The Peptide Recognized by HLA-A68.2-restricted, Squamous Cell Carcinoma of the Lung-specific Cytotoxic T Lymphocytes Is Derived from a Mutated Elongation Factor 2 Gene

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ABSTRACT

The identification of naturally processed tumor peptides that can stimulate a tumor-specific, CTL response is crucial to the development of a vaccine-based, immunotherapeutic approach to cancer treatment. One type of cancer in which a tumor-specific, CTL response has been observed is squamous cell carcinoma of the lung. In this system investigated here, the tumor-specific peptides are HLA-A68.2 restricted. Immunoaffinity chromatography was used to isolate the HLA-A68.2 molecules from the tumor cell line, and peptide was eluted with acid from the HLA-A68.2 molecules and subjected to three rounds of separation by reversed-phase high performance liquid chromatography (RP-HPLC). To determine which fractions contained the peptide recognized by the tumor-specific CTLs, an aliquot of each RP-HPLC fraction was added to the autologous, B-lymphoblastoid cell line, and the cells were then tested as targets for tumor-specific CTLs. After the third round of RP-HPLC, mass spectrometry was used to sequence individual peptide candidates, and a peptide with a m/z of 497 was identified as the active peptide. Collision-activated dissociation of m/z 497 allowed identification of the peptide sequence as ETVSEQSNV. With the exception of a single amino acid difference (glutamic acid versus glutamine as the sixth position in the peptide), this peptide is identical to residues 581 to 589 of elongation factor 2. The PCR revealed that at least one peptide fraction contained the peptide recognized by the CTLs. The objective of the work described was to determine: (a) the identity of the peptide that was recognized by the CTL; (b) the protein origin of the antigenic epitope; and (c) whether this antigen is expressed in additional lung cancer cell lines.

INTRODUCTION

Lung cancer is expected to account for 178,100 (13%) of all new cancer cases in 1997. Because the disease typically metastasizes to distant locations before diagnosis, a combination of radiation therapy, chemotherapy, and surgery is frequently used in an attempt to eradicate the disease. In spite of advances in these treatment areas, the combined 5-year survival rate for all stages of the disease is only 14%. The development of a new therapeutic approach to lung cancer that would be effective against both the primary lesion and distant metastases would, therefore, have a significant impact on disease mortality. One such potential approach to lung cancer treatment is CTL-mediated immunotherapy. CTL-mediated immunotherapy is based on the observation that the immune system can make an effective response to specific antigens expressed on autologous tumor cells. It has been shown that human CTLs recognize sarcomas (1), renal cell carcinomas (2), colorectal carcinomas (3), ovarian carcinomas (4, 5), pancreatic carcinomas (6, 7), squamous tumors of the head and neck (8), and squamous carcinomas of the lung (9, 10). The largest number of reports of human tumor-reactive CTLs, however, concern melanoma (reviewed in Ref. 11). The ability of tumor-reactive CTLs to mediate tumor regression, both in humans (12) and in animal models (13-15), suggests that efforts directed at increasing CTL activity would likely have a beneficial effect with respect to cancer treatment.

Although CTLs can be directly stimulated with tumor cells, vaccination of a patient population would be greatly expedited if the antigen that is recognized by the CTLs could be identified and concentrated. Unlike the antigens that are recognized by antibodies, the antigens recognized by the T-cell receptor on a CTL are small peptides, 8-10 amino acids in length, that are bound to class I MHC-encoded molecules (16). In humans, both tumor-specific and tumor-associated T-cell epitopes have been identified in melanoma (17-28), ovarian carcinoma (29-32), breast carcinoma (32), non-small cell lung carcinoma (10), and pancreatic carcinoma (7). Because initial studies of peptide immunization in human melanoma patients have yielded promising results (33, 34), the identification of additional peptides that act as T-cell epitopes for these and other cancers is of critical importance for the development of a vaccine-based, immunotherapeutic approach to cancer treatment. Toward this end, we have developed previously a CTL line that recognizes an autologous squamous cell cancer of the lung (9). Antibody-blocking experiments demonstrated that the CTLs recognized antigen in association with HLA-A68, and reconstitution experiments with peptide derived from HLA-A68 and separated by RP-HPLC demonstrated that at least one peptide fraction could reconstitute the epitope recognized by the CTLs. The objective of the work described was to determine: (a) the identity of the peptide that was recognized by the CTL; (b) the protein origin of the antigenic peptide; and (c) whether this antigen is expressed in additional lung cancer cell lines.

