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A Single Retroviral Gag Precursor Signal Peptide Recognized by FBL-3 Tumor-Specific Cytotoxic T Lymphocytes

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Several dominant T-cell receptors of cytotoxic T-lymphocyte (CTL) clones specific for FBL-3 tumor antigen were clonally amplified in mixed lymphocyte tumor cell cultures derived from an individual immune mouse. Every CTL clone analyzed had a common specificity for a single epitope in the precursor to cell membrane-associated nonstructural *gag*-encoded protein, Pr75^{gag}, which can be minimally identified by nine amino acid residues, SIVLCCLCL. This epitope is located within the hydrophobic signal sequence motif that mediates translocation of the protein into the endoplasmic reticulum. These novel observations suggest that expression of Pr75^{gag} in FBL-3 tumor cells led to the amplification of CTLs which recognize the signal sequence of the nonstructural *gag*-encoded glycoprotein precursor.

The cytotoxic T-lymphocyte (CTL) response to tumor antigen(s) induces a complex series of cellular events and plays a critical role in the elimination of tumors (2, 22, 30). To better understand such an immune response, we have isolated an anti-T-cell receptor (TCR) antibody (monoclonal antibody [MAb] N9-127) which discriminates a subset within tumor antigen-specific T cells and have shown specific TCR expression at an appreciably high frequency in $CD8^+$ T cells responding to a syngeneic Friend murine leukemia virus (F-MuLV) complex (FV)-induced erythroleukemia (FBL-3) generated in C57BL/6 (B6) mice (28). This dominant TCR idiotype ($127Id^+$) is characterized by conserved α- and β-chain amino acid sequences, which are normally varied within the V(D)J junctional nucleotide sequences (17). In the present report, we also isolated several CTL clones carrying dominant TCRs which were characterized by either Southern hybridizations of mixed lymphocyte tumor cell culture (MLTC) or cytofluorometric analysis with anti-V region antibodies. Thus, we suspected that the dominant TCRs with a limited target specificity are selected by a strong growth advantage.

Viral proteins that appear on the surface of FV-infected cells are encoded by the *env* and *gag* genes of each of the two component viruses of FV, helper F-MuLV and replication-defective spleen focus-forming virus (15, 51). The major retroviral proteins expressed by FV-induced leukemia are products of the *env* and *gag* genes of F-MuLV (31, 41). Previous studies have shown the participation of both *env* and *gag* gene-encoded cell surface proteins for CTL recognition (18, 23, 38). Although a peptide sequence in the *env* gene was identified as a CTL epitope (40), the viral *gag* epitope(s) remains to be investigated.

We exploited the use of recombinant vaccinia viruses (rVVs) expressing individual F-MuLV genes and showed that the vast majority of CTLs have a common specificity for the leader sequence of Gag precursor protein Pr75^{gag}. We further mapped the epitope by using synthetic peptides and identified a nine-residue peptide as a minimal CTL epitope restricted by the D^b molecule. Surprisingly, consensus H-2D^b-binding motifs were not well represented in this hydrophobic signal sequence carrying three cysteine residues, SIVLCCLCL.

MATERIALS AND METHODS

In vivo immunization with tumor cells. Six- to eight-week-old B6 mice were inoculated with 5×10^6 FBL-3 tumor cells of B6 origin by subcutaneous injection into the dorsal region. After complete tumor regression, mice were given up to four intraperitoneal booster doses of 5×10^6 tumor cells at 2-week intervals.

Isolation of T-cell clones. Class I major histocompatibility complex (MHC) (H-2D^b)-restricted CTL clones specific for FBL-3 tumor cells were isolated from the immune spleen cells initially on a two-cell-per-well basis from a limiting dilution culture of day 7 MLTC cells and recloned at 0.5 cells per well as described previously (25, 28). Cytofluorometric analyses were performed as described previously (28). Antibodies used were MAb RR3-16 (anti-$V_{\alpha 3}$) (53), MAb KJ25 (anti-$V_{\beta 3}$) (36), MAb 44-22-1 (anti-$V_{\beta 6}$) (32), MAb F23.1 (anti-$V_{\beta 8}$) (50), and MAb RR3-15 (anti-$V_{\beta 11}$) (5).

Southern blot analysis. Southern blot analyses were performed as described previously (28). DNA probes used were $J_{\beta 2}$ (2.3-kb *Eco*RI fragment), $V_{\alpha 11}$ (2.2-kb *Hin*dIII fragment subcloned from V_α of hybridoma AN6.2, a gift from L. Hood) (24), and $V_{\alpha 3}$ (0.5-kb *Apa*LI cDNA fragment prepared from CTL clone B413-2.3) (17).

