Polymorphism within the Herpes Simplex Virus (HSV) Ribonucleotide Reductase Large Subunit (ICP6) Confers Type Specificity for Recognition by HSV Type 1-Specific Cytotoxic T Lymphocytes

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A panel of herpes simplex virus type 1 (HSV-1)-specific, CD8⁺, major histocompatibility complex class I (*H-2K^b*)-restricted cytotoxic T-lymphocyte (CTL) clones was derived from HSV-1-immunized C57BL/6 (*H-2^b*) mice in order to identify the HSV-1 CTL recognition epitope(s) which confers type specificity. HSV-1 × HSV-2 intertypic recombinants were used to narrow the region encoding potential CTL recognition epitopes to within 0.51 to 0.58 map units of the HSV-1 genome. Using an inhibitor of viral DNA synthesis and an ICP6 deletion mutant, the large subunit of ribonucleotide reductase (ICP6, RR1) was identified as a target protein for these type-specific CTL. Potential CTL recognition epitopes within RR1 were located on the basis of the peptide motif predicted to bind to the MHC class I *H-2K^b* molecule. A peptide corresponding to residues 822 to 829 of RR1 was shown to confer susceptibility on *H-2K^b*-expressing target cells to lysis by the type 1-specific CTL. On the basis of a comparison of the HSV-1 RR1 epitope (residues 822 to 829) with the homologous sequence of HSV-2 RR1 (residues 828 to 836) and by the use of amino acid substitutions within synthetic peptides, we identified HSV-1 residue 828 as being largely responsible for the type specificity exhibited by HSV-1-specific CTL. This HSV-1 RR1 epitope, when expressed in recombinant simian virus 40 large T antigen in primary C57BL/6 cells, was recognized by the HSV-1 RR1-specific CTL clones. These results indicate that an early HSV protein with enzymatic activity provides a target for HSV-specific CTL and that type specificity is dictated largely by a single amino acid.

Herpes simplex virus (HSV) causes primary infection of the epithelium followed by the establishment of a latent infection in the sensory ganglia (57). The ability of HSV to persist in a latent state makes immunological control difficult. Therefore, experimental approaches to control HSV infection have focused on preventing the establishment of latency and limiting the virus spread after reactivation from the latent state. There is evidence that components of both humoral and cell-mediated immunity function in limiting the severity of HSV infection and the establishment of latency (45, 61). While antibody-mediated mechanisms have been shown, in part, to control HSV infection, adoptive transfer studies have demonstrated a key role for CD8⁺ cytotoxic T lymphocytes (CTL) in mediating protection against both primary infection and the establishment of latent HSV infection (9, 10, 28, 62). Thus, the identification of the HSV-encoded proteins and their corresponding CTL recognition epitopes that provide the targets for HSV-specific CTL is important in designing strategies to elicit the most effective CTL-mediated responses for controlling HSV infections. Although the mechanisms of major histocompatibility complex (MHC) restriction, antigen processing, peptide-mediated MHC assembly, transport of the class I-peptide complex to the cell surface, presentation of viral peptides by the MHC molecules, and recognition of the peptides by the T-cell receptor (TCR) (76) are well understood, approaches to se-

lectively induce and enhance CD8⁺ T-cell-mediated immune responses to individual CTL determinants that can confer protection against infection need to be explored.

HSV encodes approximately 77 polypeptides (41, 42, 57); however, only a few of these have been identified as providing a target for HSV-specific CTL. For example, glycoprotein D (gD) and gC have been identified as target proteins for human CTL (35, 79), while gB, gD, ICP4, and ICP27 have been identified as target proteins for murine-derived CTL (3, 30, 40, 74). Recently, studies have focused on identifying MHC-restricted CTL recognition epitopes within these proteins (4, 11, 24). By examining the amino acid sequences of HSV-encoded polypeptides for the presence of peptide motifs which have been predicted to bind the MHC class I molecules, it became evident to us that a large majority of the HSV polypeptides possess potential class I-binding epitopes, depending on the MHC class I allele; however, fewer of these may be recognized by CTL. Since CTL have been shown to recognize peptides derived from proteins irrespective of their function or location within the cell (76), the range of potential CTL epitopes that may be effective in inducing a CTL-mediated immune response is abundant. For example, CTL have been shown to recognize large T antigen of simian virus 40 (SV40) (65) as well as influenza virus nucleoprotein (71, 77), respiratory syncytial virus nucleoprotein (2), the immediate-early protein pp89 of murine cytomegalovirus (56), and the Epstein-Barr virus nuclear antigens (47). In addition, influenza virus and human immunodeficiency virus polymerase enzymes are both capable of providing targets for recognition by CTL (7, 26).

To define relevant HSV CTL determinants that might induce immune protection, we have established a panel of

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H-2K^b-restricted CTL clones (11) from a population of splenic lymphocytes obtained from C57BL/6 mice immunized with HSV type 1 (HSV-1) (9). These CTL clones have been used as probes for identifying the HSV gene product(s) that may contain peptides which provide epitopes for CTL recognition. We have previously shown that one such group of clones is HSV-1/HSV-2 cross-reactive and recognizes amino acid residues 498 to 505 of HSV-1 gB (11). The other group exhibits HSV-1 type specificity (11). We now show that these type 1-specific *H-2K^b*-restricted CTL clones recognize an epitope within the large subunit of the HSV-1 ribonucleotide reductase (RR1). Using the peptide motifs predicted to bind the MHC *H-2K^b* molecule, we were able to localize this CTL epitope to amino acids 822 to 829 of HSV-1 RR1. Furthermore, a single amino acid change from an arginine at position 7 (P7) in the HSV-1 RR1 peptide to a methionine in the corresponding HSV-2 peptide was shown to dictate the HSV-1 specificity of the RR1 epitope.