MATERIALS AND METHODS

Cell Lines. VBT2, a squamous cell carcinoma of the lung, was established previously in culture from a metastatic lesion in a 45-year-old African-American male (9). All of the material from this patient was obtained following informed, written consent. The lung cell cancer lines CALU-1 (epidermoid), Sk-Mes-1 (squamous), and Sk-Lu-1 (adenocarcinoma) were obtained from the American Type Culture Collection. The cells were maintained in RPMI 1640 containing 5% FBS and 2 mM l-glutamine. VBT2-EBV, a B-lymphoblastoid cell line obtained from the same patient as were the VBT2 tumor cells (9), was maintained in RPMI 1640 containing 10% FBS and 2 mM...
l-glutamine. Hymz.C1R, a class I MHC-negative cell line, was transfected previously with the genes encoding HLA-A*0201 (C1R-A2.1), HLA-A*6801 (C1R-A68.1), and HLA-B*0702 (C1R-B7) and maintained in RPMI 1640 containing 5% FBS, 2 mm l-glutamine, and 300 μg/ml of G418.

**CTT Lines.** VBT2-specific CTLs were restimulated in vitro with autologous tumor as described previously (9).

**Immunopurification of Peptides & Associated Proteins.** VBT2 cells were grown in 10-chamber Nunc cell factories (Fisher, Pittsburgh, PA). The cells were harvested by treatment with 0.45% trypsin and 0.32 mM EDTA, washed two times in PBS solution (pH 7.4), and stored as cell pellets at −80°C. Aliquots of 3−10 x 10⁶ cells were solubilized at 5−10 x 10⁶ cells/ml in 20 mM Tris (pH 8.0), 150 mM NaCl, 1% 3-(cholamidopropyl)dimethylammonio)-1-propanesulfonate, 18.5 μg/ml iodoacetamide, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM EDTA, 0.2% sodium azide, and 17.4 μg/ml phenylmethylsulfonyl fluoride for 1 h. This and all subsequent steps were performed with ice-cold solutions and at 4°C. The lysates were then centrifuged at 100,000 x g, the pellets discarded, and the supernatants passed through a 0.22 μm filter. The supernatants were then passed over a series of columns with the first containing Affi-gel Hg 2+ Hydrazide (Bio-Rad, Hercules, CA) and the second containing the HLA-A2/A68-specific monoclonal antibody, CR1−351, covalently coupled to the Affi-gel Hg 2+ matrix. In some experiments, Sepharose 4B was substituted for Affi-gel Hg 2+ in the precolumn. The second column was then sequentially washed with 20 column volumes of 20 mM Tris (pH 8.0), 150 mM NaCl, 20 column volumes of 20 mM Tris (pH 8.0), 1.0 M NaCl, and 20 column volumes of 20 mM Tris (pH 8.0). The peptides were eluted from the column with 5 column volumes of 10% acetic acid. The isolated HLA-A68 molecules were then boiled for 5 min to further dissociate any bound peptide from the heavy chains. The peptides were then separated from the copurifying class I heavy chain and β2-microglobulin by centrifugation on a Ultrafree-CL membrane with a nominal molecular weight cutoff of M, 5000 (Millipore, Bedford, MA).

**Peptide Fractionation.** The peptide extracts were fractionated by RP-HPLC using an Applied Biosystems (ABI) model 140B system. The extracts were concentrated by vacuum centrifugation from ~20 ml to 250 μl and injected onto either a Brownlee (Norwalk, CT) C18 Aquapore column (2.1 mm x 3 cm; 300 Å; 7 μm) or a Higgins (Mountain View, CA) C18 Haisil column, and an ABI model 785A UV absorbance detector. The column was 300 μm x 3 cm; 300 Å; 7 μm) or a Higgins (Mountain View, CA) C18 Haisil column, and an ABI model 785A UV absorbance detector. The column was a 1.6-kb product from genomic DNA. After heating PCR components to 94°C, 50 ng of genomic DNA or cDNA corresponding to 50 ng of reverse transcription was added to each tube. Cycling conditions were 94°C for 15 s, 62°C for 1 min, and 72°C for 1 min. The annealing temperature was reduced to 60°C for cycle 2 and 58°C for cycles 3-30, with a final extension of 72°C for 1 min. The PCR products were gel purified, and 20-40 ng of product were sequenced directly using the individual amplification primers.