TCR cDNA cloning and sequencing. The TCR cDNA for CTL clone SB14-11 was isolated from a cDNA library with the C_α and C_β probes and subcloned into pHSG399 as described previously (17). cDNAs of other CTL clones were amplified by PCR (42) with the PCR primers of $V_{\alpha 11}$ (AATTTTACGACCACCAT GAGGGCT) (3, 24), $V_{\beta 14}$ (ACCATAAAGGGGAAATCAAGCCCT) (4, 24), $V_{\alpha 3}$ (CAGCTGAGATGCAAGTATTCCTAC) (19), $V_{\beta 3}$ (ATCCCTGAAAAG GGACATCCAGTT) (7), C_α (CTCGGTCAACGTGGCATCACAGGG) (43), and $C_{\beta 2}$ (ATGGAACTGCACATTGGCAGCGGAA) (27). Nucleotide sequences were determined by the dideoxy-chain-termination method (44) with C_α primer CAGGCAGAGGGTGCTGTCCTG and C_β primer TGTTTGCAATC TCTGCTTTTG.

Preparation of rVVs. rVVs were constructed by standard methods with the transfer plasmid pSC11SB (29). Cloned fragments of the *gag* gene of F-MuLV (strain FB29) are shown in Fig. 3. Vaccinia virus recombinant r20-6v was constructed to express the entire Pr65^{gag} starting from the ATG initiation codon by replacing the 5′ portion of the *gag* gene with a synthetic oligonucleotide as previously described (29). In recombinant ΔAB, the Pr65^{gag} region from *Afl*II (position 642) to *Bst*EII (position 2451) is deleted. These resulting plasmids were

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transfected into monkey CV-1 cells that had been infected with a wild-type vaccinia virus to accomplish homologous recombination. Three independent clones of rVV ΔAB were selected both for their lack of the viral thymidine kinase activity and for the presence of cotransferred bacterial β-galactosidase activity. All resultant rVVs were plaque purified three times. All rVV-infected cells expressed Gag proteins of expected antigenicity and molecular masses (29). Cells infected with rVVs containing the CTG initiation codon at position 355 also showed Gag antigenicity both on the surface of live cells and in the cytoplasm of fixed cells (29). rVVs expressing the influenza virus hemagglutinin gene (V36) (49) or the F-MuLV *env* gene (p4-4) (12) have been previously described.

Peptide synthesis. Peptides were synthesized as described previously (20). In brief, the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase method (11) was used, and synthesis began from the C-terminal amino acid anchored on Wang resin. Since the coupling of amino acids was not efficient for the leader sequence, especially for IVLCC, every peptide was synthesized manually and each coupling was monitored by ninhydrine reaction. However, the peptides containing the hydrophobic leader sequence were insoluble in aqueous buffers. Therefore, two lysine residues were added to the C terminus and a third lysine was added to the N terminus to improve the solubility.

Assay for cytotoxic activity. Since FBL-3 carries endogenous retroviral antigens, cells of a heterologous species (monkey) were favored as an indicator cell line for the cytotoxic assay of synthetic peptides. A transfected monkey CV-1 cell line was established by introducing the restricting MHC class I gene, H-$2D^b$ (1), by cotransfecting with pSV2neo. Selection was conducted at 2 mg of G418 per ml (geneticin sulfate; Gibco Laboratories, Grand Island, N.Y.), and stable G418-resistant transfectants, CV-$1D^b$, were isolated.

To induce antigen-specific lysis of rVV-infected target cells, CV-$1D^b$ cells (2×10^6) were infected overnight in a 6-cm plastic dish with either of the rVVs at a multiplicity of infection of 2. Infected or noninfected CV-$1D^b$ cells were incubated for 1 h with 3.7 MBq of ^{51}Cr, washed three times, and then plated into V-bottom 96-well plates at 10^4 cells per well. ^{51}Cr-labeled CV-$1D^b$ cells were preincubated for 30 min with a synthetic peptide in a total volume of 200 μl containing 2.5 mM 2-mercaptoethanol and 2.5 mM acetic acid. For competition experiments, the target cells were preincubated for 15 min with the competitor peptide before addition of the antigenic peptide.

After a 6-h incubation with varying numbers of effector CTLs, 100 μl of supernatant was removed from each well, and radioactivity was measured in a gamma counter. The percent specific ^{51}Cr release was taken as a measure of CTL lytic activity and calculated by the following formula: 100 × (experimental release − spontaneous release)/(total release in detergent − spontaneous release). The data shown are the means of duplicate determinations with spontaneous release values of <20%.

Nucleotide sequences. The nucleotide sequence data for TCR reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers D45219-22, D45225, and D45226.

