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Functional Analysis of Amino Acid Residues Encompassing and Surrounding Two Neighboring H-2D^b-Restricted Cytotoxic T-Lymphocyte Epitopes in Simian Virus 40 Tumor Antigen

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Simian virus 40 tumor (T) antigen contains three H-2D^b- and one H-2K^b-restricted cytotoxic T lymphocyte (CTL) epitopes (sites). Two of the H-2D^b-restricted CTL epitopes, I and II/III, are separated by 7 amino acids in the amino-terminal one third of T antigen. In this study, we determine if the amino acids separating these two H-2D^b-restricted CTL epitopes are dispensable for efficient processing and presentation. In addition, the importance of amino acid residues lying within and flanking the H-2D^b-restricted epitopes I and II/III for efficient processing, presentation, and recognition by site-specific CTL clones was determined by using T-antigen mutants containing single-amino-acid substitutions between residues 200 and 239. Using synthetic peptides in CTL lysis and major histocompatibility complex class I stabilization assays, CTL recognition site I has been redefined to include residues 206 to 215. Substitutions in amino acids flanking either site I or site II/III did not affect recognition by any of the T-antigen-specific CTL clones. Additionally, the removal of the 7 residues separating site I and site II/III did not affect CTL recognition, thus demonstrating that these two epitopes when arranged in tandem in the native T antigen can be efficiently processed and presented to CTL clones. Differences in fine specificities of two CTL clones which recognize the same epitope (Y-1 and K-11 for site I and Y-2 and Y-3 for site II/III) have been used in conjunction with synthetic peptide variants to assign roles for residues within epitopes I and II/III with respect to TCR recognition and/or peptide-major histocompatibility complex association.

Major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) recognize viral and tumor antigens in the form of peptides, usually 8 to 10 amino acids in length, which are processed in the cytosol and then translocated into the endoplasmic reticulum by the TAP 1/TAP 2 transporter (32, 56, 79). The peptides associate with nascent MHC class I molecules and β2-microglobulin to form a trimolecular complex which is transported to the cell surface. The peptide-MHC complex is then recognized by the T-cell receptor (TCR) in an MHC-restricted manner (32, 79, 81). The magnitude of the T-cell immune response to CTL epitopes presented on MHC class I antigens is dependent on the availability of peptides generated during processing and transport into the endoplasmic reticulum, the affinity for the MHC molecules, and the affinity of TCR for the MHC-peptide complex.

Processing events can be influenced by the amino acid residues which surround a CTL epitope. It has been demonstrated that an H-2L^d-restricted CTL epitope, encoded in the murine cytomegalovirus genome, transferred to the amino terminus of hepatitis B virus core protein, and expressed in vaccinia virus, was not processed efficiently (20). Translocation of this epitope to other locations in the hepatitis B virus core protein did not affect its processing and presentation (20). Another example of the role of flanking residues was demonstrated by using the H-2K^d-restricted influenza virus nucleoprotein epitope defined by residues 147 through 155. The addition of two residues (Thr-157 and Gly-158) to the 147-through-155 epitope (without Arg-156; 147-158/R$^-$) greatly diminished recognition of the epitope expressed in cells as a vaccinia virus minigene construct (24). However, the 147-158/R$^-$ sequence was efficiently recognized when expressed from within the full-length nucleoprotein (missing Arg-156) or when target cells were coinfected with vaccinia constructs expressing a 147-158/R$^-$ minigene and a dipeptidyl carboxypeptidase angiotensin-converting enzyme (23). Hahn and coworkers (36), by saturation mutagenesis of residues surrounding two overlapping H-2K^d-restricted influenza virus hemagglutinin epitopes (residues 204 through 213 and 210 through 219), found that a substitution of glycine for valine at position 214 (Val-214→Gly) abolished the recognition of the epitope spanning residues 204 through 213 by a specific CTL clone. Substitutions at other positions outside of the 204-through-213 epitope did not affect CTL recognition. In another study, Hahn et al. (35) demonstrated that translocation of influenza virus hemagglutinin residues 202 through 221 (which include these two overlapping H-2K^d-restricted epitopes) to six different locations in a fragment of influenza virus hemagglutinin did not affect the processing and presentation of either epitope. As yet, no general rules have been identified that will predict whether flanking residues will noticeably affect processing and presentation of CTL epitopes from proteins expressed as self or introduced as a result of virus infection.

In order to explore whether the flanking residues surrounding CTL epitopes affect processing and presentation of CTL epitopes, we carried out mutagenesis of DNA encoding simian virus 40 (SV40) T-antigen residues 200 through 239. This region of T antigen contains two adjacent H-2D^b-restricted CTL epitopes separated by 7 amino acids. SV40 T antigen, a 708-amino-acid transforming DNA virus oncoprotein, is unusual in

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that it contains at least three (I, II/III, and V) H-2D^b-restricted epitopes (19, 64, 65). Epitope I was defined by residues 207 through 215 (AINNYAQKL), epitope II/III was defined by residues 223 through 231 (CKGVNKEYL), and epitope V was defined by residues 489 through 497 (QGINNLDNL) (19). The CTL recognition sites I, II/III, and V are recognized by CTL clones Y-1 and K11, Y-2 and Y-3, and Y-5 respectively (7, 19, 64, 65). Generating amino acid substitutions within the CTL recognition epitopes would aid in defining the role of individual residues in presentation to and recognition by the CTL clones. Previous studies (18, 45) identified only a few residues in the T-antigen epitopes I and II/III that interact with the TCR. The SV40 T-antigen system has the advantage of having multiple H-2D^b-restricted CTL epitopes in the protein which provide an internal control for recognition of CTL epitopes processed from mutant T antigens. Additionally, the use of two sets of independently derived CTL clones (Y-1 and K11, specific for site I, and Y-2 and Y-3, specific for site II/III) which differentially recognized each CTL site allowed us to define residues within the epitopes that interact with the TCR. Our results show that amino acid substitutions only within epitopes I and II/III of SV40 T antigen influenced CTL recognition and that changes in flanking residues surrounding these two sites did not influence presentation of these epitopes to CTL clones.

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides used were synthesized by the Macromolecular Core Facility of the Pennsylvania State University College of Medicine. Two types of oligonucleotides were used; one contained random nucleotide substitutions and the other contained specific nucleotide substitutions. The random oligonucleotide pools used were 5′-CTT ACT ccA caC agG caT agA gtG tcT gcT atT aaT AAC TAT-3′ and 5′-ATT AAT aaC taT gcT caA aaA ttG tgT acC ttT agC TTT TTA-3′. Lowercase letters indicate nucleotide positions of potential variation. When synthesizing these oligonucleotides, residues occupying the positions indicated by lowercase letters were added from a nucleotide pool which contained 92.5% of the wild-type nucleotide and 2.5% of each other nucleotide. Synthesis of oligonucleotides in this manner was expected to yield a population of oligonucleotides in which 22% contained no nucleotide substitution, 34% contained one substitution, and 25% contained two nucleotide substitutions at any position indicated by a lowercase letter. Mutagenesis with the random oligonucleotide pools yielded nine mutant plasmids.

Oligonucleotide site-directed mutagenesis. Mutation of the DNA encoding SV40 T antigen was accomplished by three methods. These methods included a modified Kunkel procedure (International Biotechnologies Inc., New Haven, Conn.), the Amersham oligonucleotide-directed in vitro mutagenesis system, version 2 (Amersham Corporation, Arlington Heights, Ill.), and Promega Altered Sites mutagenesis system (Promega Corporation, Madison, Wis.). Plasmids pSelect/Δ200-206, pSelect/Δ216-222, pSelect/206SP (in which T antigen carries a 206-S→P mutation), pSelect/211YA, pSelect/215LV, pSelect/232ML, pSelect/232MQ, pSelect/231LA:232ML, and pSelect/231LA were obtained by oligonucleotide-directed site-specific mutagenesis, using the Altered Sites mutagenesis system (Promega). Nine mutant T-antigen-containing plasmids were obtained by mutagenesis with an oligonucleotide pool which contained random nucleotide substitutions in a region specific for T-antigen residues of interest (see above), using either the Kunkel or Amersham procedures. Mutants were identified and confirmed by DNA sequencing (Sequenase version 2; United States Biochemicals Corp., Cleveland, Ohio). Other plasmids encoding large T-antigen mutants containing amino acid substitutions have been reported previously (18, 45, 58, 61, 62). The mutant T-antigen plasmids used in this study are listed in Table 1.

Generation of mutant SV40 T-antigen-expressing cell lines. The T-antigen-encoding plasmids were transfected into primary C57BL/6 (H-2^b) mouse embryo fibroblasts or adult kidney cells by the calcium phosphate precipitation method (34) as modified by Wigler et al. (77) and Tevethia (68). Individual foci were expanded into permanent cell lines. Cell lines were tested for the expression of T antigen by indirect immunofluorescence with the monoclonal antibody PAb 901, which recognizes an epitope in the carboxy terminus of T antigen (70). Genomic DNA isolated from the majority of cell lines was tested for the presence of the correct T-antigen coding sequence by PCR amplification and sequence analysis of the region encoding T-antigen residues 200 through 239 (45).