**Molecular Genotyping.** Oligonucleotides were made with an Applied Biosystems 392 DNA/RNA synthesizer, and dye terminator DNA sequencing reactions were analyzed with an Applied Biosystems 370 automated DNA sequencer. Total RNA and genomic DNA was isolated simultaneously from cultured cells with a commercial RNA/DNA kit (Qiagen, Santa Clarita, CA). First-strand cDNA was synthesized by reverse transcribing 1 μg of total RNA with oligo(dT) using the SuperScript system (Life Technologies, Inc., Gaithersburg, MD). PCR amplifications were done with the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN) using 1.5 mM MgCl2.

**Peptide Synthesis.** The peptides were synthesized using a Gilson (Madison, WI) AMS 422 multiple peptide synthesizer. Ten μmol quantities were synthesized using conventional Fmoc amino acids, resins, and chemistry. Peptides were purified by RP-HPLC using a 4.6 mm x 100 mm POROS (Perseptive Biosystems, Cambridge, MA) column and a 10−50% acetonitrile in 0.1% TFA gradient.

**Mass Spectrometric Analyses.** Active RP-HPLC fractions were screened by on-line RP-HPLC/electrospray ionization mass spectrometry using an in house-produced microcapillary column and a Finnigan-MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan, San Jose, CA). Approximately 1% of the active RP-HPLC fraction was loaded onto a section of 185 μm (i.d.) x 75 μm (i.d.) fused silica packed with 10−12 cm of 10-μm C18 particles. Peptides were directed into the mass spectrometer using a 10−100−60% acetonitrile in 0.1 M acetic acid gradient. Ions were formed by electrospray ionization, and mass spectra were recorded by scanning between mass to charge ratios (m/z) 300 and 1400 every 1.5 s.

The active second dimension HPLC fraction was analyzed using an effluent splitter on the microcapillary HPLC column. In this experiment, the column (360 μm o.d. x 100 μm i.d. with a 25-cm C18 bed) was run with a zero dead volume tee (Valco, Houston, TX) to two pieces of fused silica of different lengths (25 μm and 40 μm i.d.). Peptides were eluted with a 34-min gradient of 0−60% acetonitrile. The 25-μm capillary directed one-fifth of the HPLC effluent into the wells of a microtiter plate for use in a CTT epitope reconstitution assay, whereas the remaining four-fifths of the effluent was directed into the mass spectrometer, with mass spectra recorded as described above. Peptide sequences were determined by CAD tandem mass spectrometry as described (35).

**Peptide Synthesis.** Peptides were synthesized using a Gilson (Madison, WI) AMS 422 multiple peptide synthesizer. Ten μmol quantities were synthesized using conventional Fmoc amino acids, resins, and chemistry. Peptides were purified by RP-HPLC using a 4.6 mm x 100 mm POROS (Perseptive Biosystems, Cambridge, MA) column and a 10−50% acetonitrile in 0.1% TFA gradient.

**Oligonucleotide primers for PCR amplification of EP2 were designed to flank the area corresponding to the purified VBT2 peptide antigen and were based on the published human cDNA sequence (36). Primers DEP2-1 (coding strand position 1610−1632; 5′-CAT CGA GGA GTG CCG AGA GCA C) and DEP2-2 (coding strand, position 1925−1903; 5′-TCC CAT TCG TAC TCC TGG GCC AG) were used to amplify at a 315-bp product from cDNA or a 1.6-kb product from genomic DNA. After heating PCR components to 94°C, 50 ng of genomic DNA or cDNA corresponding to 50 ng of reverse transcribed total RNA was added to each tube. Cycling conditions were 94°C for 15 s, 62°C for 1 min, and 72°C for 1 min. The annealing temperature was reduced to 60°C for cycle 2 and 58°C for cycles 3−30, with a final extension of 72°C for 1 min. PCR products were gel purified, and 20−40 ng of product were sequenced directly using the individual amplification primers.