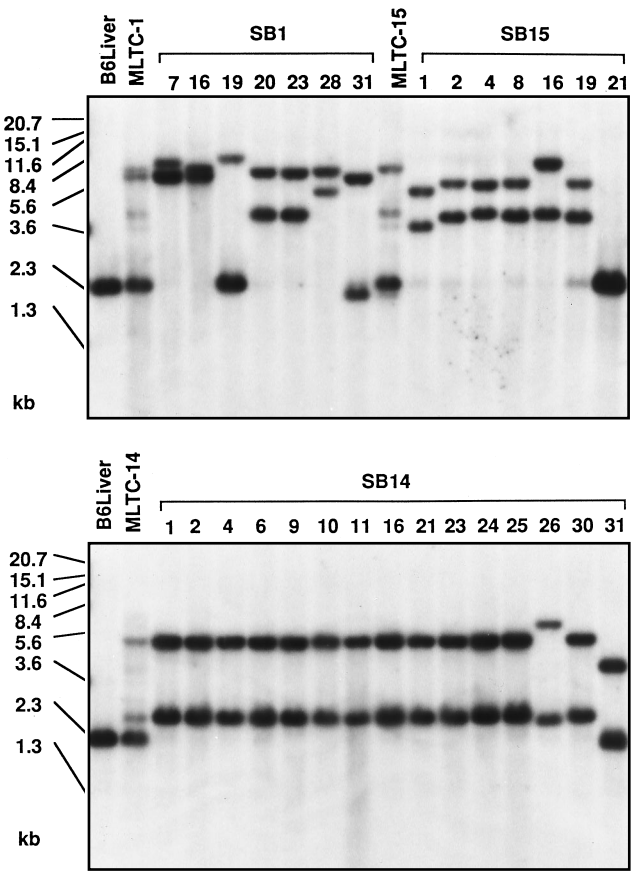

FIG. 1. Southern blot analysis of a T-cell clone series, SB1, SB14, and SB15, isolated from individual MLTC-1, -14, and -15, respectively. DNA was digested with *Eco*RI, subjected to electrophoresis, and hybridized with $J_{\beta 2}$ probe.

RESULTS

Predominance of T cells sharing similar TCR rearrangements in MLTC. A significant proportion (11%) of the CTL clones isolated from FBL-3-specific MLTC cells carried the idiotype ($127Id^+$) which was defined by MAb N9-127 and characterized by a specific combination of TCR α- and β-chain genes ($V_{\alpha 1}$ $J_{\alpha 112\text{-}2}$/$V_{\beta 10}$ $D_{\beta 2.1}$ $J_{\beta 2.7}$) (17, 28). In order to see whether this biased TCR usage seen in individual MLTC bulk cultures is supported at the level of TCR gene rearrangements, we performed a Southern blotting analysis. We prepared MLTC from 22 mice immunized four times with FBL-3 cells. Rearrangements at the β-chain loci were examined with the $J_{\beta 2}$ probe. Several distinctly rearranged bands of β chain were observed as shown in MLTC-1, -14, and -15 (Fig. 1).

We isolated 30 T-cell clones from each limiting dilution culture of MLTC-1, -14, and -15. Most were $CD8^+$ T cells with a small number of $CD4^+$ clones (four for MLTC-1; one for MLTC-14; none for MLTC-15). As expected from the frequency of $127Id^+$ cells in the MLTC measured by cytofluorometry (19.8% for MLTC-1; 1.2% for MLTC-14; 1.0% for MLTC-15), the frequency of such $127Id^+$ clones was 8/30 for MLTC-1, 1/30 for MLTC-14, and 0/30 for MLTC-15, respectively. We subjected several $127Id^-$ T-cell clones to Southern blot analysis. Rearranged bands observed in bulk MLTC were more or less reproduced on a clonal level (Fig. 1). Three distinctly rearranged bands of MLTC-1, 6.8, 6.2, and 4.2 kb, were reproduced in four, three, and two of seven clones, respectively. Two clones (SB1-20 and 1-23) faithfully reproduced both distinct bands (6.8 and 4.2 kb) of MLTC-1. $127Id^+$ clone SB1-28, included as the reference, reproduced a 5.1-kb *Eco*RI band representing $V_{\beta 10}$ $D_{\beta 2.1}$ $J_{\beta 2.7}$. Two rearranged bands of MLTC-15 (4.3 and 3.8 kb) were reproduced in SB15 clones, but the 7.4-kb major band was not. Both distinctly rearranged bands of MLTC-14 (5.4 and 2.8 kb) were reproduced in 13 of 15 clones, whereas the remaining clones, SB14-26 and 14-31, reproduced the minor bands 6.2 and 4 kb, respectively. Every clone was positive for CD8 except clone SB14-31 ($CD4^+$).

Predominance of $V_{\alpha 3}$/$V_{\beta 3}$ TCR usage in MLTC. The $V_{\alpha 3}$ gene was common for TCR in three $127Id^-$ CTL clones (17). We found $V_{\alpha 3}$ rearrangement in each of the 7 clones from both SB1 and SB15 and in 2 clones from 15 SB14 clones (data not shown). High-frequency $V_{\alpha 3}$ usage in FBL-3-specific CTL clones encouraged us to examine the CTL populations with antibodies specific for individual TCR V regions. We prepared FBL-3-specific MLTC cells from 11 mice after immunization twice in vivo and analyzed the T-cell population by cytofluorometry with antibodies anti-$V_{\alpha 3}$, anti-$V_{\beta 3}$, anti-$V_{\beta 6}$, anti-$V_{\beta 8}$, and anti-$V_{\beta 11}$. Table 1 shows the appearance of $V_{\alpha 3}{}^+$ and $V_{\beta 3}{}^+$ T cells in MLTC from 11 different mice (MLTC-101 to -111). As compared with the known frequency of $V_{\alpha 3}{}^+$ ([3.4 ± 0.4]%) in the $CD8^+$ splenic T cells from B6 mice (53), $V_{\alpha 3}{}^+$ T cells were selectively enriched in most MLTC. In two of these mice (MLTC-110 and -111), exceedingly high proportions of $V_{\alpha 3}{}^+$ and $V_{\beta 3}{}^+$ T cells were observed. In MLTC with low populations of $V_{\beta 3}{}^+$ cells, $V_{\beta 6}$ is enriched to 15.4% in MLTC-109 and $V_{\beta 8}$ is enriched to 43.0% in MLTC-106 (data not