MATERIALS AND METHODS

Cells and viruses. The B6/WT-3 and B6/pPVU-0 cell lines were derived from C57BL/6 (*H-2^b*) mouse embryo fibroblast cells after transformation by SV40 virus (55) and after transformation with SV40 large-T-antigen-encoding plasmid pPVU-0 (33), respectively. Both cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 0.075% NaHCO₃, 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin sulfate per ml. Vero cells are a continuous line of African green monkey kidney cells and were maintained in medium 199 supplemented with 4% heat-inactivated FBS, 4% heat-inactivated newborn bovine serum, 8% tryptose phosphate broth, 0.075% NaHCO₃, 100 U of penicillin per ml, and 100 µg of streptomycin sulfate per ml. RMA cells are Rauscher murine leukemia virus-transformed T lymphocytes from which the *H-2^b* class I loss variant RMA-S cell line was derived by ethyl methanesulfonate mutagenesis (32, 39). Both RMA and RMA-S cells were passaged in RPMI 1640 medium supplemented with 10% FBS, 5 mM HEPES, 0.225% NaHCO₃, 2 mM glutamine, 100 U of penicillin per ml, 100 µg of streptomycin sulfate per ml, and 50 µM 2-mercaptoethanol.

The HSV-1 × HSV-2 intertypic recombinants RH1G44, RS1G25, R50BG10, RB7G5, and RB7G6 containing various regions of HSV-2 DNA were kindly provided by Bernard Roizman, The University of Chicago, and have been previously described (15, 34, 46, 50, 68). The ICP6 deletion mutant of HSV-1 (ICP6Δ) and its revertant (ICP6Δ⁺3.1) (23) were kindly provided by Sandra Weller, The University of Connecticut Health Center. These viruses, along with HSV-1 strains Patton and KOS and HSV-2 strains 186 and G, were propagated in Vero cells at a multiplicity of infection (MOI) of 0.01, and virus titers were determined by plaque assay on Vero cells.

Synthetic peptides. A computer algorithm, Homol, was used to scan protein sequences for potential class I peptide-binding epitopes and was written by Douglas H. Stickle (Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine). Potential epitopes were identified by assigning relative values to each residue within an octamer. This value was dependent on whether the residue was dominant, strong, or weak as defined for the *H-2K^b*-binding motif described by Falk et al. (19). A numerical score was assigned to each octamer on the basis of the summation of values for each of the residues. A threshold score, based on previously identified CTL epitopes, was used to determine which peptides represented potential CTL epitopes within HSV-1 RR1. Peptides were synthesized at the Macromolecular Core Facility of the M. S. Hershey Medical Center by 9-fluorenylmethoxycarbonyl chemistry, using an automated peptide synthesizer (9050 MilliGen Pep-Synthesizer). The composition of each peptide was determined by the Pico Tag amino acid analysis system (Waters, a division of Milligen). Peptide stock solutions of 1 mM were made by solubilizing the lyophilized peptides in dimethyl sulfoxide (not to exceed 5% of the final volume) and adjusting the solution to the appropriate volume with unsupplemented RPMI 1640 medium.

Generation and maintenance of HSV-specific, *H-2K^b*-restricted CTL clones. The generation and maintenance of the HSV-specific, *H-2K^b*-restricted CTL clones have been described previously (11). The HSV-1-specific, *H-2K^b*-restricted CTL clones (1C2, 1D3, 1D11, 1F8, 1G5, 2D1, and 2G5) were derived by limiting dilution (11) of a polyclonal CTL population established from the spleens of HSV-1-immunized C57BL/6 mice (9). An *H-2K^b*-restricted CTL clone, 2D5, which recognizes amino acids 498 to 505 within HSV gB and is cross-reactive for the recognition of HSV-1 and HSV-2, has been described previously (11). All CTL clones were maintained in 12-well plates with 5 × 10⁵ stimulator cells (mitomycin-treated, HSV-1-infected B6/WT-3 cells), 10% (vol/vol) T-STIM (Collaborative Biomedical), and methyl-α-D-mannopyranoside (0.05 M) in a total volume of 4 ml of Iscove's modified Dulbecco's medium supplemented with 10% FBS, 0.225% NaHCO₃, 2 mM glutamine, 100 U of

penicillin per ml, 100 µg of streptomycin sulfate per ml, and 50 µM 2-mercaptoethanol (10).

Cytotoxicity assay. The standard cytotoxicity assay using ⁵¹Cr-labeled target cells has been described previously (9, 11). Briefly, target cells were infected with HSV at an MOI of 5 or 10 for various times as indicated. Target cells were labeled with 150 µCi of ⁵¹Cr for 1 h or overnight at 37°C, washed three times in supplemented Iscove's modified Dulbecco's medium, and resuspended at a concentration of 10⁵ cells per ml. In some experiments, ⁵¹Cr-labeled target cells were incubated with various concentrations of peptides for 30 min at 37°C and washed free of unbound excess peptide. Target cells (10⁴) in a volume of 100 µl were added to 100 µl of various concentrations of CTL clones in 96-well V-bottom tissue culture plates (Costar). The plates were centrifuged at 45 × *g* for 3 min, incubated at 37°C for 5 h, and centrifuged at 180 × *g* for 5 min. One hundred microliters of supernatant was removed from each well, and the amount of radioactivity released into the supernatant was counted in a Beckman gamma counter. Percent specific lysis was calculated as [(*E* - *S*)/(*M* - *S*)] × 100, where *E* is the counts per minute released from the target cells incubated in the presence of effector CTL clones, *S* is the counts per minute released from target cells in medium alone, and *M* is the maximum counts per minute released from target cells in the presence of 5% sodium dodecyl sulfate.

Detection of ICP6 by indirect immunofluorescence. B6/WT-3 cells, growing on glass coverslips, were infected with ICP6Δ or ICP6Δ⁺3.1 at an MOI of 1 for 14 h, at which time the cells were washed three times with phosphate-buffered saline (PBS) and fixed in 100% ethanol for 45 min at -70°C. Cells were reacted with monoclonal antibody 48S, specific for ICP6 (60) (kindly provided by Martin Zweig, Bio-Molecular Techniques, Inc.) or monoclonal antibody 58S, specific for ICP4 (60). After an incubation for 30 min at 37°C, the cells were washed three times with PBS and reacted with goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate (Cappel) for 30 min. After being washed with PBS, the coverslips were air dried, mounted in Cytoseal (Stephens Scientific), and examined under a fluorescence microscope.

