Phosphorylated Peptides Are Naturally Processed and Presented by Major Histocompatibility Complex Class I Molecules In Vivo

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Abstract

Posttranslational modification of peptide antigens has been shown to alter the ability of T cells to recognize major histocompatibility complex (MHC) class I–restricted peptides. However, the existence and origin of naturally processed phosphorylated peptides presented by MHC class I molecules have not been explored. By using mass spectrometry, significant numbers of naturally processed phosphorylated peptides were detected in association with several human MHC class I molecules. In addition, CD8 T cells could be generated that specifically recognized a phosphorylated epitope. Thus, phosphorylated peptides are part of the repertoire of antigens available for recognition by T cells in vivo.

Key words: MHC class I • posttranslational protein processing • phosphopeptides • mass spectrometry • cytotoxic T lymphocytes

Introduction

MHC class I molecules are expressed on the surface of almost all nucleated cells and are responsible for the presentation of antigenic peptides to CD8 CTLs. The presented epitopes are typically generated from proteins which are synthesized in the cytosol, degraded by proteases, and transported into the lumen of the endoplasmic reticulum via the transporter associated with antigen processing (TAP). Then, the peptides are loaded onto newly synthesized MHC class I molecules and trafficked to the cell surface (1, 2). By using mass spectrometry (MS), it has been demonstrated that as many as 10,000 different peptide species are presented by individual class I MHC alleles. These range in abundance from 1 to >4,000 copies per cell (3). The peptide sequences in these complex mixtures can also be determined by MS (4–6). Most importantly, this technique facilitates direct identification of peptide antigens that are associated with class I or class II MHC molecules on the cell surface (4, 5, 7–9).

By using MS, several MHC class I–associated peptides have been found to contain posttranslational modifications. These modifications include deamidation of asparagine (10, 11), cysteinylation of cysteine (12–14), and glycosylation (15). Posttranslational modification alters the ability of T lymphocytes to recognize MHC class I– or class II–restricted epitopes (10, 12–17). Recently, Hogan et al. (18) identified the only published example of a naturally processed and presented phosphorylated peptide associated with any MHC molecule. However, the ability of this phosphorylated peptide to be recognized by T lymphocytes was not investigated. More recently, Andersen et al. (19) demonstrated that synthetic phosphorylated MHC class I peptide epitopes can be transported by TAP, can bind to MHC class I molecules, and can be recognized by specific CTLs. Since the complexity and origin of phosphorylated peptides presented by MHC molecules are unknown, we have used MS to explore this issue. We have also evaluated the ability of these naturally processed phosphorylated peptides to stimulate specific immune responses.

Materials and Methods

Cell Lines and Transfectants. All cell lines were grown in growth medium consisting of RPMI 1640 supplemented with 5%
FCS with SerXtend (Irvine Scientific). JY (HLA-A*0201, -A*0201, -B7, -B7, -DR4, -DR6), ABB (HLA-A2, -A3, -B7, -B35, -DR01, -DR103), and Ruppen (HLA-A1, -A2, -B8, -B27) are all EBV-transformed B lymphoblastoid cell lines (B-LCLs). VTB2 (HLA-Asy,-A*6802, -B35, -Cw4) is a squamous lung carcinoma that has been described previously (18, 20). T2-B7 is an HLA-A*0201 human B-LCL with a deletion in the MHC including the genes for TAP1, TAP2, LMP2, and LMP7 (21, 22) which has been transfected with HLA-B7*0702. T2-B7 was grown under selection in growth medium supplemented with 300 μg/ml hygromycin B. A transfectant of the B-LCL C1R expressing chimeric MHC class I molecule consisting of α1 and α2 domains of HLA-A*0201 and α3 domains of H-2D (AAD) has been described previously (23, 24), and was maintained under selection in growth medium supplemented with 300 μg/ml G418.