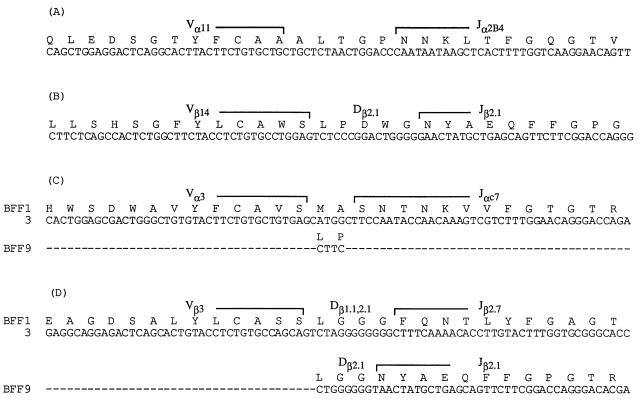

FIG. 2. Nucleotide sequences of TCR α (A) and β (B) cDNA from clone SB14-11 and TCR α (C) and β (D) cDNA from clones BFF1, -3, and -9. Assigned protein sequence is shown by the one-letter code. Hyphens indicate identity with the BFF1 cDNA sequence. Sequences compared are $V_{\alpha 11}$ (3, 24), $J_{\alpha 2B4}$ (3), C_α (43), $V_{\beta 14}$ (4, 24), D_β (47), $J_{\beta 2}$ and $C_{\beta 2}$ (27), $V_{\alpha 3}$ (19), $J_{\alpha C7}$ (14), and $V_{\beta 3}$ (7).

shown). No specific enrichment of $V_{\beta 11}$ was observed in these MLTC cells. We isolated 23 T-cell clones from limiting dilution cultures of MLTC-111 cells. Eight clones were $V_{\alpha 3}{}^+$ $V_{\beta 3}{}^+$, seven clones were $V_{\alpha 3}{}^+$ $V_{\beta 3}{}^-$, and eight clones were $V_{\alpha 3}{}^-$ $V_{\beta 3}{}^-$ (data not shown). Consistent with these results, 41 of 99 127Id$^-$ CTL clones isolated from a separate group of five mice were $V_{\alpha 3}{}^+$. Among these, 13 clones were $V_{\beta 3}{}^+$ (data not shown).

TCR cDNA of CTL clones predominated in MLTC. We cloned cDNA for the α and β chains of SB14-11, a dominant SB14 CTL clone, and determined its nucleotide sequences. Clone SB14-11 was found to contain a TCR encoded by $V_{\alpha 11}$ $J_{\alpha 2B4}$ and $V_{\beta 14}$ $D_{\beta 2.1}$ $J_{B2.1}$ gene rearrangements as shown in Fig. 2A and B. This sequence data provided us with probes

TABLE 1. Frequency of $V_{\alpha 3}{}^+$ and $V_{\beta 3}{}^+$ T cells in MLTC

MLTC no.	% Positive cells in MLTC blasts	
	$V_{\alpha 3}$	$V_{\beta 3}$
101	22.2	6.8
102	15.0	2.8
103	48.7	5.7
104	17.6	3.2
105	19.2	5.8
106	3.4	2.7
107	12.6	7.5
108	7.9	5.4
109	20.7	4.8
110	45.5	22.2
111	67.9	35.6
Mean ± SD	25.5 ± 18.0	9.3 ± 9.6

useful for Southern blot analysis of the α chain for other SB14 clones. SB14 clones that share the $J_{\beta 2}$ rearrangements shown in Fig. 1 were then examined with a $V_{\alpha 11}$ probe. Whereas four bands (20, 9.2, 5.2, and 4.3 kb) cross-hybridizing to $V_{\alpha 11}$ were discernible in the germline DNA, a 2.8-kb rearrangement was identified in *Hin*dIII digests of every dominant SB14 CTL clone (data not shown). We selected five more SB14 clones, SB14-4, -14-9, -14-10, -14-23, and -14-24, and determined the cDNA junctional sequence after amplification by PCR. Nucleotide sequence diversities generated at the junctional region of both α and β chains and the assigned amino acid sequences were identical in all six of these SB14 clones (data not shown).

Three of eight $V_{\alpha 3}{}^+$ $V_{\beta 3}{}^+$ CTL clones from MLTC-111 were selected, and the cDNA sequence was determined after amplification with a set of PCR primers, $V_{\alpha 3}/C_\alpha$ and $V_{\beta 3}/C_\beta$ (Fig. 2C and D). Two clones (BFF1 and -3) shared an identical sequence composed of $V_{\alpha 3}$ $J_{\alpha c7}$ and $V_{\beta 3}$ $D_{\beta 1.1}$ $D_{\beta 2.1}$ $J_{\beta 2.4}$.