Maintenance of SV40-transformed cell lines. The cell lines used in this study are listed in Table 1. The cell line B6/K-0 was generated by transfection of primary C57BL/6 kidney cells with the wild-type T-antigen-expressing plasmid pPVU-0 (66). B6/Scl-7 is a spontaneously immortalized B6/MEF cell line which does not express any T antigen (70). B6/K-1,4,5 was generated by sequentially coculturing the B6/K-0 cell line with CTL clones Y-1, Y-4, and Y-5 (67). The B6/K-1,4,5 cell line has lost the expression of all the SV40 T-antigen CTL epitopes recognized by our H-2^b-specific CTL clones. B6/K-3,1,4 was generated by sequentially coculturing the B6/K-0 cell line with CTL clones Y-3, Y-1, and Y-4 and maintains site V expression (64, 66). All of the cell lines used in this study were maintained in Dulbecco modified Eagle medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 0.075% (wt/vol) sodium bicarbonate (19, 65, 69).

Maintenance of cytotoxic T lymphocytes. The generation and maintenance of the CTL clones Y-1, Y-2, Y-3, Y-5, and K-11 have been described previously (11, 64, 65). Briefly, CTL were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 10 mM HEPES, 5×10^{-5} M β-mercaptoethanol, and 25 μg of pyruvic acid per ml. CTL clones were stimulated with gamma-irradiated (10,000 rads) T-antigen-expressing cells (B6/WT-19 for all clones but Y-5; clone Y-5 was stimulated with B6/K-3,1,4 cells). CTL clones Y-1, Y-2, Y-3, and Y-5 were maintained in 7.5 U of human recombinant interleukin-2 (kindly provided by Amgen, Thousand Oaks, Calif.) per ml. The CTL clone K-11 was maintained in RPMI medium supplemented with 10% (vol/vol) rat T-Stim culture supplement (Collaborative Biomedical, Bedford, Mass.) and 5% (vol/vol) 1 M methyl α-D-manno-pyranoside (Sigma Chemical, St. Louis, Mo.).

Cytotoxicity assay. The ability of the CTL clones to recognize and lyse target cells was determined by the ^{51}Cr release assay, according to the procedure described previously (19, 64, 65, 69). In brief, target cells treated with 30 to 40 units of gamma interferon (kindly provided by H. M. Shepard, Genentech Inc., San Francisco, Calif.) per ml were labeled with ^{51}Cr at 37°C for 1 h. The target cells were washed three times to remove unincorporated ^{51}Cr. The target cells (1×10^4) were then dispensed into the wells of a V-bottom 96-well plate (Costar, Cambridge, Mass.) and were reacted with CTL clones at various effector-to-target cell ratios. After incubation at 37°C for 4 to 5 h, the supernatant was removed and the amount of radioactivity released was determined by counting in a Gamma 8500 counter (Beckman Instruments, Fullerton, Calif.). Percent specific lysis was calculated as [(Exp. − Spon.)/(Max. − Spon.)] × 100. Percent wild-type lysis was calculated as [(%spec. lysis of mutant cell line)/(%spec. lysis of B6/K-0)] × 100. The negative-control cell line B6/K-1,4,5 exhibited less than 5% specific lysis in all experiments. Cells expressing the wild-type T antigen were included in all experiments.

Synthetic peptides. Peptides were synthesized by the Pennsylvania State University College of Medicine Macromolecular Core Facility as previously described (18). The composition of peptides LT205-215 and LT207-215 was confirmed by the Pico Tag amino acid analysis system (Waters, a division of MilliGen). The sequence of selected peptides was confirmed by an automated sequencer (477A Applied Biosystem) coupled to a 120-A amino acid analyzer. The peptides were solubilized in dimethyl sulfoxide and adjusted to the proper concentration with RPMI medium. The peptides were used to pulse target cells for use in the cytotoxicity assay as previously described (19) or in the MHC class I stabilization assay (see below).

Western blot analysis. Steady-state levels of T antigen in various cell lines were determined by Western blot (immunoblot) analysis essentially as previously described (43).

MHC class I molecule stabilization assay. The relative binding efficiencies of the synthetic peptides for the MHC class I molecule were determined by measuring the levels of expression of MHC class I molecules on the surface of the cell line RMA/s. The RMA/s cell line is a Rauscher virus-induced T-cell lymphoma which has a low surface expression of MHC class I molecules (42, 46). Increased expression of surface MHC class I molecules can be induced by incubation of RMA/s cells in the presence of a haplotype-specific peptide or at low temperatures (47, 72, 73). RMA/s cells (3×10^5 to 6×10^5) were incubated in 1 ml of supplemented RPMI medium with various concentrations of peptides for 14 to 24 h as previously described (31, 63). Expression of H-2D^b molecules was determined by fluorescence-activated cell sorter (FACS) analysis. Cells were washed twice in phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide (FACS buffer). Cells were then incubated for 45 min on ice with 0.1 ml of the anti-D^b monoclonal antibody 28-14-8 (55). After being washed with FACS buffer, the cells were incubated with 0.1 ml of fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 45 min on ice. The cells were washed, fixed in 1% paraformaldehyde in PBS, and analyzed for the level of MHC class I molecule surface expression (31, 63).

RESULTS

Expression of mutant SV40 T antigen in primary B6/MEF and B6 kidney cells. Amino acid substitutions of residues 200 through 239 that encompass SV40 T-antigen sites I and II/III were obtained by site-directed mutagenesis of the DNA that encodes this region of T antigen. The mutant T antigens were

TABLE 1. Cell lines used in this study

Cell line	Plasmid	Alteration within SV40 T antigen		
		Position	Amino acid substitution	Reference
B6/T200PQ	pGC1ESVpro-200PQ	200	Pro to Gln	This study
B6/T201HY	pGC1ESVpro-201HY	201	His to Tyr	This study
B6/T202RK	pBS-SV40/202RK	202	Arg to Lys	61
B6/T202RN:209NH	pGC1ESVpro-202RN:209NH	202; 209	Arg to Asn; Asn to His	This study
B6/T203NH	pBS-SV40/203NH	203	Asn to His	61
B6/T204RK	pBS-SV40/204RK	204	Arg to Lys	61
B6/T205VA	pGC1ESVpro-205VA	205	Val to Ala	This study
B6/T205VL	pBS-SV40/205VL	205	Val to Leu	61
B6/T206SP	pSelect/206SP	206	Ser to Pro	This study
B6/T206ST	pBS-SV40/206ST	206	Set to Thr	61
B6/T207AG	pBS-SV40/207AG	207	Ala to Gly	61
B6/T207AS	pGC1ESVpro-207AS	207	Ala to Ser	This study
B6/p3257	p3257	207	Ala to Val	18
B6/T208IL	pBS-SV40/208IL	208	Ile to Leu	61
B6/T209NS	pBS-SV40/209NS	209	Asn to Ser	61
B6/T209NT	pGC1ESVpro-209NT	209	Asn to Thr	This study
B6/T210NY	pGC1ESVpro-210NY	210	Asn to Tyr	This study
B6/T211YA	pSelect/211YA	211	Tyr to Ala	This study
B6/T212AG	pBS-SV40/212AG	212	Ala to Gly	61
B6/T213QH	pBS-SV40/213QH	213	Gln to His	61
B6/pML9D	pML9D (SRV9D)	214	Arg to Lys	18
B6/T215LV	pSelect/215LV	215	Leu to Val	This study
B6/T216CG	pBS-SV40/216CG	216	Cys to Gly	61
B6/T217TP	pGC1ESVpro-217TP	217	Thr to Pro	This study
B6/T218FY	pBS-SV40/218FY	218	Phe to Tyr	61
B6/T219SC	pGC1ESVpro-219SC	219	Ser to Cys	This study
B6/T220FY	pBS-SV40/220FY	220	Phe to Tyr	61
B6/T221LI	pBS-SV40/221LI	221	Leu to Ile	61
B6/T222IL	pBS-SV40/222IL	222	Ile to Leu	61
B6/T223CS	pBS-SV40/223CS	223	Cys to Ser	61
B6/pCGA	pPVU-0-CGA	224	Lys to Arg	18
B6/T225GA	pBS-SV40/225GA	225	Gly to Ala	61
B6/T226VA	pBS-SV40/226VA	226	Val to Ala	61
B6/T227NT	pBS-SV40/227NT	227	Asn to Thr	61
B6/T228(lys→asn)	pGC1ESVpro-228KN	228	Lys to Asn	45
B6/T229ED	pBS-SV40/229ED	229	Glu to Asp	61
B6/T230(tyr→phe)	pGC1ESVpro-230YF	230	Tyr to Phe	45
B6/T231LA	pSelect/231LA	231	Leu to Ala	This study
B6/T231LA:232ML	pSelect/231LA:232ML	231; 232	Leu to Ala; Met to Leu	This study
B6/T231LY:232ML	pBS-SV40/231LY:232ML	231; 232	Leu to Tyr; Met to Leu	61
B6/T232ML	pSelect/232ML	232	Met to Leu	This study
B6/T232MQ	pSelect/232MQ	232	Met to Gln	This study
B6/T233YF	pBS-SV40/233YF	233	Tyr to Phe	61
B6/T235AG	pBS-SV40/235AG	235	Ala to Gly	61
B6/T237TS	pBS-SV40/237TS	237	Thr to Ser	61
B6/T239DG	pBS-SV40/239DS	239	Asp to Ser	61
B6/TΔ216-222	pSelect/Δ216-222	Δ216-222	Deletion	This study
B6/K-0	pPVU-0		Wild type	65
B6/K-1,4,5[a]		Δ134-263; 405; 491	Deletion; Val-15→Leu; Ile to Phe	45, 51a, 67
B6/Scl-7[b]	None			70