Isolation of HLA-associated Peptides. MHC class I molecules were immunoprecipitated from B-LCLs, and their associated peptides were extracted as described previously (5, 22) with slight modifications. Between 5 × 10⁶ and 2 × 10⁷ cells were lysed in 20 mM Tris–HCl, pH 8.0, 150 mM NaCl with 1% 3-[3-Cholamidopropyl] dimethylammonio]-1-propane sulfonate (CHAPS), 20 mM Tris-HCl, pH 8.0, 150 mM NaCl with 1% 3-[3-cholamidopropyl] dimethylammonio]-1-propane sulfonate (CHAPS), 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1.100 dilutions of phosphatase inhibitor cocktails I and II (Sigma-Aldrich), in order to prevent potential dephosphorylation of peptides during extraction. After sample centrifugation, supernatants were incubated with Abs specific for MHC class I molecules (Ab GAP-A3 specific for HLA-A3; BB7.2 specific for HLA-A2.1 [25]; ME1-1.2 specific for HLA-B7 and -B27; W6/32 specific for HLA-A, -B, and -C [26]; or Bi23.23 specific for HLA-B and -C [27]) that had been bound to recombinant protein A–Sepharose fast-flow (Amersham Pharmacia Biotech). Purification of multiple MHC class I alleles from a single cell line was accomplished by sequential immunoprecipitation purification. Peptides were eluted from the MHC class I molecules with 10% acetic acid and separated into Teflon eppendorf tubes (Savillex) using a 5,000-dalton cutoff ULTRAFREE-MC filter (Millipore). Extracts were stored at −80°C.

Immunobilized Metal Affinity Chromatography Enrichment of Phosphorylated Peptides. Immunobilized metal affinity chromatography (IMAC) columns were constructed by packing 360 μm outer diameter (OD) × 100 μm inner diameter (ID) fused silica (Polymer Microtechnologies) with 8 cm POROS 20 MC (PerSeptive Biosystems). Columns were activated with 200 μl of 100 mM FeCl₃ (Sigma-Aldrich). Between 1.5 × 10⁶ and 1.4 × 10⁶ cell equivalents (c.eq.) of HLA-extracted peptide in 10% acetic acid (Sigma-Aldrich) were loaded onto the column in each experiment. To remove nonspecific binding peptides, the column was washed with a solution of 100 mM NaCl (Sigma-Aldrich) in acetonitrile (Mallinkrodt), water, and glacial acetic acid (25:74:1 vol/vol/vol; Sigma-Aldrich). This column was then connected to a second fused silica column packed with 6 cm of 5–20 μm C₁₈ (YMC) via 1 cm of 0.012-in ID × 0.060-in OD Teflon tubing (Zeus). Phosphorylated peptides were eluted to the pre-column with 5 μl of 50 mM Na₂HPO₄ (pH 9.0; Sigma-Aldrich) and rinsed with several column volumes of 0.1% acetic acid to remove salts before MS analysis.

Liquid Chromatography/MS/MS Parameters on the LCQ Ion Trap Mass Spectrometer. IMAC-enriched phosphorylated peptides were analyzed by a procedure described previously (28) using nanoflow-HPLC/microelectrospray ionization (μESI)/MS/MS on an LCQ ion trap mass spectrometer (Finnigan). In brief, precolumns were connected via 0.012-in ID × 0.060-in OD Teflon tubing to an analytical column with a laser-pulled ESI emitter tip (2–4 μm in diameter). Peptides were eluted into the mass spectrometer with an HPLC (model 140B; Applied Biosystems) gradient (consisting of 5–60% B in 40 min; A = aqueous 0.1 M acetic acid, B = 70% acetonitrile with 0.1 M acetic acid). The mass spectrometer was operated in data-dependent mode and cycled through a single MS and five MS/MS experiments every 12–15 s. MS/MS experiments (collision energy 40%) were performed on the five most abundant ions (3-dalton window; precursor m/z ± 1.5 daltons) in each full-scan mass spectrum.