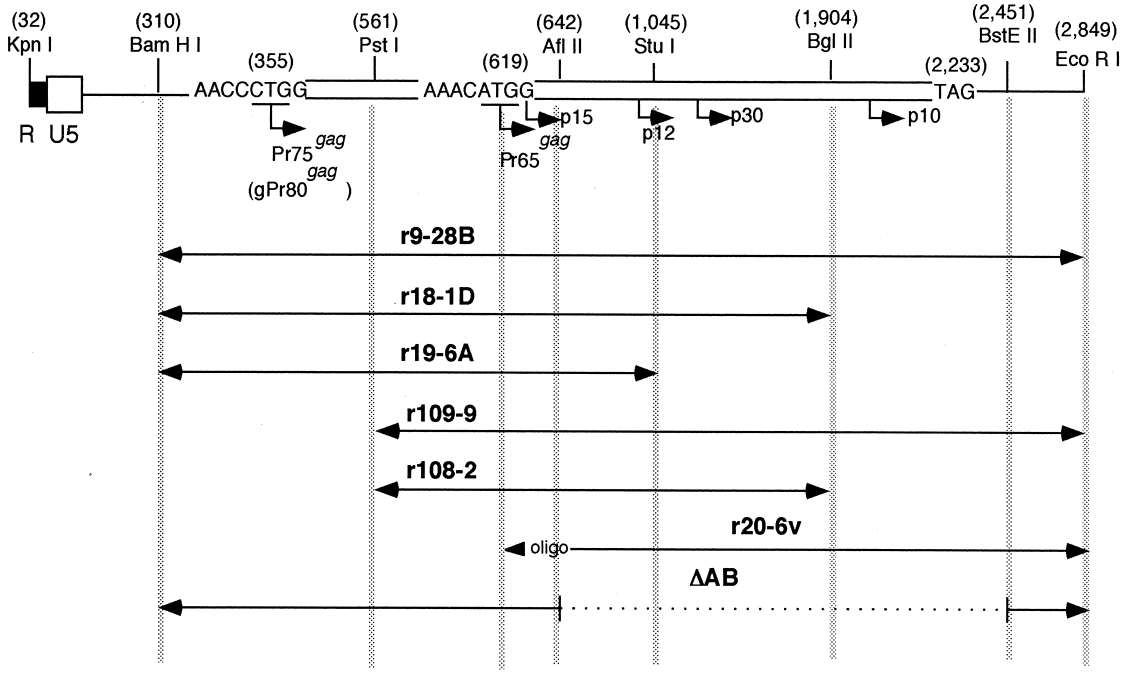

FIG. 3. A diagrammatic representation of the F-MuLV *gag* gene and construction of the rVVs. Details are described in Materials and Methods. Base numbering of the *gag* gene is according to the complete sequence of F-MuLV FB29 (34).

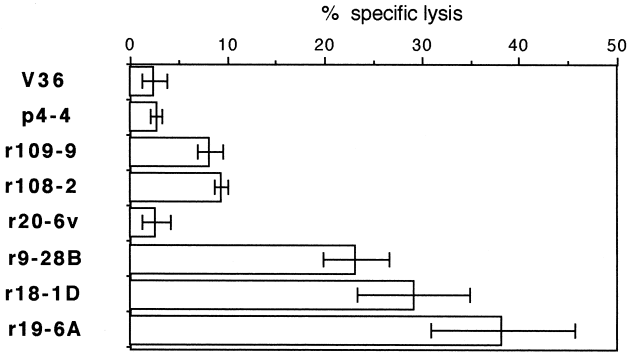

FIG. 4. Viral target antigen specificity of polyclonal CTL preparations from MLTC. Cytotoxicity assays on CV-1D^b cells infected with rVVs expressing different *gag* regions were performed at an effector/target (E/T) ratio of 90:1. Control targets included rVV expressing influenza virus hemagglutinin (V36) and an entire *env* gene (p4-4). The results represent the mean of three separate experiments performed independently with three donor B6 mice. Error bars represent standard errors of the mean.

Another clone, BFF9, contained the structure of $V_{\alpha 3}$ $J_{\alpha c7}$ and $V_{\beta 3}$ $D_{\beta 2.1}$ $J_{\beta 2.1}$. The junctional nucleotide sequence of $V_{\alpha 3}$ $J_{\alpha c7}$ was different in these two groups.