[a] B6/K-0 variant selected in vitro.
[b] No T antigen; spontaneously immortalized B6/MEF clone.

expressed in B6/MEF or in B6 kidney cells by transfection with plasmid DNA as previously described (18, 43, 45, 68, 70). Cell lines expressing mutant T antigens were established from individual transformed foci. All cell lines were found to be positive for T-antigen expression when tested by indirect immunofluorescence with the monoclonal antibody PAb 901, which recognizes an epitope in the carboxy terminus of T antigen. These results (data not shown) confirmed the nuclear localization of the mutant T antigens and indicated that the reading frame of mutant T antigens was not altered. These mutant T antigens retained their ability to transform primary cells into continuous cell lines. Determination of steady-state levels of mutant T antigen by Western blot analysis showed that all cell lines accumulated similar amounts of T antigen (Fig. 1), suggesting that the stability of the mutant proteins was comparable to that of the wild-type T antigen. These cell lines expressing mutant T antigens with amino acid substitutions between residues 200 through 239 were then tested for their ability to be lysed by CTL clones specific for site I (Y-1 and K-11) and site II/III (Y-2 and Y-3).

Optimal site I consists of 10 residues. Previous work had shown that residues 207 through 215 (AINNYAQKL) defined the minimal site I. Site I was defined as residues 207 through 215 because the peptide LT207-215 sensitized targets for lysis

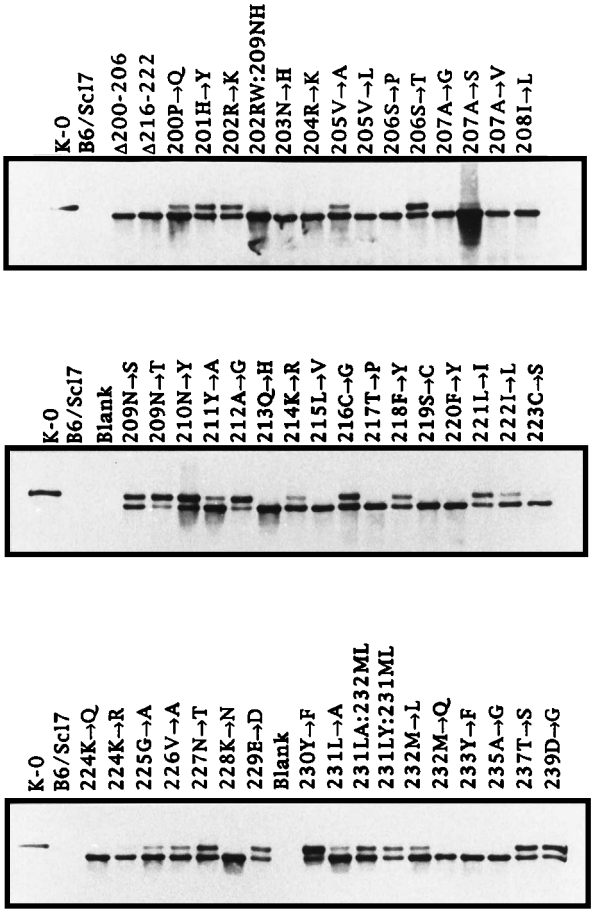

FIG. 1. Western blot analysis of cell lines expressing T antigens containing amino acid substitutions affecting residues 200 through 239. Cells were lysed and extracts were precleared with an irrelevant antibody. Volumes of extract representing 100 μg of total protein were immunoprecipitated with the monoclonal antibody PAb 901 and subjected to Western blot analysis. Substitutions affecting T-antigen amino acid residues are indicated by single-letter amino acid designations above the respective lanes; for each single mutant, the position affected and the wild-type residue at that position are followed by an arrow and the substituted residue. Double mutations within T antigen are indicated without arrows; designations for substituted positions are separated by a colon. Wild-type T antigen is represented by lanes labeled with K-0. B6/Scl7 cells express no T antigen. Blank lanes contained no extract.

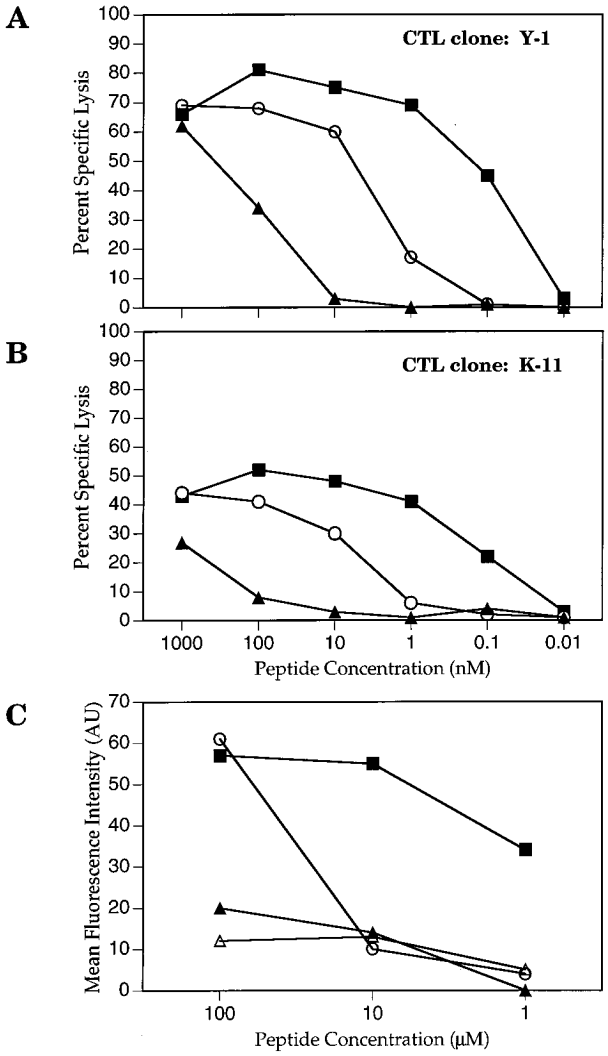

FIG. 2. Dose-response curves from CTL lysis and MHC class I stabilization assays utilizing synthetic peptides representing site I. B6/K-1,4,5 cells were pulsed with various concentrations of the synthetic peptides LT205-215 (○), LT206-215 (■), and LT207-215 (▲) and were incubated with the CTL clone Y-1 (A) or K-11 (B) in a standard ^{51}Cr release assay. CTL clones and targets were combined at an effector-to-target ratio of 10:1. (C) Stabilization of H-2D^b MHC class I complexes on the surface of RMA/s cells was determined by flow cytometry following incubation of RMA/s cells in the presence of various concentrations of the synthetic peptides LT205-215 (○), LT206-215 (■), LT207-215 (▲), and LT404-411 (△). Peptide-treated cells were incubated with an H-2D^b-specific monoclonal antibody (28-14-8) followed by a fluorescein isothiocyanate-conjugated secondary antibody. Mean fluorescence intensity is given in arbitrary units (AU). The fluorescence of RMA/s cells incubated without peptides was assigned a value of zero. In the same assay, the mean fluorescence intensity of RMA cells incubated without added peptides was 101 AU.

by site I-specific CTL clones and was 9 amino acids in length, which was consistent with the length determined for native H-2D^b-binding peptides (19). However, H-2D^b-binding peptides contain an invariant Asn at anchor position 5 (P5), whereas LT207-215 contained a Tyr at P5 and an Asn at P4 (26). The addition of residue 206 to the 207-through-215 peptide would place Asn-210 at P5 (SAINNYAQKL), thus satisfying the requirement for efficient binding of the peptide to the H-2D^b molecule (26, 31, 56). Earlier results had shown that peptide LT205-215 was presented more efficiently to CTL clone Y-1 than the minimal peptide LT207-215 (19). To determine whether the optimal site I might include residues 206 through 215, synthetic peptides (LT205-215, LT206-215, and LT207-215) were compared by a peptide dose-response lysis assay and a surface MHC class I molecule stabilization assay. The data in Fig. 2A and B show the dose-response curves for the peptides LT205-215, LT206-215, and LT207-215 obtained from chromium release assays utilizing the CTL clones Y-1 and K-11, respectively. The peptide LT206-215 was the most efficient in sensitizing target cells to lysis by CTL clones Y-1 and K-11.

