30), does not block binding of C1R TL.J to the CD8α(+) monolayer. Interestingly, the rat anti-TLA mAb HD168 also does not block the binding, although cell surface staining of transfectants that express L^d/TLA chimeric molecules indicates that both HD168 and TL.m4 recognize an epitope encoded at least partially by the $T18^d$ α1 and α2 domains (data not shown). All three TLA-specific mAbs have no effect on binding of either C1R A2.1 or C1R parent cells to the monolayers. Anti-CD8 mAbs 66.2 and 51.1, which were previously shown to block HLA-A2.1-mediated binding to CD8α(+) monolayers (28, 29), also block TLA-mediated binding (Fig. 2 and data not shown). This CD8 mAb blocking data argues that either TLA binds epitopes on human CD8α equivalent to those bound by human class I, as was also previously suggested for mouse transplantation antigens K^b and D^p (29), or that these antibodies sterically hinder more than one binding domain.

The blocking of TLA-CD8α adhesion by TL.m4 mAb is specific but could be due to the downregulation of TLA cell-surface expression upon antibody binding. Previously, downmodulation of surface TLA was shown to be dependent on a number of factors including temperature, with low temperature inhibiting modulation; cellular metabolism, with modulation inhibited by NaN_3; and the cell-type expressing TLA, with downmodulation most readily detected in T cell leukemia lines (41, 42). However, flow cytometric analysis of C1R TL.J transfectants at 4°C and at 37°C in the buffer used for cell-cell binding assays showed no difference in peak

or mean channel fluorescence intensity as compared with TL.m4 staining used to determine high-expressing transfectants (data not shown). Also, when observed in the fluorescence microscope, there was no evidence for either 'capping' or decreased antibody staining under the conditions used in the binding assays compared to conditions normally used to determine cell-surface expression by flow cytometry (data not shown). The lack of TLA modulation could be due to the use of the β-actin promoter in expressing TLA, the presence of NaN_3 in the assay buffer, the inability of exogenously introduced TLA genes in C1R B cells to be inhibited, or perhaps a combination of these and other factors.

Cytotoxic T Lymphocyte (CTL) Adhesion to Plate-Bound Thymus Leukemia Antigen (TLA) Glycoprotein. Recently it was demonstrated that CD8-mediated binding of CTLs to purified mouse class I transplantation antigens is activated via a TCR-mediated signal (33). In the absence of a TCR stimulus, which can be generated by soluble anti-TCR mAbs, CTL binding to purified, immobilized class I protein cannot be detected. In the presence of soluble anti-TCR mAb, a significant fraction of the CTLs bind to any immobilized K- or D-locus protein. Binding is independent of both the CTL's TCR specificity and polymorphic class I determinants, and is blocked by anti-Lyt-2 antibodies (33). Purified TLA was used to determine if it could function as a CD8 ligand in this TCR-activated adhesion system. Unlike the cell-cell adhesion assay, this assay measures interactions with mouse CD8 α/β heterodimers expressed at physiologic levels on T lymphocytes, and allows a semiquantitative comparison of
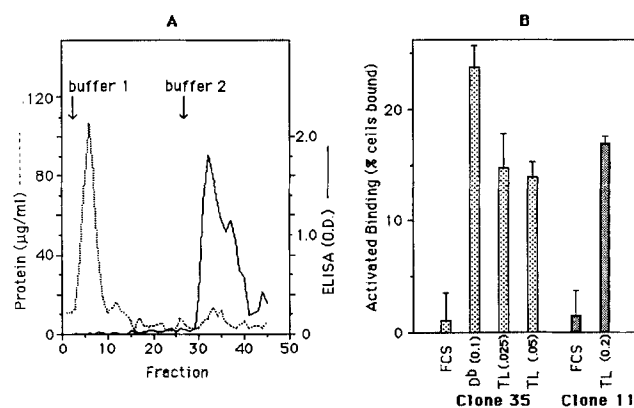