Localization of CTL epitope with rVVs carrying F-MuLV gene segments. To allocate the epitope of dominant CTL clones specific for FBL-3 tumor antigen to the F-MuLV genome, we used a panel of rVVs expressing portions of the F-MuLV genome (Fig. 3). Syngeneic MLTC cells generated in response to immunization with FBL-3 cells were assayed for their ability to recognize and lyse rVV-infected CV-1D^b cells. The results shown in Fig. 4 demonstrated that MLTC cells lysed target cells expressing the entire *gag* region (r9-28B) but not those expressing the entire *env* region (p4-4). Unexpectedly, cells infected with rVV expressing the entire *gag*-encoded precursor protein of virion core Pr65^{gag} (r20-6v) were not susceptible to lysis by FBL-3-specific CTLs. Only the cells infected with rVVs that contained the 5′-leader sequence upstream of the ATG start codon for Pr65^{gag} (r9-28B, r18-1D, and r19-6A) were lysed by MLTC cells despite deletions of the 3′ portion of the Pr65^{gag} gene in the latter two rVVs. Interestingly, the rVVs with the truncated Pr75^{gag} gene lacking the CTG (position 355) initiation codon (r109-9 and r108-2) induced a low but significant cell lysis by FBL-3-specific MLTC cells. These data thus suggest that the *gag* region from positions 561 (*Pst*I site) to 619 (ATG initiation codon for Pr65^{gag}) may encode at least a portion of the antigenic specificities that are recognized by anti-FBL-3 CTLs.

The localization of the epitope for representative CTL clones predominantly in FBL-3-specific MLTC was further examined with rVVs expressing F-MuLV genes (Fig. 5 and 6). CTL clones SB14-11 and BFF-9 were isolated in the present study, and two other clones, B413-4 and B5-10, carried the 127Id$^+$ idiotype which was defined by MAb N9-127 (17, 28). Each clone exhibited the same specificity as FBL-3-specific MLTC cells. To confirm the CTL target specificities of the 5′-leader sequence, we constructed a new rVV ΔAB carrying only the 5′-leader sequence by removing the *Afl*II (position 642)-*Bst*EII (position 2451) fragment of an entire structural *gag* region from r9-28B. As shown in Fig. 6, cells infected with rVV ΔAB were lysed efficiently by the 127Id$^+$ clones, B413-4 and B5-10.

Determination of CTL epitope with synthetic peptides. Studies with rVVs localized the CTL epitope within the 5′-leader sequence of Pr75^{gag} shown in Fig. 7. The significant but decreased cytotoxicity against target cells infected with rVVs r109-9 and r108-2 may indicate the critical peptide length. In these two rVVs, the first three hydrophobic residues of the signal sequence (L-67, V-68, and C-69) are deleted. We synthesized nested sets of peptides downstream of C-69 (Fig. 7) and analyzed them for their ability to sensitize target cells to lysis by the representative 127Id$^+$ CTL clone B413-4 (Fig. 8). Peptide 1 of the sequence farthest downstream was ineffective for sensitizing target cells. Peptides 3, 4, and 5 sensitized CV-1D^b target cells efficiently, leading to significant lysis by the 127Id$^+$ CTL clone. Peptides 2 and 6 with endpoints at I-72 and C-78, respectively, gave only background levels of lysis, which implies that the minimal epitope is a nonamer, SIVLCCLCL

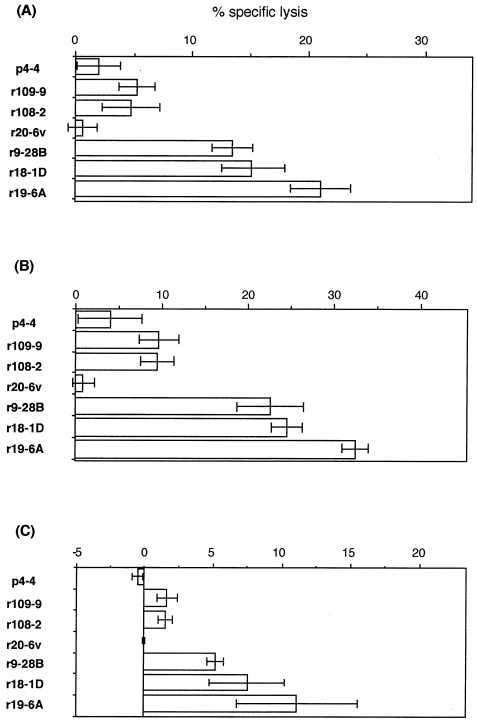

FIG. 5. Viral target antigen specificity of CTL clones SB14-11 (A), BFF9 (B), and B413-4 (C). Cytotoxicity assays on CV-1D^b cells infected with rVVs were performed at an E/T ratio of 30:1. Experiments were repeated three times. Other indications are described in the legend to Fig. 4.

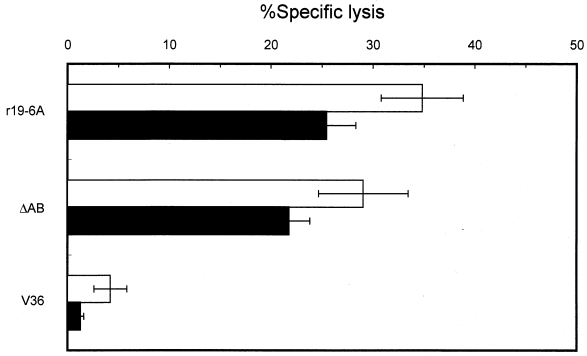

FIG. 6. Cytotoxicity assays of effectors, B413-4 (open bar) and B5-10 (closed bar), on CV-1D^b cells infected with rVVs expressing Pr75^{gag} signal sequence, r19-6A and ΔAB. E/T ratio is 33:1. Other indications are described in the legend to Fig. 4.