The relative binding efficiencies of the peptides LT205-215, LT206-215, and LT207-215 to H-2D^b molecules were determined by using the RMA/s stabilization assay. Incubation of MHC class I-deficient RMA/s cells with H-2^b-restricted peptides leads to an increase in surface class I molecule expression (15, 31, 63). Various concentrations of site I peptides were incubated with RMA/s cells, and H-2D^b surface expression was determined as described in Materials and Methods. The results in Fig. 2C show that the peptide LT206-215 induced higher

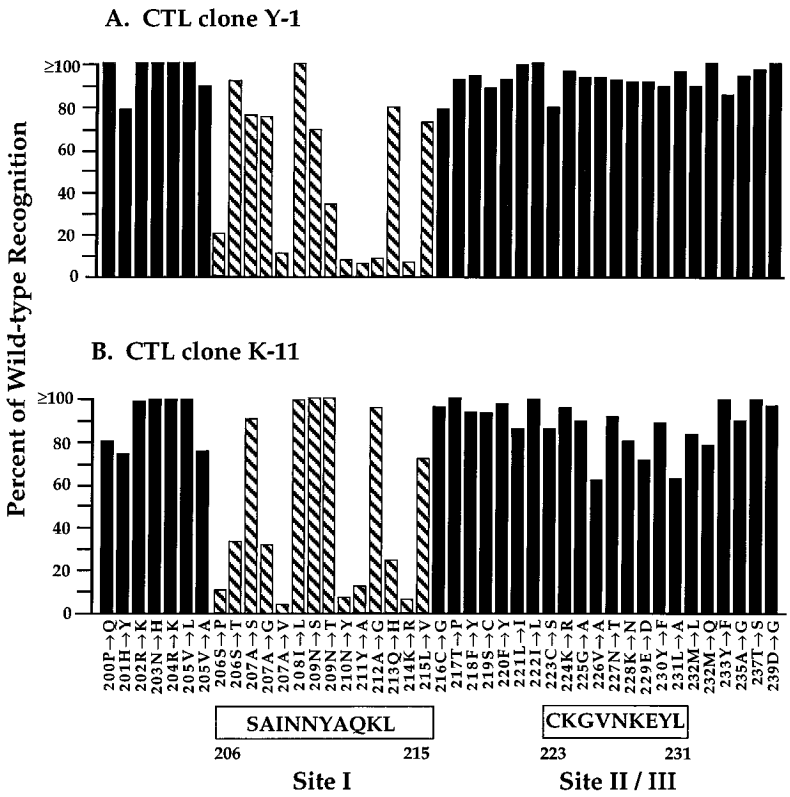

FIG. 3. Recognition by CTL clones Y-1 and K-11 of H-2^b fibroblast cell lines expressing T-antigen derivatives with substitutions in amino acid residues within or flanking site I. The data are average percents wild-type recognition (see Materials and Methods). Values for lysis by the CTL clones are shown for cell lines expressing T antigens with amino acid substitutions affecting residues 200 through 239. CTL clones and targets were combined at effector-to-target ratios of 10:1 or 5:1. The amino acid sequences and boundaries of the wild-type epitopes are indicated. The number of experiments ranged from 3 to 10 for each cell line. ■, lysis values for cell lines expressing T antigens bearing substitutions in residues which flank site I; ▧, lysis values for cell lines expressing T antigens bearing substitutions within site I.

levels of H-2D^b expression than the peptides LT205-215 and LT207-215 at the two lower concentrations tested. At the highest concentration assayed, 100 μM, the peptides LT206-215 and LT205-215 induced similar levels of H-2D^b molecules. In repeated assays, the peptide LT207-215 was found to stabilize relatively low levels of H-2D^b molecules on the surface of RMA/s cells (see below). For comparison, the peptide LT404-411 was included in this and other experiments as a negative control for H-2D^b stabilization. Residues 404 through 411 in SV40 T antigen define the minimal site IV epitope, which is H-2K^b restricted (51a). Together, CTL lysis and MHC class I stabilization results indicate that LT206-215, a 10-mer, represents an optimal site I peptide.

Role of amino acids flanking site I and site II/III in recognition by site-specific CTL clones. The effects of amino acid substitutions flanking site I on recognition by CTL clones Y-1 and K-11 are shown in Fig. 3. The results show that none of the amino acid substitutions in residues flanking site I (residues 200 through 205 or residues 216 through 239) affected the ability of the site I-specific CTL clone Y-1 to efficiently recognize and lyse target cells expressing mutant T antigens (Fig. 3A). Similar results were obtained with the other site I-specific CTL clone, K-11 (Fig. 3B). Amino acid substitutions at positions 236 and 238 were not made and thus could not be tested.

Of particular interest is the substitution at residue 216 (C→G) in T antigen which immediately flanks the carboxy terminus of site I. In three studies in which a negative effect of flanking residues was demonstrated, a glycine residue was located immediately adjacent to the inefficiently recognized epitope (9, 20, 36). The results from our study show that a substitution of glycine for cysteine at position 216, which immediately flanks site I (residues 206 through 215) in T antigen, had no negative effect on the ability of Y-1 or K-11 to recognize the B6/T216CG cells (B6 cells expressing T antigen carrying a C-216→G mutation). It appears that the presence of a glycine residue immediately flanking a CTL epitope does not noticeably affect antigen processing in all cases.

The effect on recognition by CTL clones Y-2 and Y-3 of cells expressing T antigen with amino acid substitutions flanking site II/III (223 through 231) is shown in Fig. 4. None of the amino acid substitutions outside of epitope II/III affected the ability of the site II/III-specific CTL clone Y-2 (Fig. 4A) or CTL clone Y-3 (Fig. 4B) to efficiently recognize and lyse the mutant T-antigen-transformed cells. When results from the analysis of substitutions affecting the region including residues 200 through 239 are taken together, our results confirm that sites I and II/III in T antigen are processed and presented independently. That is, substitution of a residue which flanks one epitope yet lies within the second epitope did not abrogate the recognition by epitope-specific CTL clones directed at the first epitope. Therefore, the ability of SV40 T-antigen CTL epitopes I and II/III to be processed and presented to CTL appears to be independent of the flanking residues and of the adjacent H-2D^b-restricted CTL epitope.

Processing and presentation of site I and site II/III expressed in tandem in T antigen. The lack of a negative effect of substitutions in the residues flanking either site I or site II/III on recognition by the site-specific CTL clones indicates

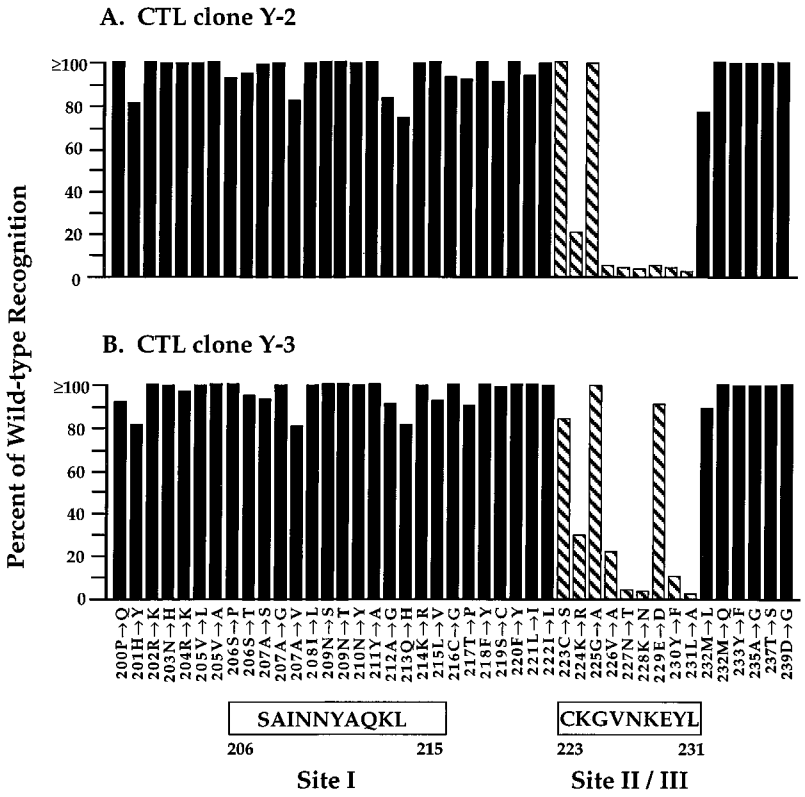

FIG. 4. Recognition by CTL clones Y-2 and Y-3 of H-2^b fibroblast cell lines expressing T-antigen derivatives with substitutions in amino acid residues within or flanking site II/III. The data are average percents wild-type recognition. CTL clones were used at effector-to-target ratios varying from 20:1 to 5:1. For further details, see the legend to Fig. 3. ■, lysis values for cell lines expressing T antigens bearing substitutions in residues which flank site II/III; ▨, lysis values for cell lines expressing T antigens bearing substitutions within site II/III.

that the flanking sequences may be dispensable for efficient expression of sites I and II/III. Indeed, a previous study from our laboratory has demonstrated that site I (contained in residues 205 through 215 and flanked by nonnative linker residues), when translocated to two different locations within T antigen (at amino acid positions 350 and 650), was processed, presented to, and recognized by the CTL clone Y-1 (29). It was of interest to determine whether either of the two CTL epitopes, I and II/III, when expressed in tandem would be efficiently processed and presented to the epitope-specific CTL clones. This was approached by constructing a T-antigen derivative with a deletion of residues 216 through 222, which separate CTL epitopes I and II/III (Fig. 5A). CTL clone Y-5, which recognizes the H-2D^b-restricted epitope V (residues 489 through 497), was used as a positive control for the cell line B6/TΔ216-222, which expresses a mutant T antigen carrying the Δ216-222 deletion. The results in Fig. 5B show that the B6/TΔ216-222 cell line was recognized by CTL clones Y-1, K11, Y-2, Y-3, and Y-5 at an efficiency comparable to that of cells expressing the wild-type T antigen. These results demonstrate that two independent CTL epitopes can be efficiently processed and presented when arranged in tandem in a large protein, thus showing that intervening residues are dispensable.