**Oligonucleotide primers for PCR amplification were designed to synthesize a fragment that could distinguish between the HLA-A*6801, A*6802, A*6803, A*6804, and A*6805 alleles of HLA-A68. Primers 55A68 (coding strand position 160−185; 5′-GAC ACC CTC CTC GTG TCC ACC CTG TTC CTC GCC AGC) and 33A68 (non-coding strand position 549−526; 5′-GTA GGC TTC CCA CTG TCG CAC CAC) were used to amplify a 389-bp product from VBT2 first-strand cDNA. The PCR product encompasses the portion of the αα-α domain that contains the nucleotide differences that determine the HLA-A68 subtypes.5 Numbering of nucleotides begins from the start of the open reading frame. PCR cycling conditions were as described above, and the PCR product was directly sequenced with the amplification primers in addition to two other internal primers, SA68 (coding strand position 198−223; 5′-CCA GAG GAT GAA GCC GCC GCC GCC GGT) and 3A68

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5 American Society for Histocompatibility and Immunogenetics Website (www.swwed.edu/home_pages/ASHL/ashl.htm).
RESULTS

**VTB2 Expresses the HLA-A6802 Subtype of HLA-A68.** To determine which HLA-A68 subtype is expressed by VTB2, PCR amplification was used to amplify the gene encoding the molecule, and the resulting amplification product was directly sequenced (data not shown). The nucleotide sequence exactly matched the HLA-A*6802 gene.

**Identification of the Peptide That Reconstitutes the VTB2 Epitope.** To identify the antigen(s) present on a squamous cancer of the lung that are recognized by VTB2-specific CTLs, HLA-A68.2 molecules were purified by immunoaffinity chromatography from 5.5 x 10^10 VTB2 tumor cells. The HLA-A68.2/peptide complexes were eluted from the column with acid and dissociated from one another by boiling. The peptide was separated from the class I MHC-encoded molecules and β2-microglobulin by membrane filtration, concentrated, and then fractionated by RP-HPLC using HFBA as the organic modifier. A CTL epitope reconstitution assay with VTB2-EBV target cells demonstrated that only fractions 10 and 11 were capable of sensitizing the target cells for lysis by the VTB2-specific CTLs (Fig. 1A).

Fraction 11 from the first dimension fractionation was then further fractionated by RP-HPLC using TFA as the organic modifier. A CTL epitope reconstitution assay with VTB2-EBV and the VTB2-specific CTLs demonstrated that fraction 10 of the second dimension separation contained the active peptide (Fig. 1B). Because the mixture of peptides in fraction 10 was still relatively complex, the fraction was further separated with an on-line microcapillary RP-HPLC column that was connected to a splitter that diverted 80% of the effluent to the mass spectrometer for analysis and 20% of the effluent to a 96-well microtiter plate for use in a CTL epitope reconstitution assay. When the fractions from this third dimension separation were tested for their ability to reconstitute the epitope that is recognized by the VTB2-CTL, it was found that activity was present in fractions 13–15 (Fig. 2).

The relative ion abundances of the peptides found in fractions 13–15 were then correlated with the biological activity observed in those same fractions. On the basis of this analysis, it was determined that the abundances of four ion species, represented as the doubly-charged m/z (M + 2H) (2+), matched the measured biological activity (Fig. 2). Two of the candidate ions were successfully sequenced and were identified as m/z 400, ETAPAAPAA, and m/z 477, RVASpPTSGV, where “p” indicates a phosphorylated serine. Sequence information from two additional candidates, m/z 497 and 485, could not be obtained. Peptides corresponding to m/z 400 and 477 were synthesized and were identified as m/z 400, ETVSEQSNV, and m/z 477, RVASpPTSGV, where “p” indicates a phosphorylated serine. Sequence information from two additional candidates, m/z 497 and 485, could not be obtained. Peptides corresponding to m/z 400 and 477 were synthesized and sequenced and were identified as m/z 400, ETAPAAPAA, and m/z 477, RVASpPTSGV, where “p” indicates a phosphorylated serine. Sequence information from two additional candidates, m/z 497 and 485, could not be obtained. Peptides corresponding to m/z 400 and 477 were synthesized and tested in a CTL epitope reconstitution assay but were not recognized by the VTB2-specific CTL (data not shown).