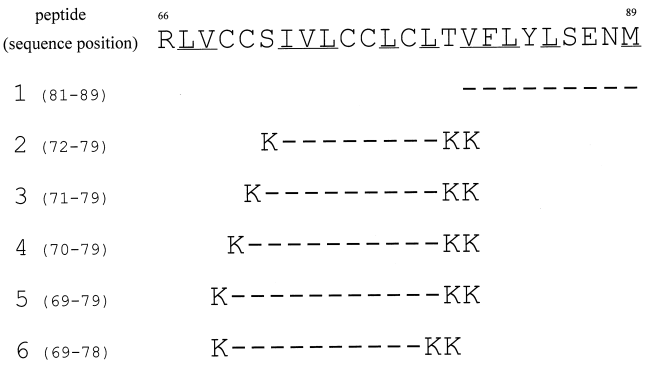

FIG. 7. Amino acid sequence of signal peptide of membrane protein precursor Pr75^{gag} and the peptides used for cytotoxicity assays on CV-1D^b cells. Neutral nonpolar residues are underlined. M-89 is encoded by the ATG (position 619) initiation codon for Pr65^{gag}. Synthesized sequences are shown by bars. Every peptide except peptide 1 was flanked by lysine residues (K) at both ends.

(peptide 3). Presentation of peptide 3 was inhibited by an excess of influenza virus NP366-374 peptide (39, 52) containing H-2D^b-binding motif (xxxxNxxxM) (Fig. 8). This result demonstrates that, although SIVLCCLCL does not fulfill the D^b-binding motif, it is actually binding to D^b and is recognized by the CTL.

DISCUSSION

The F-MuLV *gag* gene codes for two different Gag precursor proteins (10). The precursor to virion core structural proteins is Pr65^{gag}, which is myristylated on the N-terminal glycine and proteolytically cleaved into four proteins in the mature virion: p15, p12, p30, and p10, from the N terminus to the C terminus. Alternatively translated Pr75^{gag} precursor protein gives rise to an intermediate gPr80^{gag} glycosylated molecule, and further processing yields the gP95^{gag} and gP85^{gag} products expressed on the surface of infected cells. The translation of Pr65^{gag} starts at the usual ATG initiation codon at position 619, while that of Pr75^{gag} is believed to start at an upstream CTG codon at position 355 within a favorable initiation context (35). In the present study, we expressed the entire F-MuLV *gag* gene or its shorter fragments with or without the CTG initiation site in CV-1D^b cells and showed that CTL epitope is encoded in the leader sequence of Pr75^{gag}. Our study confirmed previous results identifying the Gag membrane protein gP85^{gag} as a target molecule recognized by Moloney MuLV-specific CTL clones (54).

In the previous studies (20, 45), we isolated FBL-3 tumor-specific helper T cells reactive to the determinants on F-MuLV envelope (Env) protein. Expression of the Env gp70 on FBL-3 cell surfaces was confirmed by membrane immunofluorescence with MAbs 48 and 720 (6, 37) (data not shown). However, no CTL response directed against Env was observed among MLTC cultures induced by FBL-3. This may be due to the presence of immunodominant CTL epitope of the leader sequence of Pr75^{gag}.

rVVs r108-2 and r109-9 contained the fragment of Pr75^{gag} leader sequence downstream of the *Pst*I site, which was utilized to construct these rVVs. There is no usual ATG codon or alternative CTG codons within a favorable initiation context in the truncated leader sequence. Nevertheless, both r108-2 and r109-9 reproducibly sensitized target cells to lysis by FBL-3-specific CTLs, while rVV r20-6v that lacked the leader sequence upstream of the ATG initiation codon for Pr65^{gag} did not. In addition, induction of protective immunity against FV infection in vivo was more remarkable with the rVVs r108-2 and r109-9 than with r20-6v (29). These results suggest that the portion of leader sequence downstream of the *Pst*I site is translated in cells infected with the rVVs r108-2 and r109-9. Although translation initiation at non-AUG triplets is shown in mammalian cells (33), actual mechanisms of the translation initiation in the truncated leader sequence are currently unknown. We constructed the new two rVVs, expressing a 16-mer, V-68 to L-83, flanked by the ATG initiation codon and stop codon, and expressing a 70-mer, L-1 to C-70. Only the rVV expressing the 16-mer peptide containing the minimum 9-mer target showed full cytolytic activity (unpublished results).