Figure 3. Cloned CTLs are activated by anti-TCR mAb to bind to affinity-purified TLA glycoprotein. (A) Purification of TLA by elution from an anti-TLA HD168 mAb affinity column. Buffer 1, used to remove nonspecifically adherent proteins, and buffer 2, used to specifically elute mAb bound TLA from the column, are described in Materials and Methods section. Eluted fractions were assayed for total protein content (· · · ·) and for TLA (———). Fractions 30 to 40 were pooled yielding an enriched preparation of approximately 25 μg/ml TLA. (B) CTL binding assay. The amount of input class I-enriched total protein per well, in μgs, is expressed in parentheses on the abscissa. Control wells were coated with 2% FCS. Results are expressed as % of cells bound as a result of activation. Activated binding (%) = % cells bound in the presence of anti-TCR mAb − % cells bound in the absence of anti-TCR mAb. Background binding in the absence of soluble F23.1 (35) mAb was 14% for C35 and 4% for C11. Error bars depict the standard deviations of triplicate samples.
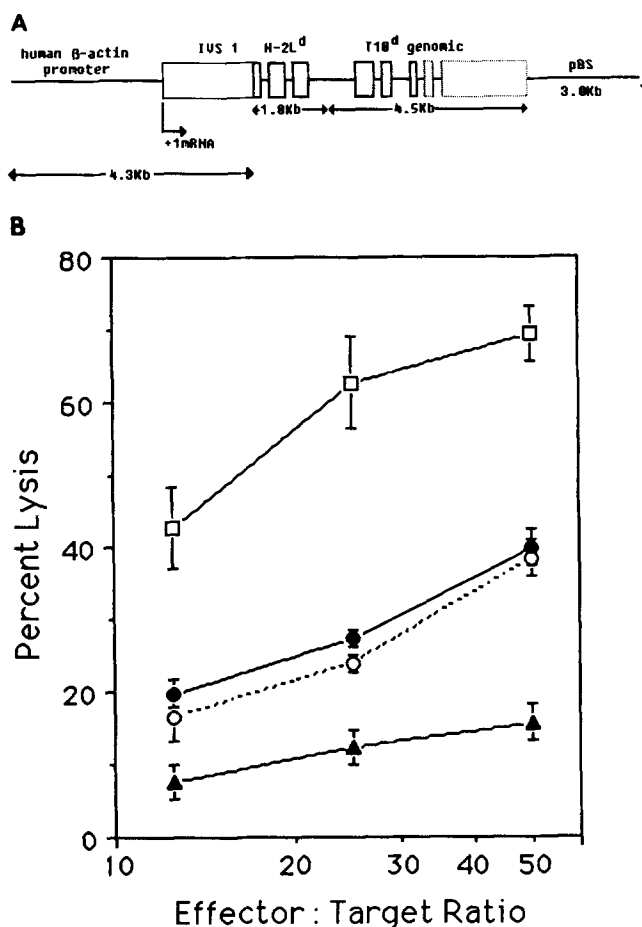
A

B

Effector : Target Ratio

Figure 4. The chimeric L^d/TLA MHC class I molecule supports L^d alloreactive target cell lysis. (A) Construct pHβAprLT18^d is depicted, not to scale. This construct was used to create the L^d/TLA transfectant while the plasmid pL^d neo was used to create the L^d transfectant (not depicted). pBS is the cloning vector pBluescript KS+. (B) Cytotoxicity assay using L cell transfectant and P815 mastocytoma (H-2^d) target cell lines. (▲): mock (pSV2neo) transfectant; (O): L^d transfectant; (●): L^d/TL transfectant; and (□): P815 mastocytoma cells. Harvested supernatants, representing one-half each well's total volume, were assayed for ^{51}Cr content. Results are expressed as the percent lysis as a function of E/T ratio. Percent lysis (%) = % total ^{51}Cr release - % non-specific ^{51}Cr release. Error bars represent the standard deviations of triplicate samples.

TLA binding with binding results obtained for other class I molecules. TLA glycoprotein was purified from the CIR TL.J transfectant using an HD168 mAb affinity column (Fig. 3 A). Contamination with HLA class I molecules is unlikely from CIR cells and, furthermore even if present, previous results suggest that human class I molecules probably would interact poorly with CD8 expressed on mouse CTLs (43). The purified TLA was directly immobilized on plastic microtiter wells. As previously demonstrated for K and D class I proteins (31), the immobilized TLA retained serologic determinants (Fig. 3 A) and remained associated with human β_2-microglobulin based on ELISA using the anti-β_2-microglobulin mAb BBM.1 (data not shown). Two different Vβ8$^+$, K^b alloantigen-specific CTL clones were examined for binding. In both cases, purified TLA was observed to function as a ligand for TCR-activated adhesion following stimulation with soluble anti-Vβ8 mAb F23.1 (Fig. 3 B). The percent of T cells bound was in the same range as that obtained using D- (Fig. 3 B) and K-locus encoded class I proteins as ligands (reference 33 and M. Mescher, unpublished results) at approximately equivalent protein concentrations used to coat the wells. These data suggest that the affinity of the TLA-CD8 interaction is similar to the affinity of K or D for CD8.