MHC class I *H-2K^b* stabilization assay. Stabilization of surface class I *H-2K^b* expression was detected by incubating 6 × 10⁵ RMA-S cells for 16 h at 37°C with various concentrations of peptide. RMA-S cells are defective in the transport of peptides from the cytosol into the endoplasmic reticulum (69). Peptide-pulsed RMA-S cells were stained with two different primary antibodies against the *H-2K^b* molecule, EH144 (52) and 28-13-3 (48) (kindly provided by Sebastian Joyce, The Pennsylvania State University College of Medicine). Following incubation with the primary antibody at 4°C for 30 min, the cells were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel). In addition, RMA and non-peptide-pulsed RMA-S cells were stained for class I *H-2K^b* surface expression. Fluorescence was detected on a Epics V flow cytometer/sorter (Coulter Electronics, Inc.).

Generation of a B6 cell line expressing a chimeric SV40 large T antigen containing the HSV-1 RR1 822-829 epitope. A B6 cell line expressing a chimeric SV40 large T antigen was generated as described previously (22). Briefly, oligonucleotide pairs representing HSV-1 RR1 residues 822 to 829 were annealed and ligated into pPVU-0:E350, which had been linearized with *Eco*RI. The orientation and authenticity of the insert were confirmed by PCR analysis and DNA sequencing. Primary B6 mouse embryo fibroblasts were immortalized with the chimeric SV40 T antigen as described previously (64). An immortalized cell line, B6/T350 HSV-1 RR1(822-829), was generated and shown to express SV40 T antigen when examined by indirect immunofluorescence using the monoclonal antibodies PAb419 and PAb901 (66), which recognize the N and C termini, respectively, of T antigen.

RESULTS

Localizing the region of the HSV-1 genome which encodes potential HSV-1-specific CTL epitopes, using HSV-1 × HSV-2 intertypic recombinants. We have previously reported the isolation of a panel of *H-2K^b*-restricted, HSV-1-specific CTL clones (1C2, 1D3, 1D11, 1F8, 1G5, 2D1, and 2G5) (11). HSV-1 × HSV-2 intertypic recombinant viruses were used to narrow the region of the HSV-1 genome encoding potential epitopes on the basis of their ability to render infected cells susceptible to lysis by these CTL clones. Two representative HSV-1-specific CTL clones, 1D11 and 1F8, and an HSV-1 × HSV-2 cross-reactive CTL clone, 2D5, were reacted with B6/WT-3 cells infected with the intertypic recombinants as well as HSV-1 and HSV-2. The results in Fig. 1 show that the HSV-1-specific CTL clones recognized and lysed target cells infected with recombinants RH1G44, RS1G25, R50BG10, and RB7G5 and HSV-1-infected cells. The recognition of cells infected with these intertypic recombinant viruses eliminated the regions of the HSV-1 genome from 0.35 to 0.43, 0.45 to 0.51, 0.58 to 0.72, and 0.82 to 1.0 map units (m.u.) as potentially encoding

FIG. 1. Susceptibility of HSV-1 x HSV-2 intertypic recombinant virus-infected B6/WT-3 cells to lysis by the HSV-1 specific, *H-2K^b*-restricted CTL clones 1D11 and 1F8. The approximate location of HSV-2 DNA within the HSV-1 x HSV-2 intertypic recombinant genomes is schematically represented. HSV-1 DNA sequences are represented by black bars, and HSV-2 DNA sequences are represented by white bars. B6/WT-3 (*H-2^b*) cells were infected with wild-type HSV-1, HSV-2, or an intertypic recombinant (RH1G44, RS1G25, R50BG10, RB7G5, or RB7G6) at an MOI of 10 and harvested 4 h postinfection. The infected and uninfected B6/WT-3 cells were labeled with 150 μ Ci of 51 Cr and used as target cells in a 5-h 51 Cr release assay. The HSV-1/HSV-2 cross-reactive, gB-specific CTL clone 2D5 was included as a positive control for the recognition of HSV-1 x HSV-2 intertypic recombinant-infected target cells. The results are shown for an effector-to-target cell ratio of 2:1.

the HSV-1-specific epitopes, as the presence of the corresponding HSV-2 DNA did not abrogate recognition by the HSV-1 CTL clones. The lack of recognition of cells infected with recombinant RB7G6, which encodes HSV-2 sequences from 0.47 to 0.61 m.u., suggested that the HSV-1-specific CTL epitope is encoded within the region from 0.47 to 0.61 m.u. The HSV-1/HSV-2 cross-reactive CTL clone 2D5 was capable of recognizing cells infected with each of the recombinant viruses. Together, these results suggest that the epitopes recognized by the HSV-1-specific CTL are encoded within the region from approximately 0.51 to 0.58 m.u. From these results for the intertypic recombinant viruses, the HSV-1 proteins encoded by genes UL36, UL37, UL38, and UL39 were predicted to contain the CTL epitope for HSV-1-specific CTL clones.

Effects of DNA and RNA synthesis inhibitors on recognition of HSV-1-infected target cells by the HSV-1-specific CTL. Expression of the UL36 gene product, VP1/2, which is a true late structural protein, is dependent on viral DNA replication (44). Likewise, expression of the UL38 gene product, a true late protein involved in capsid assembly, is inhibited when viral DNA replication is blocked (75). To determine if the epitope recognized by the HSV-1-specific CTL is derived from either of these proteins, the effect of phosphonoacetic acid (PAA), a DNA synthesis inhibitor, on the expression of this CTL epitope was investigated. B6/WT-3 cells were infected with HSV-1 in the presence or absence of PAA (300 μ g/ml) as described in the legend to Fig. 2, used as target cells against 1D11 and 1F8 CTL clones, and screened for the expression of gC, a true late glycoprotein, by fluorescence flow cytometry. The results in Fig. 2 showed that the cells infected with HSV-1 in the presence of PAA were recognized as efficiently as the non-PAA-treated, HSV-1-infected cells. Although the expression of the UL36 and UL38 gene products within the target cells was not determined, the block in viral DNA synthesis was detected by the loss of the expression of gC at the surface of the PAA-treated, HSV-1-infected cells (data not shown). These results suggest that the *H-2K^b*-restricted, HSV-1-specific CTL clones

do not recognize the HSV-1 true late proteins encoded by UL36 and UL38.