To determine whether the m/z 497 and 485 peptide candidates or other ion species of lesser abundance were the peptide recognized by the VTB2-specific CTL, a new aliquot of peptides from an additional 5 x 10^10 VTB2 tumor cells was prepared. These peptides were subjected to two dimensions of RP-HPLC separation as described above, and the CTL epitope reconstitution assay demonstrated activity in the same peaks. Rather than performing the third dimension RP-HPLC separation on-line with the mass spectrometer, an off-line RP-HPLC fractionation with triethylamine acetate as the organic modifier was chosen in an attempt to further simplify the mixture of peptides in any given fraction. A CTL epitope reconstitution assay with the third dimension fractions demonstrated that fractions 16 and 17 had activity (data not shown). Mass spectrometry analysis of these fractions revealed that the abundance of the m/z 497 ion strongly correlated with the biological activity observed in the CTL epitope reconstitution assay. Analysis of the fragments obtained from the CAD of the m/z 497 candidate allowed determination of the peptide sequence as ETVSEQSNV (Fig. 3).

The sequence of the m/z 497 peptide, ETVSEQSNV, is identical to the sequence of amino acids 581 to 589 of EF2 (EF2_{581-589}) with the exception of a single amino acid difference at position 586, which is a glutamic acid in EF2 (36). Therefore, test peptides were synthesized that corresponded to EF2_{581-589} and the same sequence with a glutamine substituted for glutamic acid at position 586 (EF2_{581-589} (Q586G)). When both of these peptides were tested in a CTL epitope reconstitution experiment, the EF2_{581-589} peptide, but not the EF2_{581-589} peptide, was found to sensitize VTB2-EBV for lysis, thus identifying the peptide that is recognized by the VTB2-specific CTLs (Fig. 4). Half-maximal lysis with EF2_{581-589} was achieved at ~50 pm, whereas a 100,000-fold higher concentration of EF2_{581-589} was generally required for a similar level of activity (Fig. 4A). In a single experiment in which an E:T of 20:1 rather than 10:1 was used, the
EF2<sub>581-589</sub> peptide was capable of sensitizing VBT2-EBV for levels of lysis comparable with that found with the EF2<sub>581-589(ES58Q)</sub> peptide, although a 50-fold higher concentration of peptide was required to achieve half-maximal lysis (Fig. 4A).

Recognition of EF2<sub>581-589(ES58Q)</sub> on Class I MHC Molecules Other Than HLA-A68.2. To determine whether the EF2<sub>581-589(ES58Q)</sub> peptide is presented on class I MHC molecules other than HLA-A68.2, peptide titration experiments were also performed on cell lines expressing HLA-A2.1 (C1R-A2.1), HLA-A68.1 (C1R-A68.1), and HLA-B7 (C1R-B7; Fig. 4B). C1R-A68.1 sensitization required 10-100-fold more peptide than did VBT2-EBV to achieve half-maximal lysis. Although no peptide concentration was capable of sensitizing C1R-A2.1 for lysis, high concentrations of peptide weakly sensitized C1R-B7 for lysis.

Identification of the Gene That Encodes the m/z 497 Peptide.
Because the difference between the EF2<sub>581-589(ES58Q)</sub> peptide and the EF2<sub>581-589</sub> peptide can be accounted for by a single bp substitution in the gene encoding for EF2, a mutation in the EF2 gene of VBT2 was the most likely explanation for the origin of the m/z 497 peptide. PCR amplification with primers specific for the EF2 gene was used, therefore, to amplify cDNA and genomic DNA from both VBT2 and VBT2-EBV. Amplification of cDNA from both cell lines yielded a product that was consistent with the predicted fragment size of 315 bp, whereas amplification of genomic DNA gave a 1.6-kb product that contained a 1.3-kb intron (data not shown). DNA sequencing of the amplified products from VBT2 indicated the presence of both a G and a C at the first bp position of the codon encoding Glu586 (GAG) in the EF2 gene, whereas sequencing of the same products from VBT2-EBV revealed only the presence of a G (data not shown). The DNA sequence chromatograms of the heterozygous mutation displayed the same intensity of the two overlapping peaks (G and C) in both the cDNA and genomic DNA samples, demonstrating that the two alleles are equally expressed at the mRNA level. The substitution of a C for a G at this position would result in the synthesis of an EF2 protein encoding Glu<sub>586</sub>→Gln<sub>586</sub>[EF2<sub>581-589(ES58Q)</sub>] and indicates that this variant form of EF2 is the source protein from which the m/z 497 peptide is derived.