Most secretory proteins contain a signal sequence of 16 to 30 amino acid residues that initiates transport across the endoplasmic reticulum membrane (26). Usually, a signal sequence has one or more positively charged amino acids near the N terminus, followed by a continuous stretch of 6 to 12 hydrophobic residues. We identified a nine-residue CTL epitope, SIVLCCLCL (peptide 3), embedded in a typical signal peptide sequence as shown in Fig. 7. This signal sequence is conserved between F-MuLV and Moloney MuLV (34, 46). Consistent with this fact, FBL-3-specific CTL clones showed cross-reactivity to MBL-2 lymphoma cells induced by Moloney MuLV complex (28). This 9-mer peptide was solubilized for in vitro cytolytic assay by adding terminal lysine residues to decrease hydrophobicity. However, expected negative influence of carboxyl-terminal additions to antigenic peptides (13) was not drastic, possibly because of the presence of a labile carboxypeptidase N present in serum with specificity for terminal lysine or arginine substrates (48).

Naturally processed peptide fragments bound to the H-2D^b molecule showed a consensus sequence of nine residues, xxxxNxxxM, in which the conserved residues Asn-5 and Met-9 are likely to be involved in anchoring the peptide to H-2D^b molecules (13). Asn at position 5 was invariant, and position 9 contained a strong signal for Met, an intermediate one for Ile, and a weak one for Leu. However, invariant anchor residue Asn at position 5 is substituted by Cys in the FBL-3-specific

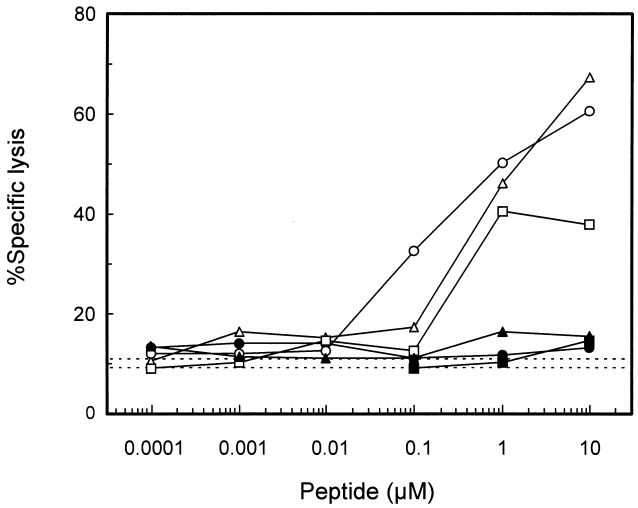

FIG. 8. Titration of synthetic peptides 2 (▲), 3 (□), 4 (○), 5 (△), and 6 (●) with B413-4 CTL clones and CV-1D^b target cells. Results are shown as percentage of specific lysis at an E/T ratio of 10:1. The competition experiment for peptide 3 was performed in the presence of 50 μM influenza virus NP366-374 peptide (■). The upper broken line shows background level with NP366-374, and the lower broken line shows that without peptides.

CTL epitope (peptide 3) identified in the present study. Cys may be equivalent to Asn as a neutral polar amino acid. Less abundant Cys and Trp residues were not detected by sequencing of self-peptides eluted from MHC molecules possibly because of technical limitations (13). FBL-3-specific CTL epitopes derived from the signal peptide domains may be novel peptides processed by a second pathway distinct from the known cytoplasmic pathway as suggested by studies using an antigen-processing mutant cell line (16).

Although there is wide variability of biased usage of TCRs among different MLTC cultures induced by FBL-3, they showed the same level of cytolytic activity in vitro. According to the current tertiary structure model for TCR-MHC peptide interactions (9), the surface of an MHC protein-peptide complex could interact with the combining site of a TCR such that the CDR1 and CDR2 regions on V_α and V_β contact the side chains of the MHC α-helices and the centrally located CDR3 regions [encoded by the V(D)J junctions] contact the bound peptide. A single amino acid substitution (G to D), which changes MAb N9-127-mediated blocking susceptibility of the TCR, resided in the central N region of CDR3 of V_β chain in clones 8 and B5-10 (17). TCR sequences of FBL-3-specific CTL clones analyzed so far (16) (Fig. 2) showed the conserved residues in CDR3 regions, G in V_β chain and N in V_α chain, respectively. The minimum epitope 9-mer peptide recognized by these CTL clones, ^{71}SIVLCCLCL^{79}, shows an alternate structure composed of neutral polar (S and C) and nonpolar (I, V, and L) residues as shown in Fig. 7. According to the previously identified H-2D^b-binding motif (13), C-75 and L-79 may be assigned as anchor residues. By analogy with the TCR-peptide-MHC class II interaction model (21), we suggest that hydrophobic nonpolar residues I, V, and L on the peptide may interact with the similar G residue conserved in the V_β chain and that the peptide C (or S) residue may interact by hydrogen bonds with the neutral polar N residue conserved in the V_α chain.

The biological role of glycosylated Gag-Pol precursor Pr75^{gag} initiated at an alternative CTG codon(s) located upstream of the usual ATG initiation codon for Pr65^{gag} has long been puzzling. Recently, Corbin et al. (8) demonstrated that glycosylated Gag expression is essential for complete in vivo spreading and for pathogenicity of FV. If the expression of glycosylated Gag products on FV-induced tumor cell surfaces is advantageous for in vivo cell proliferation, a highly immunogenic portion of the leader peptide inevitably induces effective CTL responses which may be critical for disease control.

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