To further extend the observation that the UL36 and UL38 gene products were unlikely to provide the epitope(s) recog-

FIG. 2. Recognition of HSV-1 infected, PAA-treated B6/WT-3 cells by the HSV-1 specific, *H-2K^b*-restricted CTL clones 1D11 and 1F8. B6/WT-3 cells were either treated with PAA (300 μ g/ml) during infection with HSV-1 at an MOI of 5 or infected without PAA treatment. Uninfected B6/WT-3 cells were treated in a similar fashion. The cells were harvested 14 h after infection or treatment and used as targets in a standard 51 Cr release assay at an effector-to-target cell ratio of 1:1.

TABLE 1. Recognition of HSV-1-infected, act D-treated B6/WT-3 cells by CTL clones 1D11 and 2D5

Infection	Time (min) of addition of act D relative to time of infection ^a	% Specific lysis by CTL clones ^b	
		1D11	2D5
HSV-1	-30	1	5
	0	2	5
	+30	22	8
	+60	44	19
	+90	69	40
	+120	71	46
	+150	72	42
	+180	76	50
	None	87	72
None	— ^c	11	6
	None	5	15

^a B6/WT-3 cells were treated with 10 μ g of act D per ml 30 min prior to infection with HSV-1 at an MOI of 5, at the time of infection, or at 30-min intervals postinfection. The act D-treated cells were harvested 11 h postinfection for use in a standard ⁵¹Cr release assay. Act D-treated cells were maintained in the presence of 5 μ g act D per ml throughout the assay.

^b At an effector-to-target cell ratio of 2:1.

^c —, act D was added for a total of 11 h.

nized by the HSV-1-specific CTL, experiments were performed to determine the kinetics of expression of this HSV-1-specific CTL epitope after HSV-1 infection of target cells. B6/WT-3 cells were treated with 10 μ g of actinomycin D (act D) per ml for 30 min prior to, at the time of, or at 30-min intervals after infection with HSV-1. The ability of HSV-1-infected, act D-treated cells to be lysed by 1D11 was determined in a standard cytotoxicity assay. The results presented in Table 1 demonstrate that cells treated with act D prior to or at the time of infection with HSV-1 were not recognized by CTL clone 1D11, suggesting that input virions were not providing the epitope for recognition by the HSV-1-specific CTL. However, target cells infected with HSV-1 for 60 min before the addition of act D were recognized efficiently by CTL clone 1D11. The levels of MHC class I mRNA in act D-treated cells have been shown to be stable (72); therefore, the availability of MHC class I molecules should not have affected the recognition of the act D-treated, HSV-1-infected target cells. Since the expression of UL36 and UL38 gene products is not detectable until 8 to 9 h after infection (44, 75), it is not likely that either protein provides the epitope recognized by the HSV-1-specific CTL. These results suggest that the HSV-1-specific CTL epitope is likely to be located within proteins encoded by UL37 and UL39, both of which are produced early in infection. The UL39 gene encodes the ribonucleotide reductase large subunit (ICP6, RR1), which is part of the HSV ribonucleotide reductase which reduces ribonucleotides to deoxyribonucleotides for the production of precursors necessary for viral DNA synthesis (20, 54). The UL37 gene encodes a protein of unknown function (58).

Recognition of an HSV-1 ICP6 deletion mutant by the HSV-1-specific CTL. To determine whether ICP6 provides the epitope for recognition by the HSV-1-specific CTL clones, HSV-1 mutant ICP6 Δ , which does not express ICP6, and its revertant, ICP6 Δ +3.1 (23), were tested for the ability to provide a target against HSV-1-specific CTL clones. ICP6 Δ contains a 2.9-kb deletion within the coding region of ICP6, including the translation initiation codon, and therefore does not express ICP6. The revertant of ICP6 Δ , ICP6 Δ +3.1, was generated from marker rescue of ICP6 Δ (23). To confirm that B6/WT-3 cells infected with ICP6 Δ do not express ICP6, B6/

WT-3 cells were infected with ICP6 Δ or ICP6 Δ +3.1 and screened for the expression of ICP6 by immunofluorescence using monoclonal antibody 48S as described in Materials and Methods. ICP6 Δ -infected B6/WT-3 cells did not express detectable levels of ICP6 (Fig. 3A) but did express detectable levels of ICP4 (Fig. 3B), thus confirming the ICP6 Δ infection of the cells. The expression of ICP6 (Fig. 3C) and ICP4 (Fig. 3D) was detected in B6/WT-3 cells infected with the ICP6 Δ +3.1 and showed characteristic cytoplasmic and nuclear staining patterns, respectively. In addition, ICP6 was not detected in [³⁵S]methionine-labeled extracts from ICP6 Δ -infected B6/WT-3 cells (data not shown).

B6/WT-3 cells infected with either ICP6 Δ , ICP6 Δ +3.1, or wild-type HSV-1 or HSV-2 were tested against a panel of HSV-1-specific CTL clones (1C2, 1D3, 1D11, 1F8, 1G5, 2D1, and 2G5) and the HSV-1/HSV-2 cross-reactive clone 2D5, which recognizes an epitope within HSV gB. The data in Fig. 4 show that cells infected with ICP6 Δ were not recognized by the HSV-1-specific CTL clones. These CTL clones, however, recognized the cells infected with ICP6 Δ +3.1. As expected, the HSV-1-specific CTL clones failed to lyse HSV-2-infected cells, thus demonstrating their type specificity. The absence of recognition of the cells infected with ICP6 Δ by the HSV-1-specific CTL clones was not due to a lack of virus infection, since these cells were lysed by the HSV gB-specific CTL clone 2D5. These results indicate that the epitope recognized by the HSV-1-specific CTL clones is located within ICP6 (RR1).