Effects of amino acid substitutions within SV40 sites I and II/III on recognition by site-specific CTL clones. Residues within an epitope can play three roles; they can (i) bind to the MHC class I molecule, (ii) interact with the TCR, and/or (iii) act as a spacer residue (4, 8, 13, 18, 40, 45, 53, 60, 80). We have attempted to define the role of each residue in sites I and II/III

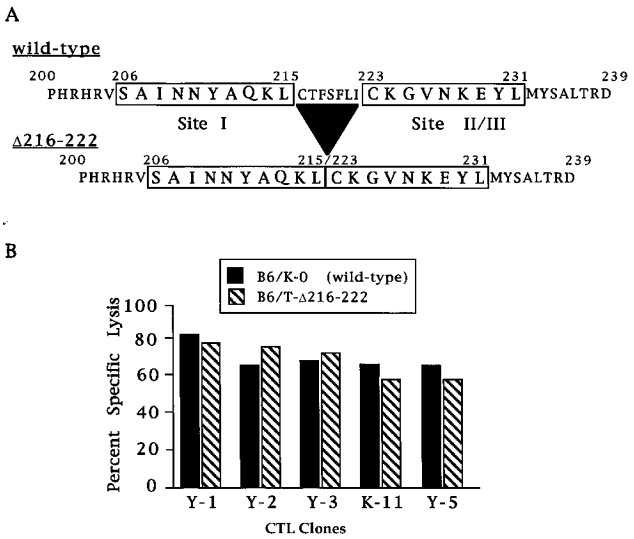

FIG. 5. Recognition by SV40 T-antigen-specific CTL clones of a cell line expressing a T antigen with a deletion of residues 216 through 222. (A) The deletion of SV40 T-antigen residues 216 through 222 (ΔCTFSFLI) places in tandem the carboxy-terminal Leu-216 residue of site I and the amino-terminal Cys-223 residue of site II/III. (B) Percent specific lysis by SV40 T-antigen-specific CTL clones of a cell line expressing the T antigen mutant with a deletion of residues 216 through 222 is shown. Percents specific lysis were determined by a standard 4.5-h ^{51}Cr release assay. CTL clones and targets were combined at an effector-to-target ratio of 10:1.

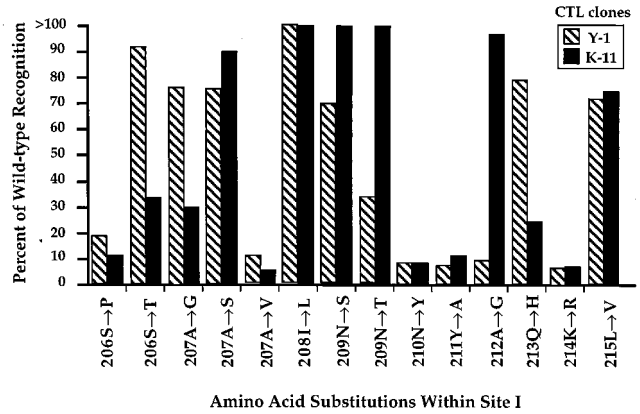

FIG. 6. Comparison of lysis by CTL clones Y-1 and K-11 of cell lines expressing T-antigen derivatives bearing substitutions in amino acid residues within site I. Average percent wild-type recognition by the CTL clones is presented for cell lines expressing T-antigen derivatives bearing an amino acid substitution within site I (residues 206 through 215). The data presented are taken from Fig. 3.

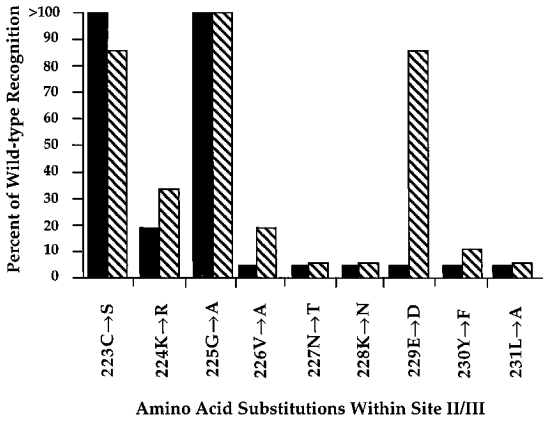

FIG. 7. Comparison of lysis by CTL clones Y-2 and Y-3 of cell lines expressing T-antigen derivatives bearing substitutions in amino acid residues within site II/III. Average percent wild-type recognition by the CTL clones is presented for cell lines expressing T-antigen derivatives bearing an amino acid substitution within site II/III (residues 223 through 231). The data presented are taken from Fig. 4. ▧, recognition by CTL clone Y-2; ■, recognition by CTL clone Y-3.

in the formation of the peptide-MHC class I molecule-TCR complex. Identification of residues within an epitope critical for binding of the TCR has been accomplished by using independently derived CTL clones which are specific for the same epitope (60). Previous studies have shown a diversity in the primary structure of the alpha and beta chains of the TCR of multiple CTL clones that recognize the identical epitope (1, 14, 25, 37, 44, 74, 78). These variations in TCR sequence may result in minor differences in how each TCR binds to the MHC class I-peptide complex. Exploiting the differences in CTL recognition can point to residues within the antigenic epitopes that interact with the TCRs. In the present study we have utilized two unique CTL clones specific for each epitope. CTL clones Y-1 and K-11 recognize site I, and site II/III is recognized by CTL clones Y-2 and Y-3 (7, 65). A substitution in T antigen that is recognized by one CTL clone and abrogates recognition by a second CTL clone specific for the same peptide suggests that the substituted residue is important for TCR interaction (60). It also implies that the amino acid substitution does not dramatically affect peptide-MHC class I binding.

The effects of amino acid substitutions in site I and site II/III on recognition by the site-specific CTL clones are presented in Fig. 3 and Fig. 4. For comparison, differential effects observed for CTL clones which recognize the same epitope are illustrated in Fig. 6 and 7. Site I-specific CTL clones Y-1 and K-11 are differentially affected by the amino acid substitutions S-206→T, A-207→G, N-209→T, A-212→G, and Q-213→H (Fig. 6). A substitution of threonine for serine at residue 206 prevented recognition by the CTL clone K-11 but had no effect on recognition by CTL clone Y-1. Conversely, the substitution of threonine for asparagine at residue 209 reduced recognition by Y-1 but had no effect on the ability of K-11 to recognize this target. Substitutions of residues 212 and 213 of T antigen indicated that the these residues most likely interact with the TCR. Replacement of alanine with glycine at position 212 or of glutamine with histidine at position 213 abrogated recognition by only one of the two site I-specific CTL clones.

We examined three amino acid substitutions at position 207 for their effect on recognition by the CTL clones K-11 and Y-1. Of these substitutions, A-207→S had no effect on the recognition by either of the two site-specific CTL clones. Replacement of alanine with valine at position 207 abrogated recognition by both CTL clones, confirming our previous observation (18). The substitution A-207→G affected only recognition by the CTL clone K-11.

The differential effects of amino acid substitutions in site II/III on recognition by the CTL clones Y-2 and Y-3 are shown in Fig. 7. The results show that a conservative Glu→Asp substitution at residue 229 had no effect on the recognition of this target by Y-3 and yet caused the loss of recognition by Y-2. This dichotomy implies that residue 229 is important for the TCR interaction of CTL clone Y-2. Previous work showed that the substitutions at residue 224 in SV40 T antigen (lysine to arginine or glutamic acid) resulted in the loss of recognition by CTL clones Y-2 and Y-3 (18). However, the replacement of lysine with glutamine at residue 224 was recognized by CTL clone Y-3 and not by Y-2. This suggested that the substitution with glutamine at residue 224 of T antigen does not prevent peptide-MHC class I binding, as this mutant T antigen was recognized by CTL clone Y-3. It was concluded that residue 224 is important for TCR interaction (18). Previous results obtained from the analysis of CTL clones Y-2 and Y-3 escape variants have indicated that residues 228 and 230 are important for TCR interaction (45). The substitutions Lys-228→Asn and Tyr-230→Phe prevent recognition by the CTL clones Y-2 and Y-3, and synthetic 9-mer peptides corresponding to the substituted epitopes are able to associate with H-2D^b molecules, as demonstrated in peptide competition experiments with the wild-type epitope II/III peptide (LT223-231).