CD8-Dependent Cytotoxicity. To determine if the TLA-CD8 interaction can facilitate a CD8-dependent immune response, a chimeric class I gene was constructed with the peptide antigen-binding α1 and α2 domains encoded by L^d, and the α3, transmembrane, and cytoplasmic domains encoded by T18^d (Fig. 4 A). L^d was chosen because primary, alloreactive L^d-specific CTLs are CD8 dependent and blockade with anti-L^d or anti-Lyt2 mAbs (44). As class I-CD8 binding is mediated by the α3 domain by (3, 29), CD8 interactions with the chimeric molecule should depend on TLA sequences.

Before testing the L^d/TLA molecule as an alloantigen for primary CTLs, its ability to bind CD8 was assessed. CIR transfectants that express the L^d/TLA chimeric class I molecule bind human CD8α(+) CHO monolayers as efficiently as HLA-A2.1-positive and TLA-positive transfectants in the cell-cell adhesion assay (data not shown).

Table 1. *Blocking of Primary Cytotoxic T Lymphocyte (CTL) Lysis with Anti-Lyt-2 mAb*

Cell type	Percent lysis					
	50:1*	50:1*‡		25:1*	25:1*‡	
Mock (pSV2neo)	15.7 ± 2.5	8.8 ± 0.8	(43.9)	12.4 ± 2.4	3.9 ± 1.1	(68.5)
L^d	38.5 ± 2.6	12.4 ± 1.8	(67.8)	23.9 ± 1.1	9.5 ± 0.4	(60.2)
L^d/TLA	39.9 ± 2.5	16.0 ± 3.8	(59.9)	27.4 ± 0.7	11.4 ± 0.3	(58.4)
P815 (H-2^d)	69.3 ± 3.9	31.8 ± 1.9	(54.1)	62.7 ± 6.4	18.3 ± 4.1	(70.1)

* Effector/target ratio.
‡ % lysis with mAb 41-3.48 incubation.
Parentheses indicate % inhibition by mAb blocking.
mAB 41-3.48 blocking of anti-H-2^d primary CTL lysis of four L cell target lines. The % inhibition is calculated as [(% lysis unblocked - % lysis blocked)/ % lysis unblocked] × 100.

Table 2. *Minimal Major Histocompatibility Complex (MHC) α3-Domain CD8 Binding Sequence*

	Cluster 1 (direct binding)							Cluster 2		Cluster 3	
	(223	224	225	226	227	228	229)	(233	235)	(245	247)
HLA-A2.1	D	Q	T	Q	D	T	E	T	P	A	V
T18^d	E	L	I	Q	D	T	E	T	P	A	V
Q7^d	E	L	T	Q	D	T	E	T	P	A	V
H-2K^b	E	L	I	Q	D	M	E	T	P	A	V
Consensus*	Q/E	Q/L	T(I)	Q	D	T/M	E	T	P	A	V

The amino acid positions listed are those that comprise the minimal α3 domain binding site as determined for HLA-A2.1 by Salter et al. (3). The amino acid at each position for four class I molecules and the consensus sequence are denoted by the standard single-letter code and were obtained from a compilation by Klein and Figueroa (18). *Consensus denotes a sequence based upon 23 class I human and mouse α3 domains with the amino acid before the slash mark at each position found in the human and that after the slash mark found in the mouse. The parentheses at position 225 indicates that a minority of both human and mouse class I molecules have I instead of T.

Primary anti–H-2^d CTLs were generated by in vitro culture of H-2^b splenocytes with irradiated BALB/c (H-2^d) stimulators and tested for H-2^d-specific cytotoxicity using P815 (H-2^d) mastocytoma targets (Fig. 4 *B*). A small amount of cytolysis was observed against mock-transfected L cell targets, perhaps due to cross-reactivity of primed effectors that can also recognize H-2^k targets. L^d-specific cytotoxicity was measured using L cell (H-2^k) transfectant targets that express equivalent amounts of either L^d or the L^d/TLA chimeric molecule. Transfectants expressing L^d/TLA are lysed as efficiently as those expressing L^d at all of the effector:target ratios tested (Fig. 4 *B*). Furthermore, the level of inhibition of target lysis by incubation with the anti-CD8 mAb 41-3.48 is similar if the target cells express either the L^d/TLA molecule or the L^d alloantigen (Table 1). In sum, these results suggest that the TLA α3 domain is capable of delivering the appropriate signal(s) through CD8 binding for a CTL response, and that the *T18^d*-encoded α3, transmembrane, and cytoplasmic domains do not impede the normal functioning of a class I molecule during this alloantigenic response.