Localizing the HSV-1-specific CTL epitope within RR1. In an attempt to define the distinct epitope within HSV-1 RR1 recognized by the HSV-1-specific CTL clones, the amino acid sequences of both HSV-1 RR1 and HSV-2 RR1 were examined for peptide sequences predicted to bind the MHC *H-2K^b* molecule. This prediction was achieved by using a computer algorithm based on the previously described *H-2K^b*-binding motif (19). The HSV-1 RR1 and HSV-2 RR1 protein sequences are unique at the amino terminus but are highly homologous at the carboxy terminus (43, 63). We identified 15 potential *H-2K^b*-binding motifs within HSV-1 RR1 and HSV-2 RR1. Of these 15 potential motifs, 10 were identical in amino acid sequence between HSV-1 and HSV-2 and 5 differed by either one or two amino acids. Two peptides, differing by two residues from their HSV-2 counterparts and corresponding to amino acids 474 to 482 (Ala-Leu-Asp-His-Tyr-Asp-Cys-Leu-Ile) and 822 to 829 (Gln-Thr-Phe-Asp-Phe-Gly-Arg-Leu) of HSV-1 RR1, were synthesized (Fig. 5A). B6/WT-3 target cells pulsed with either of the peptides were used in a cytotoxicity assay against the HSV-1 RR1-specific CTL clones 1D3, 1D11, and 1F8, in addition to the HSV-1 gB 498-505-specific CTL clone 2D5. The results presented in Fig. 5B indicate that the peptide corresponding to amino acids 822 to 829 of HSV-1 RR1 rendered target cells susceptible to lysis by the HSV-1 RR1-specific CTL clones 1D3, 1D11, and 1F8. In addition, other HSV-1 specific CTL clones, 1C2, 1G5 2D1, and 2G5, also recognized the HSV-1 RR1 822-829 peptide (data not shown). The cells pulsed with the peptide corresponding to amino acids 474 to 482 of HSV-1 RR1 were not lysed by the HSV-1 RR1-specific CTL. As expected, the 2D5 CTL clone recognized cells pulsed with the HSV-1 gB 498-505 peptide only. These results indicate that the epitope recognized by the HSV-1 RR1-specific CTL corresponds to amino acids 822 to 829 of HSV-1 RR1.

Polymorphism at P7 and P1 in HSV-1 RR1 822-829 confers type specificity. The HSV-1 RR1 8-mer epitope 822-829 (Gln-Thr-Phe-Asp-Phe-Gly-Arg-Leu) and the corresponding HSV-2 RR1 sequence 829-836 (Arg-Thr-Phe-Asp-Phe-Gly-Met-Leu) differ at P1 and P7. It was of interest to determine if

FIG. 3. Expression of ICP4 and ICP6 in B6/WT-3 cells infected with ICP6 Δ or ICP6 Δ +3.1. (A) Cells infected with ICP6 Δ and screened for the expression of ICP6 by using a monoclonal antibody to ICP6 (48S) as described in Materials and Methods. (B) Cells infected with ICP6 Δ and screened for the expression of ICP4 by using a monoclonal antibody to ICP4 (58S). (C and D) Cells infected with ICP6 Δ +3.1 and screened for the expression of ICP6 by using 48S (C) or for the expression of ICP4 by using 58S (D).

one or both amino acid changes dictated the HSV-1 specificity of this CTL epitope. We synthesized three additional peptides (Fig. 6A) which corresponded to either the HSV-2 RR1 peptide (HSV-2 RR1 829-836; Arg-Thr-Phe-Asp-Phe-Gly-Met-Leu) or the HSV-1 RR1 epitope containing the HSV-2 RR1 residue at P1 [HSV-1 RR1 822-829 (822 gln $\rightarrow$ arg); Arg-Thr-Phe-Asp-Phe-Gly-Arg-Leu] or at P7 [HSV-1 RR1 822-829 (828 arg $\rightarrow$ met); Gln-Thr-Phe-Asp-Phe-Gly-Met-Leu]. These peptides, in addition to HSV-1 RR1 822-829, were used at concentrations varying from 10 μ M to 100 pM to pulse B6/WT-3 cells in a standard cytotoxicity assay against the HSV-1 RR1 822-829-specific CTL clone, 1D11. The results in Fig. 6B show that the HSV-1 RR1 peptide sensitized target cells for lysis by 1D11 at all the concentrations tested, whereas the HSV-2 RR1 peptide was unable to sensitize the target cells even at the highest concentration. HSV-1 RR1 (828 arg $\rightarrow$ met), which contains a substitution of methionine at P7, did not render cells susceptible to lysis by CTL clone 1D11. A substitution in the HSV-1 RR1 peptide at P1 (822 gln $\rightarrow$ arg) rendered target cells susceptible to lysis by CTL clone 1D11 at concentrations ranging from 10 μ M to 10 nM. However, at lower concentrations of 1 nM and 100 pM, this peptide (822 gln $\rightarrow$ arg) was ineffective at sensitizing target cells for lysis by the CTL clone (Fig. 6B). These data suggest that the presence of methionine at residue 835 in HSV-2 RR1 contributes to the insusceptibility of HSV-2-infected target cells by the HSV-1-specific CTL clone. The presence of arginine at residue 829 in HSV-2 RR1 may also contribute in a minor way to the abro-

gation of recognition of HSV-2-infected cells by the HSV-1 RR1-specific CTL clones.