Expression of the Mutant EF2 Gene in Lung Tumors Other Than VBT2. cDNA was prepared from three additional lung cell carcinomas (CALU-1, Sk-Mes-1, and Sk-Lu-1) to determine whether the EF2<sub>581-589(ES58Q)</sub> peptide is widely expressed in other lung cell
carcinomas. PCR amplification and sequencing of the EF2 gene indicated that the mutated form of the EF2 gene was not present in three additional lung cell carcinoma lines (data not shown).

Cell Surface Abundance of the EF2581-589(E586Q) and EF2581-589 Peptides. Assuming that a 50% loss of peptide occurs at each RP-HPLC fractionation step, back-calculation from the relative abundances of the EF2581-589(E586Q) and EF2581-589 peptides indicates that the peptides are present on the cell surface at ~100 and 10 copies/cell, respectively.

DISCUSSION

The identification of antigens that stimulate a tumor-specific CTL response is critical to the development of a vaccine-based immunotherapeutic approach to cancer. To be most effective, the vaccine may need to consist of a pool of peptides, the sum total of which should be capable of binding to the most prevalent class I MHC molecules in the population and thus capable of stimulating a CTL response in a broad spectrum of patients with a particular malignancy. To date, the broadest collection of peptides that has been identified comes from melanomas (17–28), although peptides have also been identified for other cancers including ovarian (29–32), breast (32), pancreatic (7), and lung (10) carcinomas. In an effort to expand the available number of peptides that are available to treat lung cancer, we sought to identify the antigenic peptide recognized by a previously characterized tumor-specific CTL line that recognizes a squamous cell carcinoma of the lung (9).

The results presented here unambiguously identified the HLA-A68.2-associated peptide that is recognized by the VBT2-specific CTL as ETVSEQSVN. With the exception of a single amino acid difference, this peptide is identical to amino acids 581 to 589 of EF2. Thus, the most likely origin of the m/z 497 peptide is a mutated EF2 gene. This possibility was confirmed by sequencing the DNA obtained after PCR amplification with EF2-specific primers. The results showed that the VBT2 tumor cells contain a heterozygous mutation in both the genomic and cDNA encoding the EF2 gene, and that this same mutation is not present in the autologous B-LCL line, VBT2-EBV. Although this mutation appears to be expressed in the tumor but not other cells of this patient, it is not clear whether the altered protein is associated with tumorogenesis. Functionally, EF2 is responsible for the GTP hydrolysis-dependent translocation step of protein synthesis (37), and whether the observed amino acid substitution leads to altered protein function, or is related to the tumorogenesis process in general, is not known at this time. Sequence analysis of the EF2 gene from three other lung tumor cells did not reveal the presence of the E586Q substitution; thus, the mutation is not a prerequisite for tumor formation. This does not obviate, however, the possibility that a survey of a larger number of lung carcinomas would reveal the presence of a mutation in additional lines, or that a mutated EF2 gene could act as an oncogene. Although the epitopes recognized by most tumor-specific CTL have been shown to be shared among tumor cells of the same type, other instances of epitopes being derived from mutated gene products have been reported previously (38–40).

Although both the EF2581-589(E586Q) and EF2581-589 peptides can be detected on the surface of VBT2, the former peptide is ~10-fold more abundant than the latter peptide. One possible explanation for this difference is that an amino acid difference at position six of the peptide may affect the affinity of the peptide for the HLA-A68.2 molecule. This seems to be an unlikely possibility, however, because the side chain of the amino acid residue at position 6 is solvent accessible and does not contact the HLA-A68 molecule (41). An alternative explanation for the difference in abundance is that the E586Q substitution in EF2581-589(E586Q) results in a protein structure that is more sensitive to proteosome-mediated degradation or that is more readily transported to the lumen of the endoplasmic reticulum by the transporter associated with antigen processing.

The EF2581-589(E586Q) peptide sensitized cells for half-maximal lysis at ~50 pM, a concentration that is similar to that of other peptide epitopes that have been identified (26, 27). Conversely, only low activity was typically observed with the naturally occurring EF2581-589 peptide, although one experiment with a high E:T ratio demonstrated substantial levels of lysis at high peptide concentrations. This raises the possibility that the VBT2-specific CTL could cross-react on normal tissue. The fact that VBT2-EBV is not recognized by the CTL indicates that this is not likely to be a problem because the naturally occurring levels of EF2581-589 are insufficient to trigger CTL recognition.