Discussion

If the hypothesis that class Ib molecules present antigens were correct, it should be possible to demonstrate that these proteins carry out functions similar to those of the class I transplantation antigens, including appropriate trafficking through the cell, loading of peptide antigen, and interaction with the TCR and CD8. It is particularly necessary to establish this for the TLA molecule, which has not been observed to participate in cell-mediated immune responses. In this report, it is demonstrated that TLA binds to human and mouse CD8, and that a chimeric molecule containing the COOH-terminal portion of TLA can support a CD8-dependent recognition of the L^d alloantigen.

Previously reported results from our laboratory showed that TLA is highly expressed by both epithelial cells and in-

traepithelial lymphocytes (IELs) in the mouse small intestine (20). IELs are greater than 80% CD8 single-positive and approximately 50% of them express the γδ TCR (45–47). The ability of TLA to bind effectively to CD8 supports the hypothesis that it may be involved in antigen presentation to either TCR γδ(+) and/or αβ(+) IELs, although a critical test of this hypothesis will require the detection of antigen-specific TLA-restricted IELs. TLA is highly expressed by immature cortical thymocytes, most of which also express CD8. It is therefore possible that TLA-CD8 interactions occur in the thymus, and these interactions may have important consequences for T cell differentiation.

Using site-directed mutagenesis, a minimal consensus sequence encompassing at least 11 amino acids in the human MHC class I α3 domain has been defined that is required for binding to transfectants that express high levels of human CD8α (reference 3 and Table 2). The α3 domain has a conformation similar to an immunoglobulin constant region homology unit, and most of the critical amino acids for CD8 binding, including positions 223-229, 233, and 235, are on an external-facing loop connecting two antiparallel β strands (3). Interestingly, many class Ib molecules possess this α3 domain minimal sequence, or sequences that deviate from it only slightly. For example, compared to HLA-A2.1, TLA contains conservative amino acid substitutions at positions 223-225 and complete identity at positions 226-229, 233, 235, 245, and 247. TLA differs from H-2K^b only at position 228 in this minimal sequence. In fact, a compilation of 23 mouse and human class I α3 domain sequences suggests that these 11 amino acids are extremely well conserved (reference 18 and see the consensus sequence of Table 2).

The relatively high degree of sequence conservation between different MHC class I α3 binding domains suggests that other class Ib proteins in addition to TLA may also bind CD8. However, it is probably not possible to infer CD8 binding from the presence of the conserved, minimal α3 domain sequence alone. The Q7^d-encoded α3 domain retains the minimal consensus sequence for CD8 binding (Table 2).

Yet, this α3 domain is incapable of supporting a primary CTL response directed at a $L^d/Q7^d(\alpha 3)/L^d$ chimeric molecule, in which sequences other than α3 are encoded by L^d (48). Secondary CTL responses directed at this chimeric molecule, which are less CD8 dependent, can be obtained. These data suggest that the $Q7^d\alpha 3$ domain is incapable of binding CD8 in a manner that allows a primary alloreactive response. Therefore, in the absence of experimental evidence, it cannot be assumed that any class Ib molecule other than TLA binds to CD8.

The hypothesis that class Ib molecules present antigens to TCR γδ-positive lymphocytes does not require that all of these molecules interact with CD8. Most γδ-positive T cells, including the few known to recognize class Ib molecules (5, 12, 13), are CD4/CD8 double-negative. These double-negative γδ T cells do not require CD8 as a co-receptor, despite the apparent evolutionary conservation of the minimal CD8 binding site in class Ib molecules. In contrast, nearly all of the TCR γδ-positive IELs of the mouse small intestine express CD8, which could act as a co-receptor if the TCRs expressed by these cells recognize an antigen presented by the TLA molecule.

References

1. Hood, L., M. Steinmetz, and B. Malissen. 1983. Genes of the major histocompatibility complex of the mouse. *Annu. Rev. Immunol.* 1:529.

2. 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.

3. 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 of site for the T-cell co-receptor CD8 on the α3 domain of HLA-A2. *Nature (Lond.).* 345:41.