Abilities of the HSV RR1 peptides to bind the MHC class I *H-2K^b* molecule. Since the two peptides HSV-2 RR1 and HSV-1 RR1 (828 arg $\rightarrow$ met) did not render cells susceptible to lysis by CTL clone 1D11 and a substitution in the HSV-1 RR1 peptide at P1 (822 gln $\rightarrow$ arg) only partially affected recognition by CTL clone 1D11, it was necessary to determine whether these peptides could bind to the *H-2K^b* molecule. To investigate this possibility, the RMA-S cell line was used. RMA-S cells express extremely low levels of class I molecules at the cell surface because of a defect in the transporter required for peptides to enter the endoplasmic reticulum (53). Since RMA-S cells cannot transport peptide into the endoplasmic reticulum, the class I molecules remain empty and are rapidly degraded (38). However, the addition of an exogenous peptide known to bind the class I molecule has been shown to enhance or stabilize the surface expression of the respective class I molecules (25, 31, 69). The ability of the HSV-1 and HSV-2 RR1 peptides and peptides carrying amino acid substitutions at P1 and P7 (Fig. 7A) to stabilize the *H-2K^b* surface expression on RMA-S cells was tested. RMA-S cells were incubated for 16 h with various concentrations of peptides ranging from 1 to 100 μ M, after which time the surface expression of *H-2K^b* was determined by fluorescence-activated cell sorter analysis. Two monoclonal antibodies specific for *H-2K^b*, EH144 and 28-13-3, were used. Since it has been shown that the ability of some MHC class I-specific antibodies to interact with class I mole-

FIG. 4. Ability of the HSV-1-specific CTL clones to lyse target cells infected with an ICP6 deletion mutant, ICP6Δ. B6/WT-3 cells were infected with HSV-1, HSV-2, ICP6Δ, or a revertant of ICP6Δ (ICP6Δ^{+3.1}) at an MOI of 10 and harvested 4 h postinfection. The infected cells, in addition to uninfected cells, were used in a 5-h ⁵¹Cr release assay as described for Fig. 1, using the HSV-1-specific CTL clones 1C2, 1D3, 1D11, 1F8, 1G5, 2D1, and 2G5. The HSV-1/HSV-2 cross-reactive, gB-specific CTL clone 2D5 was included as a positive control for the recognition of the infected target cells. The results are shown for an effector-to-target cell ratio of 1.5:1.

cles can be influenced by the peptide which is bound in the peptide-binding cleft (25), we chose two antibodies which are known to interact with the *H-2K^b* molecule at opposite ends of the peptide-binding cleft. Monoclonal antibody EH144 interacts with peptide-bound *H-2K^b*, specifically in the region encompassing the α1 helix at the carboxy-terminal end of the peptide-binding cleft (1, 25). Monoclonal antibody 28-13-3 interacts with peptide-bound *H-2K^b* in a region encompassing the α1 and α2 helices at the amino terminus of the peptide-binding cleft (25, 31). The binding of *H-2K^b*-restricted peptides containing arginine at P1 has been shown to interfere with the recognition of the *H-2K^b* molecule by monoclonal antibody 28-13-3. It is expected that the HSV-2 RR1 peptide which contains an arginine residue at P1 would similarly affect recognition of the *H-2K^b* molecule by this antibody (25). The results presented in Fig. 7B for EH144 reveal that all of the RR1 peptides were capable of binding to the *H-2K^b* molecule, resulting in the stabilization of the surface expression of *H-2K^b* on RMA-S cells as detected by antibody EH144. Differences in the stabilizing capacities of these peptides were observed only at the lowest concentration of 1 μM. The peptide designated SV40 T Ag Site II/III, which binds to the *H-2D^b* molecule (17), did not stabilize the *H-2K^b* expression. The results of a stabilization assay using antibody 28-13-3 are shown in Fig. 7C. The data show that the binding of the HSV-1 RR1 and HSV-1 RR1 (828 arg→met) peptides could be detected by antibody 28-13-3. However, as expected, the binding of HSV-2 RR1 to *H-2K^b* could not be detected because of the presence of arginine at P1. However, the presence of arginine in the HSV-1 RR1 peptide due to an amino acid substitution at P1 (822 gln→arg) did not abrogate the reactivity of antibody 28-13-3. This may be due to a change in the conformation of the *H-2K^b*-peptide complex as a result of arginine at P7 of the HSV-1 RR1 peptide allowing antibody 28-13-3 to recognize an epitope on the *K-2K^b* molecule. These results indicate that the lack of recognition of the HSV-2 RR1 and HSV-1 RR1 (828 arg→met) peptides by the HSV-1 RR1-specific CTL is not due to an inability of these peptides to bind the MHC class I *H-2K^b* molecule.

FIG. 5. Ability of HSV-1 ICP6 (RR1)-specific CTL to recognize HSV-1 RR1 822-829. (A) HSV-1 RR1 synthetic peptides and their corresponding amino acid sequences. (B) ⁵¹Cr-labeled B6/WT-3 cells were pulsed for 30 min at 37°C with HSV-1 RR1 474-482 (1 μM), HSV-1 RR1 822-829 (1 μM), or HSV-1 gB 498-505 (0.1 μM). These peptide-pulsed cells were used as target cells in a standard cytotoxicity assay. The HSV-1 RR1-specific CTL clones 1D3, 1D11, and 1F8 and the HSV-1/HSV-2 cross-reactive, gB-specific CTL clone 2D5 were used as effector cells at an effector-to-target cell ratio of 2:1.

A.	PEPTIDE NAME	PEPTIDE SEQUENCE
■	HSV-1 RR1 822-829	gln-thr-phe-asp-phe-gly-arg-leu
●	HSV-2 RR1 829-836	arg-thr-phe-asp-phe-gly-met-leu
▲	HSV-1 RR1 822-829 (822 gln → arg)	arg-thr-phe-asp-phe-gly-arg-leu
◆	HSV-1 RR1 822-829 (828 arg → met)	gln-thr-phe-asp-phe-gly-met-leu

FIG. 6. The amino acid residue at P7 of HSV-1 RR1 confers specificity for recognition by HSV-1 RR1-specific CTL clone. (A) RR1 synthetic peptides and their corresponding amino acid sequences. The amino acid substitutions in HSV-1 RR1 822-829 are indicated in *italics*. (B) ^{51}Cr -labeled B6/WT-3 cells were peptide pulsed as described for Fig. 5 with one of the peptides listed (A) at concentrations of 10, 1, 0.1, 0.01, 0.001, and 0.0001 μM and used in a standard cytotoxicity assay. The HSV-1 RR1 822-829-specific CTL clone 1D11 was used as the effector at an effector-to-target cell ratio of 3:1.