To determine whether the EF2581-589(E586Q) peptide associates with class I MHC molecules other than HLA-A68.2, we asked whether VBT2-specific CTL could recognize the peptide on HLA-A*6801, A*0201, or B*0702 expressing C1R cells. The peptide was readily recognized on HLA-A*68.1 expressing cells, although somewhat more weakly than on VBT2-EBV. Whether this is a reflection of the HLA-A68.1/peptide complex being qualitatively or quantitatively different from the HLA-A68.2/peptide complex or whether this reflects an intrinsic difference in the susceptibility to lysis of the two cell lines is not known. Weak but reproducible activity was seen in association with the HLA-B7 molecule, which is interesting in light of the fact that the EF2581-589(E586Q) peptide bears no resemblance to the peptide binding motif for HLA-B7. In spite of the relatedness of the HLA-A68.2 and -A2.1 molecules, no recognition of the peptide was seen on the latter molecule.

In earlier experiments with this system (9), two different peaks of VBT2 reconstituting activity were seen, suggesting that a minimum of two peptides were recognized by this CTL line. In the experiments described here, only a single peak of activity corresponding to the originally described, late-eluting peak was observed. The absence of the early peak most likely indicates that the original columns were overloaded with peptide, and that the two peaks of activity originally observed were most likely due to the same peptide. The late-eluting peak of activity, when subjected to two additional rounds of RP-HPLC, continued to present a single peak of activity, which indicates that only a single HLA-A68-associated peptide is recognized by the CTL. Furthermore, peptides isolated from class I MHC molecules other than HLA-A68 on VBT2 did not reveal any additional active fractions. This result stands in contrast to other systems in which it has been determined that tumor-specific CTL recognize multiple peptides on the surface of a tumor cell (27, 32, 42–44). The inability to identify more than a single active peptide in the present system could either indicate that only a single tumor-specific peptide is present on the VBT2 cells that can be recognized by CTLs, or it could indicate that a monospecific population of VBT2-specific CTLs were generated by in vitro stimulation, although multiple tumor-specific peptides may have been present.

The present results validate the feasibility of using mass spectrometry and CTL epitope reconstitution assays to identify tumor-derived peptide antigens in a system other than melanoma. The identification of a phosphorylated serine-containing peptide also demonstrates the unique advantage of using the technology to identify posttranslationally modified peptides. This latter aspect is particularly important in light of the fact that for all of the previously identified CTL epitopes that have posttranslational modifications, the modifications have had a significant impact on the ability of the CTLs to recognize those peptides (45, 46). The finding that a phosphorylated peptide can associate with a class I MHC molecule, combined with the fact that proteins in cancer cells are differentially phosphorylated in compari-
son with normal cellular proteins (47, 48), raises the possibility of using differentially phosphorylated peptides as antigens for tumor-specific CTLs.

The EF25182-590E565Q peptide represents the second epitope that has been associated with non-small cell carcinoma of the lung. A HER2/neu peptide was shown previously to be recognized by HLA-A2-restricted and HER2/neu-specific CTLs, although the peptide was altered from the naturally occurring sequence at the second position to conform to the HLA-A2-binding motif (10). The fact that CTLs have been generated against squamous cell carcinoma of the lung (42), conform to the HLA-A2-binding motif (10). The fact that CTLs have been generated against squamous cell carcinoma of the lung (42), esophagus (49), and head and neck (8, 50) raises the possibility of sharing antigens being found that would be useful in the treatment of squamous cell carcinomas of multiple origins. These studies indicate that squamous cell carcinoma of the lung is a good candidate for immunotherapeutic intervention and indicate that continuing efforts should be made to identify additional peptide antigens that can be used to stimulate a CTL response.

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IDENTIFICATION OF A TUMOR-SPECIFIC CTL EPITOPE


The Peptide Recognized by HLA-A68.2-restricted, Squamous Cell Carcinoma of the Lung-specific Cytotoxic T Lymphocytes Is Derived from a Mutated *Elongation Factor 2* Gene


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