The two predicted anchor residues in site I are the asparagine at residue position 210 (P5) and the leucine at residue position 215 (PΩ). Consistent with the motif determined for H-2D^b-binding peptides, the substitution of the central asparagine residue at position 210 with a tyrosine abrogated recognition by both CTL clones Y-1 and K-11 (Fig. 6). Crystallographic data have shown that the C-terminal anchor leucine of an H-2D^b-restricted epitope lies deep within a pocket of the antigen binding groove of the MHC class I molecule (80). The substitution of leucine at residue position 215 with valine did not affect recognition by either CTL clone Y-1 or K-11 (Fig. 6). This result may expand the H-2D^b motif (56) to include valine in addition to methionine, isoleucine, and leucine as C-terminal residues which allow for efficient peptide-MHC interactions.

Residues within the site II/III epitope predicted to be critical

for antigen binding to the MHC class I molecule are the asparagine at position 227 (P5) and the leucine at position 231 (PΩ). Residue 227 is the predicted central anchor residue (P5); as expected, substitution of asparagine with threonine abrogates recognition by both CTL clones Y-2 and Y-3 (Fig. 7). The substitution at position 231 (PΩ) of leucine with alanine abrogated recognition by CTL clones Y-2 and Y-3 (Fig. 7).

Amino acid substitutions at positions 208, 211, 225, and 226 in synthetic peptides corresponding to site I or site II/III. One limitation of the approach used in this study to define the function of the amino acids encompassing CTL epitopes was the predicament which arose when the substitution within the endogenously synthesized T antigen either had no effect or abrogated recognition by both site-specific CTL clones. Residues within either site I or site II/III whose role could not be defined because the substitution abrogated recognition by both site-specific CTL clones were those involved in the substitutions Y-211→A and V-226→A. The K-214→E substitution abrogated recognition by both Y-1 and K-11, but previous results suggested that residue K-214 interacts with the TCR (18). The substitutions I-208→L and G-225→A did not affect CTL recognition by either pair of site-specific CTL clones, probably because of the conservative nature of the substitutions. Therefore, testing of the function of these residues required less conservative substitutions. We synthesized peptides corresponding to the optimal epitopes including the appropriate substitutions (see Fig. 8 and 9) and tested these peptides in CTL recognition and MHC class I stabilization assays.

The data in Fig. 8 illustrate the ability of wild-type (LT206-215) and mutant site I (I-208→E, I-208→Q, and Y-211→A) peptides to bind to MHC class I molecules and be recognized by CTL clones Y-1 and K-11. In addition, a 10-mer site I peptide with the substitution A-207→V was tested. The I-208→E and I-208→Q peptides both demonstrated reduced binding to the MHC class I molecule. The I-208→Q peptide bound to the MHC class I molecule more efficiently than the I-208→E peptide. The efficiency of MHC class I binding of these two peptides was reflected in the levels of recognition by CTL clones Y-1 and K-11. This observation suggests that residue 208 may play a secondary role in promoting or stabilizing epitope I–H-2D^b complexes. Other examples in which substitutions at residue positions other than primary anchor positions affect peptide-MHC association have been reported (16, 54, 57, 59). The Y-211→A peptide bound to the H-2D^b molecule at near wild-type levels, did not sensitize target cells to lysis by CTL clone Y-1, and only inefficiently triggered recognition by CTL clone K-11 at higher concentrations. These data suggest that residue 211 is not involved in binding the site I peptide to the H-2D^b molecule but rather is required for interaction with the TCR of the CTL clones Y-1 and K-11.

The substitution at residue position 207 of alanine with valine in the full-length T antigen abrogated recognition by site I-specific CTL clones K-11 and Y-1 (Fig. 6). It has been previously demonstrated that the substitution of alanine with valine at position 207 in synthetic peptides (residues 205 through 219 and 205 through 215) did not significantly affect recognition by CTL clone Y-1. However, the same substitution in peptides containing residues 207 through 215 and 207 through 217 did reduce recognition by the CTL clone Y-1 at all concentrations tested, compared with the parent peptide (18). In light of the fact that we have redefined site I as a 10-mer (206 through 215; see above), we examined the effect of the A-207→V substitution in a synthetic 10-mer peptide. The results in Fig. 8A and B show that the A-207→V substitution in the 10-mer site I peptide did not dramatically reduce recognition by either of the two CTL clones. The data in Fig. 8C show that the A-207→V peptide bound well to the H-2D^b molecule.

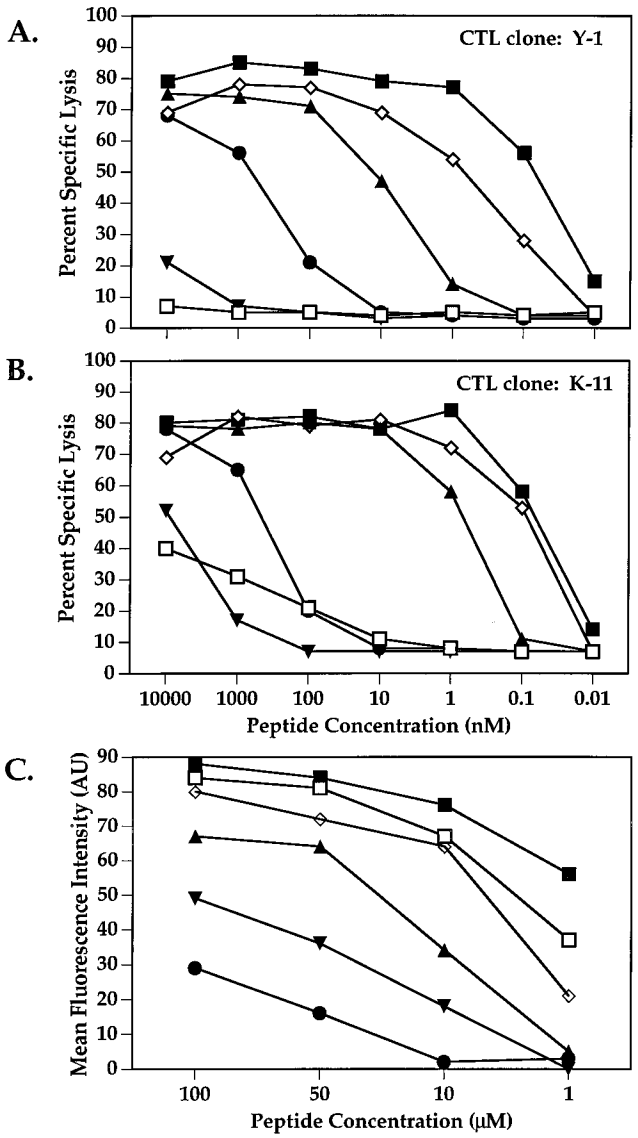

FIG. 8. Dose-response curves from CTL lysis and MHC class I stabilization assays using synthetic peptides representing site I (residues 206 through 215) and selected site I variants. B6/K-1,4,5 cells were pulsed with various concentrations of synthetic peptides and were incubated with the CTL clones Y-1 (A) or K-11 (B) in a standard ^{51}Cr release assay. CTL clones and targets were combined at an effector-to-target ratio of 10:1. Background lysis was less than 5% when irrelevant peptides were used at the highest concentrations. (C) Stabilization of H-2D^b MHC class I complexes on the surface of RMA/s cells was determined by flow cytometry following incubation of RMA/s cells in the presence of various concentrations of the same synthetic peptides (see above). For further details see the legend to Fig. 2. The mean relative fluorescence of RMA/s cells incubated without peptides was assigned a value of zero. In the same assay, the mean fluorescence intensity of wild-type RMA cells incubated without added peptides was 107 arbitrary units (AU). ■, LT206-215; ●, LT207-215; ◇, LT206-215 A-207→V; ▼, LT206-215 I-208→E; ▲, LT206-215 I-208→Q; □, LT206-215 Y-211→A.