Recognition of the HSV-1 RR1 822-829 epitope expressed within a chimeric T antigen. The region encoding HSV-1 RR1 amino acids 822 to 829 was cloned into SV40 large T antigen at position 350. This chimeric large T antigen was used to immortalize C57BL/6 mouse embryo fibroblasts, from which were derived a cell line expressing the chimeric large T antigen [B6/T350 HSV-1 RR1 (822-829)]. The ability of the HSV-1 RR1 822-829-specific CTL clone 1D11 to lyse B6/T350 HSV-1 RR1(822-829) cells was determined in a standard cytotoxicity assay (Fig. 8). The B6/T350 HSV-1 RR1(822-829) target cells were recognized efficiently by the CTL clone 1D11, as were HSV-1-infected B6/WT-3 cells. B6/pPVU-0 and B6/WT-3 target cells, both of which express only wild-type T antigen, were not recognized. These results confirm that HSV-1 RR1 822-829 serves as a target for the HSV-1-specific CTL and that such an epitope is able to be expressed and processed in the context of a heterologous protein for recognition by the HSV-1 RR1-specific CTL clone 1D11.

DISCUSSION

The results presented in this study have identified an $H-2K^b$ -restricted, HSV-1-specific CTL recognition determinant in the HSV-1 ribonucleotide reductase large subunit (ICP6, RR1). This CTL epitope has been mapped to amino acids 822 to 829 of the HSV-1 RR1, using HSV-1-specific CTL clones, HSV-1 $\times$ HSV-2 intertypic recombinants, a deletion mutant of RR1, and synthetic peptides. The results have further shown that the HSV-1 type specificity of CTL recognition of this epitope in RR1 is dictated to a large extent by the amino acid at position 828 and to lesser extent by the residue at position 822. Furthermore, the authenticity and independence of this CTL epitope from surrounding residues were confirmed by its expression in an SV40 T antigen-based vector.

Our demonstration of $H-2K^b$ -restricted, HSV-1-specific CTL clones which recognize RR1, a nonstructural protein ex-

pressed early in HSV infection, shows for the first time that a herpesvirus-encoded protein with known enzymatic function provides a target for CTL. In an infected cell, the HSV RR1 interacts with the small subunit of ribonucleotide reductase to form the biologically active enzyme which is capable of reducing ribonucleotides to deoxyribonucleotides for the production of precursors necessary for viral DNA synthesis (20, 54). The biological significance of RR1 has been demonstrated in studies showing that it is critical for efficient viral growth and pathogenesis in vivo (13, 27, 29). The recognition of nonstructural proteins by CTL has been demonstrated in other viral systems. For example, the nonstructural T antigen of SV40 and influenza virus nucleoprotein have been shown to provide a target for CTL (65, 70, 77). In addition, the immediate-early 72-kDa protein and pp89, encoded by human and murine cytomegaloviruses, respectively, have been shown to be targets for CTL (12, 56). Epstein-Barr virus nuclear antigens have also

A.	PEPTIDE NAME	PEPTIDE SEQUENCE
■	HSV-1 RR1 822-829	gln-thr-phe-asp-phe-gly-arg-leu
●	HSV-2 RR1 829-836	arg-thr-phe-asp-phe-gly-met-leu
▲	HSV-1 RR1 822-829 (822 gln → arg)	arg-thr-phe-asp-phe-gly-arg-leu
◆	HSV-1 RR1 822-829 (828 arg → met)	gln-thr-phe-asp-phe-gly-met-leu
▼	SV40 TAG SITE II/III 223-231	cys-lys-gly-val-asn-lys-glu-tyr-leu

FIG. 7. Abilities of the HSV-1 RR1 and HSV-2 RR1 peptides to stabilize surface expression of the $H-2K^b$ molecule on RMA-S cells. (A) Synthetic peptides and their corresponding amino acid sequences. The amino acid substitutions in HSV-1 RR1 822-829 are indicated in *italics*. RMA-S cells were incubated for 16 h at 37°C with 100, 50, 10, and 1 μM peptide as indicated. Following incubation with the peptides, the peptide-pulsed RMA-S cells were reacted with antibody EH144 (B) and with antibody 28-13-3 (C) and screened for surface expression of the $H-2K^b$ molecule by fluorescence-activated cell sorter analysis. The mean fluorescence intensity of the peptide-pulsed RMA-S cells relative to unpulsed RMA-S and RMA cells was determined.

FIG. 8. Recognition of the HSV-1 RR1 822-829 epitope expressed in a chimeric SV40 large T antigen. B6/T350 HSV-1 RR1(822-829), B6/pPVU-0, and B6/WT-3 cells infected with HSV-1 at an MOI of 10 for 3 h were labeled with ^{51}Cr and used in a standard cytotoxicity assay against HSV-1 RR1 822-829-specific CTL clone 1D11 at effector-to-target cell ratios of 2:1 and 1:1.

been shown to provide targets for Epstein-Barr virus-specific CTL (47). The ability of two immediate-early, nonstructural proteins, ICP4 and ICP27, to provide targets for HSV-specific CTL has been demonstrated (3, 4, 40). In other viral systems, precedence has been established for the ability of CTL to recognize epitopes derived from virally encoded enzymes. The viral polymerases of influenza virus and human immunodeficiency virus provide targets for CTL (6, 7, 26).