4. Schwartz, R.A. 1990. A cell culture model for T lymphocyte clonal analysis. *Science (Wash. DC).* 248:1349.

5. Bonneville, M., K. Ito, E.G. Krecko, S. Itohara, D. Kappes, I. Ishida, O. Kanagawa, C.A. Janeway, D.B. Murphy, and S. Tonegawa. 1989. Recognition of a self major histocompatibility complex TL region product by γδ T cell receptors. *Proc. Natl. Acad. Sci. USA.* 86:5928.

6. Stroynowski, I. 1990. Molecules related to class I major histocompatibility complex antigens. *Annu. Rev. Immunol.* 8:501.

7. Flaherty, L. 1980. Tla-region antigens. *In* The Role of the Major Histocompatibility Complex in Immunology. M.E. Dorf, editor. Garland Press, New York, NY. pp. 33–57.

8. Rothenberg, E., and J.P. Lugo. 1985. Differentiation and cell-division in the mammalian thymus. *Develop. Biol.* 112:1.

9. Rogers, J.H. 1985. Mouse histocompatibility-related genes are not conserved in other mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:749.

10. Porcelli, S., M.B. Brenner, J.L. Greenstein, S.P. Balk, C.

Terhorst, and P.A. Bleicher. 1989. Recognition of cluster of differentiation 1 antigens by human CD4⁻ CD8⁻ cytolytic T lymphocytes. *Nature (Lond.).* 341:447.

11. Vidovic, D., M. Roglic, K. McKune, S. Guerder, C. MacKay, and Z. Dembic. 1989. Qa-1 restricted recognition of foreign antigen by a γδ T-cell hybridoma. *Nature (Lond.).* 340:646.

12. Janeway, C.A. 1988. Frontiers of the immune system. *Nature (Lond.).* 333:804.

13. Strominger, J. 1989. The γδ T-cell receptor and class Ib MHC-related proteins: Enigmatic molecules of immune recognition. *Cell.* 57:895.

14. Klein, J.C., C. Benoist, C.S. David, P. Demant, K. Fischer-Lindahl, L. Flaherty, R.A. Flavell, U. Hammerling, L.E. Hood, S.W. Hunt, III, P.P. Jones, P. Kourilsky, H.O. McDevitt, D. Meruelo, D.B. Murphy, S.G. Nathenson, D.H. Sachs, M. Steinmetz, S. Tonegawa, E.K. Wakeland, and E.H. Weiss. 1990. Revised nomenclature of mouse H-2 genes. *Immunogenetics.* 32:147.

15. Boyse, E.A. 1984. The biology of Tla. *Cell.* 38:1.

16. 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.

17. 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.

18. Klein, J., and F. Figueroa. 1986. The evolution of class I MHC genes. *Immunol. Today.* 7:41.

19. Jenkins, R.N., C.J. Aldrich, N.F. Landolfi, and R.R. Rich. 1985. Correlation of Qa-1 determinants by antisera and by cyto-

toxic T lymphocytes. *Immunogenetics.* 21:215.

20. 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.* 87:9727.

21. Korber, B., L. Hood, and I. Stroynowski. 1987. Regulation of murine class I genes by interferons is controlled by regions and located both 5' and 3' to the transcription initiation site. *Proc. Natl. Acad. Sci. USA.* 84:3380.

22. 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.

23. Seeburg, P.H. 1982. The human growth hormone gene family: Nucleotide sequences show recent divergence and predict a new polypeptide hormone. *DNA.* 1:239.

24. Moore, K.W., B.T. Shen, Y.H. Sun, K.A. Eakle, and L. Hood. 1982. DNA sequence of a gene encoding a BALB/c mouse L^d transplantation antigen. *Science (Wash. DC).* 215:679.

25. Southern, P.J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Molec. Appl. Genet.* 1:327.

26. 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.

27. Stroynowski, I., A. Orn, R.S. Goodenow, M. McMillan, J. Forman, P.R. Brayton, J. Frelinger, and L. Hood. 1984. Cytotoxic T lymphocytes recognize determinants on the BALB/c-H-2L^d molecule controlled by α1 and α2 but not α3 external domains. *Immunogenetics.* 20:141.

28. 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.

29. Salter, R.D., A.M. Norment, B.P. Chen, C. Clayberger, A.M. Krensky, D.R. Littman, and P. Parham. 1989. Polymorphism in the α3 domain of HLA-A molecules affects binding to CD8. *Nature (Lond.).* 338:345.

30. 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.