The abilities of wild-type and mutant site II/III peptides to bind to MHC class I molecules and be recognized by CTL are shown in Fig. 9. Both the G-225→E and V-226→A peptides demonstrated only a slight impairment in the ability to bind to the MHC class I molecule. However, these two peptides differed markedly in their ability to sensitize targets for lysis by

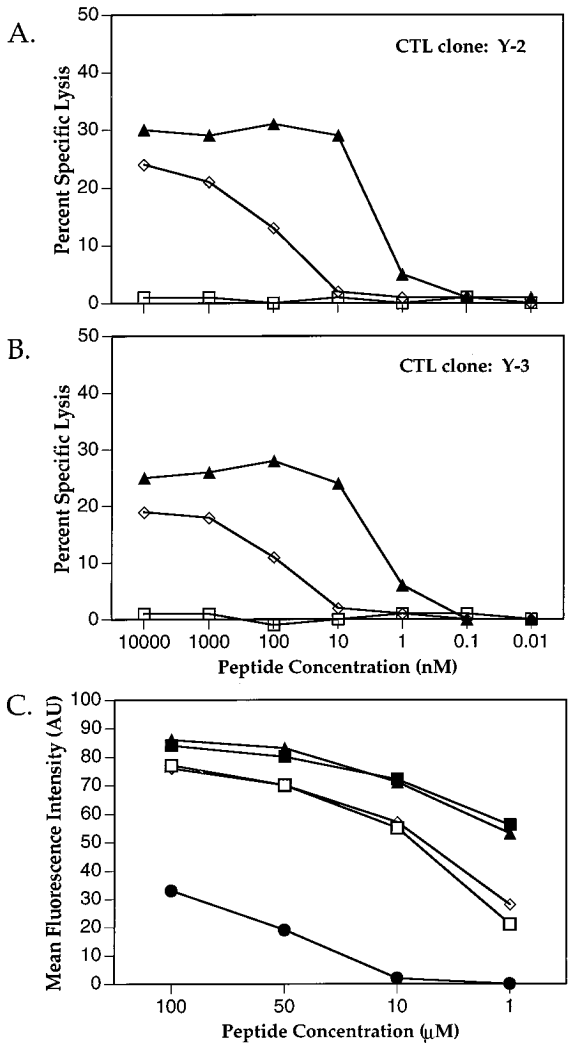

FIG. 9. Dose-response curves from CTL lysis and MHC class I stabilization assays using synthetic peptides representing site II/III (residues 223 through 231) and selected site II/III variants. B6/K-1,4,5 cells were pulsed with various concentrations of synthetic peptides and were incubated with the CTL clones Y-2 (A) or Y-3 (B) in a standard ^{51}Cr release assay. CTL clones and targets were combined at an effector-to-target ratio of 30:1. ▲, LT223-231; ◇, LT223-231 G-225→E; □, LT223-231 V-226→A. (C) Peptide-induced stabilization of H-2D^b MHC class I complexes on the surface of RMA/s cells was determined by flow cytometry. For further details see the legend to Fig. 2. The mean relative fluorescence of RMA/s cells incubated without peptides was assigned a value of zero. In the same assay, the mean fluorescence intensity of wild-type RMA cells incubated without added peptides was 103 arbitrary units (AU). ▲, ◇, and □ are as defined for panels A and B; ■, LT206-215; ●, LT207-215.

CTL clones Y-2 and Y-3. Recognition of the G-225→E peptide by the site II/III-specific CTL clones was partially affected, whereas the recognition of the V-226→A peptide was abrogated. Results obtained with the G-225→E peptide suggest that residue 225 may play a secondary role in binding to the MHC class I molecule and no direct role in TCR interaction. The lack of recognition of the V-226→A peptide despite its ability to bind to MHC class I molecules indicates that residue 226 may interact with the TCR of CTL clones Y-2 and Y-3 (Fig. 9).

DISCUSSION

Amino acid residues within an antigenic protein can have various effects on the processing and presentation of MHC class I-restricted epitopes. Examples in which the substitution of residues immediately flanking an epitope appear to affect efficient epitope processing and presentation for that epitope have been reported (9, 20, 24, 36). Previous reports have also investigated the consequences of substituting residues within an epitope for the ability of the epitope peptide to bind to the MHC class I molecule and/or interact with the TCR of reactive CTL (4, 8, 12, 18, 40, 45, 53). However, the precise boundaries of the epitope in question must be defined to allow meaningful interpretation of these types of results. The evidence presented in this study demonstrates, using two criteria, that the optimal SV40 T antigen site I epitope includes residues 206 through 215. That is, a synthetic peptide with the highest relative binding affinity for MHC class I molecules and with the ability to confer sensitivity to CTL recognition at lower concentrations should represent the optimal epitope. The optimal synthetic peptide is assumed to represent the endogenously presented epitope peptide. However, direct identification of the endogenous antigenic peptide is the only means by which such conclusions can be confirmed (26, 38, 75).

Amino acid residues which flank an MHC class I-restricted epitope can affect the processing of the epitope into a form that can be presented to CTL (9, 20, 24, 36). The systematic substitution of residues that surround two SV40 CTL epitopes has failed to demonstrate any adverse effect on antigen recognition by the CTL clones Y-1, K-11, Y-2, and Y-3. The deletion of residues 216 through 222 (Δ216-222) brings site I into juxtaposition with site II/III. Removal of these 7 intervening amino acids, which represent flanking residues for both sites I and II/III, did not noticeably decrease recognition by CTL clones directed against either epitope. This result supports the notion that the intervening amino acids are not necessary for the efficient processing and presentation of either epitope. We note that in both wild-type T antigen and the Δ216-222 construct, a Cys residue immediately flanks the carboxy terminus of site I (Fig. 5A). Since a cysteine residue flanking the carboxy terminus of site I is not absolutely required for efficient processing and for presentation to either of the site-specific CTL clones (Fig. 3), these results illustrate that the precise identity of multiple residues lying beyond this adjacent cysteine residue are also unimportant for efficient site I presentation.

One implication of our observations is that there may not be antigen processing signals which lie outside of an epitope. Antigen processing may instead have a limited specificity for residues within the epitope. MHC class I epitope peptide motifs determined for murine haplotypes consistently predict that the aliphatic residues methionine, leucine, isoleucine, and valine are preferred at the carboxy-terminal anchor residue position (26, 56). If antigen processing were directed by the carboxy-terminal residue within an epitope, then the deletion of residues separating sites I and II/III would not affect epitope recognition. In such a model, the proteasome may scan the antigen, pause, and cleave after a potential carboxy-terminal residue to generate an appropriate peptide (or precursor peptide) for transport and MHC class I binding. This model may be supported by recent results suggesting that proteasomes may preferentially cleave after hydrophobic residues and that gamma interferon modulates proteasome subunit composition and function (2, 3, 10, 21, 22, 27, 30). Another explanation for the lack of an effect when residues between two epitopes are deleted may involve the abundance or turnover rate of the antigenic protein (5, 50, 71). If the antigen is expressed at levels such that the number of MHC-peptide complexes expressed on the cell surface is in excess of that required for efficient CTL recognition (17), then a change which moderately decreases processing efficiency may not lead to reduced

CTL lysis. Expression of site I and site II/III in tandem (or with any of the flanking residue substitutions tested) may decrease the number of epitope-MHC class I molecule complexes, but the resulting levels remain above the threshold needed for efficient CTL recognition. Alternatively, if such signals do exist, their importance for efficient epitope processing and presentation may be secondary to other factors which determine the antigenicity of the test epitope. For example, access to the endoplasmic reticulum via the TAP 1/TAP 2-dependent transporter represents a step at which the sequence of the epitope peptide may control the efficiency of presentation (6, 51, 52, 56).

Our finding that two epitopes, which are presented by the same MHC class I protein, can be efficiently processed and presented when linked in tandem has important implications for vaccine design. The efficacy of a potential therapeutic agent may be increased by including multiple antigenic determinants. Linking multiple determinants in tandem minimizes the amount of genetic information required to encode the peptide epitopes. Short epitope-containing peptides can be efficiently processed and presented when expressed from minigenes carried on vaccinia vectors (33). Furthermore, efficient processing and presentation has been demonstrated for two CTL epitopes separated by several spacer residues when expressed from minigenes carried on vaccinia vectors (9, 76). In both instances, however, the pair of epitopes used was presented by different MHC molecules (D^b and L^d or L^d and D^b), and two or more amino acids separated the minimal epitopes in the primary translation products (9, 76). We report here that two CTL epitopes restricted by the same MHC molecule (H-$2D^b$) can be efficiently processed and presented to CTL clones in vitro when expressed without intervening or spacer residues from within the amino terminus of SV40 T antigen. Moreover, we find that more than two H-$2D^b$-restricted epitopes fused in tandem within the full-length T antigen (this study) or as minigenes in vaccinia virus vectors can be efficiently processed and presented to CTL clones in vitro and can be used to elicit an appropriate CTL response in vivo (51b).

Many molecular interactions important for presentation and recognition of MHC class I-restricted antigens have been identified. Sequencing of pools of peptides eluted from various specific MHC class I molecules has yielded motifs which may be used to look for potential MHC class I-binding peptides within an antigenic protein (26). Crystallographic studies have revealed the three dimensional structure of the peptide binding groove from a number of MHC class I molecules; allele-specific peptide binding preferences predicted from the crystal structures agree with information gained from analysis of epitope peptides and native peptide pools (28, 39, 48, 49, 80). We have taken advantage of the available native peptide motif and crystallographic data for the H-$2D^b$ molecule in our analysis of the presentation of T-antigen epitopes I and II/III. The binding motif and crystal structure, together with substitution mutagenesis and synthetic peptides, have allowed us to assign a probable function(s) to the residues within these two epitopes. We have demonstrated that residues within an epitope can function by affecting interaction with the H-$2D^b$ molecule or by affecting the recognition of the TCR in a direct or indirect manner, and we have provided examples of each.

Two unique CTL clones specific for each of the epitopes, I and II/III, within SV40 T antigen have been used in this study. CTL clones Y-1 and K-11 recognize site I, and clones Y-2 and Y-3 recognize site II/III. We have identified amino acid substitutions within each epitope that abrogate recognition by one clone without affecting the other CTL clone's ability to recognize the same target cells. A substitution which allows recognition by one CTL clone but prevents recognition by a second CTL clone specific for the same peptide antigen supports the conclusion that the residue which is changed is important for recognition by the TCR of the second CTL clone (60). Unaffected recognition by one CTL clone suggests that the substitution does not dramatically reduce MHC class I binding or grossly alter the conformation of the MHC-bound epitope peptide. Examples of differential recognition of an amino acid substitution within site I and site II/III by their respective SV40 CTL clones are demonstrated by this work.