The finding that RR1 provides an epitope for CTL recognition may have important implications for a potential role of these CTL in combating HSV infection early during the replication cycle. CTL have been shown to lyse vaccinia virus- as well as SV40-infected cells prior to the production of progeny virions (5, 81). It is expected that CTL specific for RR1 may be effective in lysing HSV-infected cells *in vivo* at an early time after infection, prior to HSV DNA synthesis and the production of progeny HSV, therefore limiting the severity of viral infection and perhaps reducing the degree of latent HSV in the sensory ganglia. The role of class I-restricted CTL in reducing the amount of virus at the time of HSV infection and in limiting the frequency of establishment of viral latency has been previously demonstrated (9). The mechanism of class I-restricted, CTL-mediated reduction of HSV pathogenesis may involve direct lysis of virus-infected cells and could also involve cytokines released by the CTL. Recent studies have shown that clearance of infectious HSV from the skin is dependent on the production of gamma interferon by CD8⁺ lymphocytes (62). We have collected preliminary data which show that *H-2K^b*-restricted, HSV-1 RR1-specific CTL clones synthesize gamma interferon upon antigenic stimulation (data not shown).

The studies reported here have demonstrated that the HSV-1 RR1 CTL determinant is type specific. Although type-specific T-cell responses have been previously demonstrated for HSV (11, 35, 51, 67, 78), the identities of these CTL epitopes were not known. The search for the HSV-1 RR1 epitope was made possible by the isolation of HSV-1-specific CTL clones from B6 mice immunized with HSV-1 and by the identification of RR1 as the target protein by the use of HSV-1 × HSV-2 intertypic recombinants and a deletion mutant of HSV-1 RR1 (23). The peptide motif shown to bind to the

MHC class I *H-2K^b* molecule (19) aided in the precise identification of the CTL recognition epitope within HSV-1 RR1. A number of CTL epitopes, including those of HSV, have been identified by using the MHC-binding motifs (4, 16, 36, 49). The HSV-1 RR1 822-829 peptide (Gln-Thr-Phe-Asp-Phe-Gly-Arg-Leu) is eight amino acids in length and has anchor residues at P5 (Phe) and at P8 (Leu), which conforms to the predicted *H-2K^b*-binding motif (19). The corresponding peptide sequence within HSV-2 RR1 829-836 (Arg-Thr-Phe-Asp-Phe-Gly-Met-Leu) meets all requirements for binding to *H-2K^b* molecules but is not recognized by the HSV-1 RR1-specific CTL. Experimental evidence confirmed the ability of the HSV-2 RR1 829-836 peptide to bind the *H-2K^b* molecule; therefore, the inability of the HSV-1-specific, RR1-specific CTL to recognize the HSV-2 RR1 peptide must be at the level of TcR interaction. The HSV-2 RR1 829-836 peptide differs from HSV-1 RR1 822-829 at P1 (HSV-1, Gln; HSV-2, Arg) and at P7 (HSV-1, Arg; HSV-2, Met). The amino acid difference at P7 was shown to be responsible for the abrogation of CTL recognition, as an amino acid substitution of arginine at P7 with methionine completely abrogated recognition by the HSV-1-specific CTL clone. A substitution of glutamine at P1 with arginine in the HSV-1 RR1 peptide only partially affected recognition by the HSV-1-specific CTL clone. By using an *H-2K^b*-restricted nucleoprotein epitope (N52-59) of vesicular stomatitis virus, the residues at P1 and P7 have been shown to interact with the TcR (21, 59, 80). This finding suggests that the glutamine at P1 and arginine at P7 of the HSV-1 RR1 epitope may be involved in interaction with the TcR. Therefore, differences between the HSV-1 RR1 CTL epitope and the corresponding residues in HSV-2 RR1 at P1 and P7 determine HSV-1 specificity. It is clear that the failure of HSV-1 CTL clones to recognize the HSV-2 RR1 829-836 peptide is not due to the inability of the HSV-2 RR1 peptide to bind to *H-2K^b* molecule, as this peptide stabilized the expression of *H-2K^b* molecules at the surface of RMA-S cells (Fig. 7B). It is entirely possible that the presence of arginine at P1 and methionine at P7 in the HSV-2 RR1 peptide affects the conformation of the *H-2K^b* molecule, as shown by the failure of antibody 28-13-3 to recognize an epitope on this class I molecule. Previous studies (31) have indicated that the presence of arginine at P1 in an *H-2K^b*-restricted vesicular stomatitis nucleoprotein peptide when bound to an *H-2K^b* molecule blocked the recognition of the epitope by antibody 28-13-3. Interestingly, the results obtained in this study show that substituting arginine for glutamine at P1 of the HSV-1 RR1 peptide (arginine at P1 and P7) only partially affected the reactivity of antibody 28-13-3.

The identification of class I-restricted CTL viral epitopes has led to attempts to determine their role in inducing protection against viral infections in the immunized host. In a number of cases, synthetic peptides corresponding to the CTL epitopes or the epitopes expressed from vaccinia virus vectors or in heterologous proteins have been shown not only to induce the generation of CTL but also to provide immune protection. Molecularly engineered vaccines containing an *H-2L^d*-restricted lymphocyte choriomeningitis virus (LCMV) nucleoprotein epitope (14, 73) or *H-2D^b*-restricted glycoprotein epitopes (73) have been shown to protect mice from lethal LCMV infection. In addition, LCMV nucleoprotein (*H-2L^d*) and glycoprotein (*H-2D^b*) epitopes expressed in tandem, from a recombinant vaccinia virus, conferred protection in both *H-2^d* and *H-2^b* mice (73). In other studies, immune protection against lethal murine cytomegalovirus infection (18) and sporozoite-induced malaria (37) was achieved by the induction of CTL responses to a single CTL epitope. We have previously shown that a cell line immortalized by a chimeric SV40 large T

antigen containing the HSV gB 498-505 epitope (22) is able to induce gB 498-505-specific CTL in vivo (8). Likewise, we have expressed the HSV-1 RR1-specific CTL epitope 822-829 in SV40 T antigen and have shown that T-antigen-transformed cells containing the recombinant T antigen are susceptible to lysis by the RR1-specific CTL. Studies are currently in progress to determine if this recombinant T antigen is capable of inducing RR1-specific CTL and whether CTL specific for this RR1 epitope can provide immune protection against HSV infection in B6 mice.

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