31. Kane, K.P., P. Champoux, and M.F. Mescher. 1989. Solid-phase binding of class I and II MHC proteins: Immunoassay and T cell recognition. *Molec. Immunol.* 26:759.

32. Obata, Y.T., Y.T. Chen, E. Stockert, and L.J. Old. 1985. Structural analysis of TL genes of the mouse. *Proc. Natl. Acad. Sci. USA.* 82:5475.

33. 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.).* 246:187.

34. Kane, K.P., L.P. Sherman, and M.F. Mescher. 1989. Molecular interactions required for triggering alloantigen-specific cytolytic T lymphocytes. *J. Immunol.* 142:4153.

35. Staerz, U., H. Rammensee, J. Benedetto, and M.J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptors. *J. Immunol.* 134:3994.

36. Song, E.S., R. Linsk, C.A. Olson, M. McMillan, and R.S. Goodenow. 1988. Allospecific cytotoxic T lymphocytes recognize an H-2 peptide in the context of a murine major histocompatibility complex class I molecule. *Proc. Natl. Acad. Sci. USA.* 85:1927.

37. Shen, F.-W. 1981. Monoclonal antibodies to mouse lymphocyte differentiation alloantigens. *In* Monoclonal Antibodies and T-Cell Hybridomas: Perspectives and Technical Advances. G.J. Hammerling, U. Hammerling, and J.F. Kearney, editors. Elsevier/North-Holland Press, New York, NY. pp. 25–31.

38. Chen, Y.T., Y. Obata, E. Stockert, T. Takahashi, and L.J. Old. 1987. Tla-region genes and their products. *Immunol. Res.* 6:30.

39. 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.

40. Hogan, K.T., C. Clayberger, E.J. Bernhard, S.F. Walk, J.P. Ridge, P. Parham, A.M. Krensky, and V.H. Engelhard. 1988. Identification by site-directed mutagenesis of amino acid residues contributing to serologic and CTL-defined epitope differences between HLA-A2.1 and HLA-A2.3. *J. Immunol.* 141:2519.

41. Lamm, M.E., E.A. Boyse, L.J. Old, B. Lisowska-Bernstein, and E. Stockert. 1968. Modulation of TL (thymus leukemia) antigens by Fab-fragments of TL antibody. *J. Immunol.* 101:99.

42. Chorney, M.J., H. Mashimo, Y. Bushkin, and S.G. Nathenson. 1989. Characterization of the thymus leukemia (TL) product encoded by the BALB/c T3^c gene by DNA-mediated gene transfer: Comparison of the T13^c product and BALB/c leukemia TL. *J. Immunol.* 143:3762.

43. Kalinke, U., B. Arnold, and G.J. Hammerling. 1990. Strong xenogeneic HLA response in transgenic mice after introducing an α3 domain into HLA-B27. *Nature (Lond.).* 348:642.

44. Connelly, J.M., T.A. Potter, E. Wormstall, and T.H. Hansen. 1988. The lyt-2 molecule recognizes residues in the class I α3 domain in allogeneic cytotoxic T cell responses. *J. Exp. Med.* 168:325.

45. Petit, A., P.B. Ernst, A.D. Befus, D.A. Clark, K.L. Rosenthal, T. Ishizaka, and J. Bienenstock. 1985. Murine intestinal intraepithelial lymphocytes I. Relationship of a novel Thy-1⁻, lyt-1⁻, lyt-2⁺ granulated subpopulation to natural killer cells and mast cells. *Eur. J. Immunol.* 15:211.

46. Goodman, T., and L. Lefrancois. 1988. Expression of the γδ T cell receptor on intestinal CD8⁺ intraepithelial lymphocytes. *Nature (Lond.).* 333:855.

47. Bonneville, M., C.A. Janeway, K. Ito, W. Haas, I. Ishida, N. Nakanishi, and S. Tonegawa. 1988. Intestinal intraepithelial lymphocytes are a distinct set of γδ T cells. *Nature (Lond.).* 336:479.

48. Aldrich, C.J., L.C. Lowen, D. Mann, M. Nishimura, L. Hood, I. Stroynowski, and J. Forman. 1991. The Q7 α3 domain alters T cell recognition of class I antigens. *J. Immunol.* 146:3082.

49. Martin, P.J., J.A. Ledbetter, E.A. Clark, P.G. Beatty, and J.A. Hansen. 1984. Epitope mapping of the human surface suppressor/cytotoxic T cell molecule Tp32. *J. Immunol.* 132:759.