We have relied largely on conservative amino acid substitutions in the whole T antigen expressed by target cells and in corresponding synthetic peptides to identify the function(s) of individual residues within epitopes I and II/III. An alternate approach would have been to use alanine scanning mutagenesis. The advantage of our approach is that conservative substitutions can have a dramatic effect on the ability of an epitope to be presented or recognized. For example, the conservative substitution, E-229→D, in CTL epitope II/III in T antigen resulted in the abrogation of recognition by one but not both CTL clones. The substitution of the wild-type glutamic acid with aspartic acid represents the loss of a single methylene group; an alanine substitution would have resulted in a much greater change in side chain volume and hydrophobicity. We have identified several positions within sites I and II/III for which fine specificity was demonstrated by the loss of CTL clone recognition because of a conservative amino acid substitution. However, one pitfall of the use of conservative substitutions was evident when no effect on CTL recognition was observed, such as with the substitutions I-208→L and G-225→A. Less conservative substitutions incorporated at these positions in synthetic peptides were needed to reveal their functions.

Figure 10 presents a functional assignment for each of the residues within site I and site II/III. Both site I and site II/III contain the conserved asparagine in the center of the epitope (P5) and a leucine at the carboxy terminus (PΩ); both residues are predicted to be necessary for the peptide to bind efficiently to the H-$2D^b$ molecule. Comparison of the functional assignments given to residues within site I and site II/III reveals a cluster of amino acids between the central (P5) and carboxy-terminal (PΩ) anchor residues which affect TCR interaction. Interestingly, the additional residue found in the 10-mer site I epitope is incorporated between the two anchor residues. The observation that residues between the two anchors (central asparagine and carboxy-terminal leucine) may interact with the TCR of reactive CTL is consistent with observations concerning the structure of the H-$2D^b$ molecule (41, 80). A unique ridge within the peptide binding groove of H-$2D^b$ is formed by Trp-73 on the α1 helix and Tyr-156 on the α2 helix. The result of this ridge is a loss of approximately 2.5 Å (ca. 0.25 nm) in the depth of the groove in this location (80). Residues of the bound peptide which span this ridge are expected to have a relatively large solvent-accessible surface area (80). These residues of the antigenic peptide most likely interact with the receptor of the reactive T cells, because they are readily accessible for such interactions. Therefore, the physical data on the structure of the H-$2D^b$ molecule and on the conformational effects this molecule has on bound peptides agree with the functional data presented here; both suggest that amino acids between the anchor residues are accessible and important for recognition by H-$2D^b$-restricted T cells. Likewise, position P4 of both epitopes I and II/III (T antigen residues 209 and 226) is predicted to interact with the TCR. This prediction is consistent with the solvent accessibility demonstrated for the

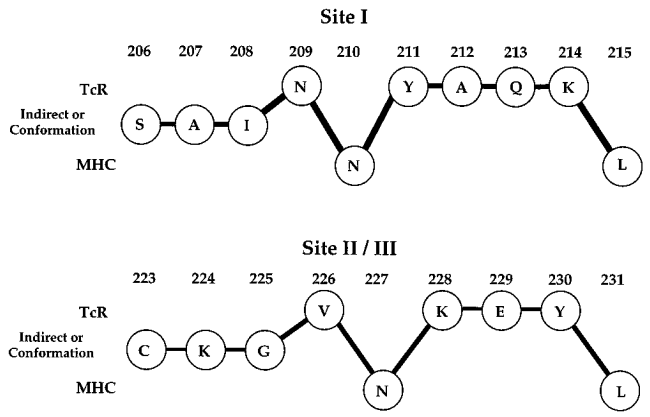

FIG. 10. Functional assignment of the residues within SV40 T-antigen site I and site II/III. Residues are assigned as either (i) interacting with the TCR (TCR); (ii) anchoring the epitope peptide to the MHC class I molecule (MHC); or (iii) functioning as a secondary MHC class I binding residue, indirectly affecting TCR interaction by affecting conformation of the peptide and/or the peptide-class I complex (Indirect or Conformation). Residue positions for which substitutions lead to measurable effects both in CTL lysis and MHC association assays may indirectly affect CTL recognition by reducing the efficiency of peptide-MHC complex formation, by altering peptide conformation within the MHC-peptide complexes, and/or by altering in a more global way the conformation of the assembled peptide-MHC complex. Assignment of a residue as one which may directly interact with the TCR involves three criteria. First, substitutions at that position should abrogate CTL recognition while allowing for efficient MHC-peptide interactions. MHC-peptide interaction is indicated by the ability of a peptide to stabilize MHC complexes on the surface of RMA/s cells, by functional competition in CTL assays, or by retained CTL recognition. Second, substitutions at the same position may differentially affect recognition by unique CTL clones which recognize the same epitope. Third, the side chain of the residue occupying the corresponding position in the H-2D^b–peptide crystal structure should be accessible to solvent. Residues important for binding to the MHC class I molecule are defined as those residues for which a substitution abrogated recognition by both site-specific CTL clones and which are predicted to be important anchor positions by the haplotype-specific epitope motif.

side chain of the residue occupying the corresponding position in the crystal structure (80).

The crystallographic data for the H-2D^b molecule support the conclusion that residues at P1 through P3 may indirectly affect recognition by the site-specific CTL clones. In the crystal structure, residues at P1 through P3 of the influenza virus nucleoprotein peptide are buried and not accessible to solvent when bound to H-2D^b (80). Our results confirm that the presence of the P1 residue 206 in a peptide representing site I is required for efficient peptide-MHC association (10a, 60, 80). A S-206→T substitution at P1 of site I did, however, differentially affect recognition by the two site I-specific CTL clones. This observation could be interpreted to indicate an interaction between this residue and the TCR. The apparent interaction with the TCR taken together with the predicted inaccessibility of this residue may in fact indicate an indirect effect of this substitution on the overall conformation of the MHC-peptide complex, leading to differential recognition by the CTL clones. From the crystal structure, P2, or the position of residue 207 in SV40 T antigen site I and of residue 224 in site II/III, is predicted to be buried under a bridge formed between side chains of residues Lys-66 and Glu-163, which are in the α1 and α2 helices of the H-2D^b molecule, respectively. It is possible that substitutions at residue position 207 or 224 of T antigen may alter the conformation of the bridge and thus affect recognition by specific CTL clones. P3 of the site I and site II/III epitopes (residues 208 and 225) may play a secondary role in the epitope's binding to the MHC class I molecule. Our results demonstrate that some substitutions at these positions prevent efficient MHC-peptide association. The extent to which MHC association is diminished for these variant peptides correlates well with the relative reduction in their ability to be recognized by CTL clones.

The A-207→V substitution in site I demonstrates a striking difference between presentation in the form of a synthetic peptide and presentation of the same sequence from within T antigen (18). In agreement with previous results, we show here that the A-207→V substitution in the context of the synthetic site I 10-mer peptide has little if any effect on MHC class I binding or recognition by the CTL clones. However, an A-207→V substitution in the endogenously produced full-length T antigen fails to be recognized. Perhaps this inconsistency points to a defect in epitope peptide processing, transport, or stability in living cells. Alternately, our peptide-MHC binding assay may not detect subtle kinetic differences in rates of association or dissociation between the A-207→V-substituted peptide and the optimal site I peptide which may be relevant for endogenous presentation (16).

In summary, this study has sought to determine the function of amino acid residues which encompass two closely linked H-2D^b-restricted SV40 T-antigen CTL epitopes. This study has provided some unique observations relevant to understanding the MHC class I antigen presentation pathway. Our results show that at least two CTL epitopes restricted by the same MHC class I allele can be linked in tandem without adversely affecting recognition by CTL clones specific for either epitope. Furthermore, we have shown that there appear to be no specific amino acids flanking either epitope which are required for efficient processing of these epitopes. Additionally, this study has indicated roles for residues within site I and site II/III. A more complete understanding of the molecular structures which restrict and potentiate T-antigen-specific recognition will require determination of the TCR structure of our epitope-specific CTL clones. For example, determination of the structure of the α and β subunits of the TCRs of Y-2 and Y-3 should explain why two CTL clones recognize the same epitope yet differ in their ability to recognize an epitope peptide altered by a substitution as minor as the loss of a methylene group. Likewise, analysis of the structure of the TCR subunits expressed on the CTL clones Y-1 and K-11 will provide information about why K-11 appears to be more sensitive to changes affecting the amino-terminal half of site I than Y-1. Structural data on the TCR will complement this study in the delineation of the role of residues within an epitope. We are currently determining the primary sequence of the TCR subunits expressed on these CTL clones. Studies such as these are essential for broadening our understanding of the molecular interactions between the peptide-MHC class I complex and the TCR, which determines the immunological response to both foreign and self antigens.

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