Identification and Sequencing of Phosphopeptide MS/MS Spectra. Spectra of candidate phosphopeptides were selected from the 800–1,000 MS/MS spectra acquired during a typical HPLC-MS/MS experiment with an in-house computer algorithm termed the Neutral Loss Tool. This program screens MS/MS spectra for signals at m/z values corresponding to the loss of phosphoric acid 49 or 98 daltons from doubly or singly charged precursor ions, respectively. This process is commonly observed in ion trap MS/MS spectra of phosphorylated peptides (29). Candidate peptides were sequenced de novo and with the aid of the database searching program MS-Tag (available at http://prospector.ucsf.edu/ucsfhtml3.4/mtagfd.htm).

Negative Ion Precursor Parameters on the TSQ-7000 Triple Quadrupole Mass Spectrometer. Phosphorylated peptides were also analyzed on a Finnigan TSQ-7000 triple quadrupole mass spectrometer operating in the negative ion mode (spray voltage, −1.6 kV). The nanoflow HPLC-μESI setup, column assembly, and HPLC gradient conditions were the same as above. The mass spectrometer was set to perform precursor scans with Q1 scanning m/z 400–m/z 800 and Q3 set to transmit ions of m/z 79. Q1 and Q3 were operated with resolutions of 2 and 1 daltons, respectively. Q2 was operated with a collision cell offset of 40 V and an argon pressure of 3 mTorr.

Peptide Synthesis. Peptides were synthesized as described (12), 9-fluorenlymethoxy carbonyl (Fmoc)-protected amino acids, phosphorylated amino acids, and resins were purchased from Novabiochem. Peptides were purified by reversed-phase HPLC and sequences were confirmed by MS/MS experiments.

Generation of Phosphopeptide-specific CTLs. C57Bl/6 mice transgenic for the AAD gene have been described previously (30). Dendritic cells (DCs) were generated as described previously (31) with some modifications. On day 7, DCs were isolated using a StemSep column after incubation of GM-CSF/IL-4–cultured bone marrow–derived cells with a mixture of Abs for the enrichment of DCs (StemCell Technologies Inc.). Enriched DCs were then incubated with CD40L–transfected NIH-3T3 cells (a gift of Dr. Rejen Lapointe, National Cancer Institute, Bethesda, MD) for 14–16 h. CD40-activated “mature” DCs were pulsed with 1 μM phosphorylated peptide for 4 h at 37°C in HBSS containing 5% FCS, washed twice, and resuspended in saline at 10⁶ cells/ml. Mice were injected in the tail vein with 10⁵ DCs in 100 μl.

Spleens from primed mice were harvested 3 wk after immunization. Separate bulk T cell cultures were established in vitro with irradiated splenocytes that had been pulsed with 10, 1, or 0.1 μM phosphorylated peptide as described previously (31). Cultures were restimulated with irradiated, peptide-pulsed autologous splenocytes every 7–10 d. All CTL lines were grown in CTL medium (consisting of RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, essential and nonessential amino acids, 50 μM β-mercaptoethanol, 15 mM Heps, and gentamicin) and 10 U/ml of IL-2 in a humidified 8% CO₂ atmosphere at 37°C.

Analysis of In Vitro Cytotoxicity and Intracellular Cytokine Production. Standard ⁵¹Cr-release assays were performed to determine CTL recognition of phosphorylated or nonphosphorylated pep-
Results

We have evaluated the repertoire of naturally processed and presented phosphorylated peptides presented by MHC class I molecules isolated from several different EBV-transformed B-LCLs. Detection of phosphorylated species in the pool of >10,000 class I MHC-binding peptides was facilitated by selective enrichment using IMAC (34, 35). The presence of phosphorylated peptides in the resulting enriched sample was confirmed by screening MS/MS spectra of individual peptide ions for an abundant loss of phosphoric acid from the precursor mass. This neutral loss is a common feature in ion trap MS/MS spectra of phosphoserine (pSer) and phosphothreonine (pThr) containing peptides, but not those containing phosphotyrosine (pTyr). When 1.5 × 10^9 c.eq. of peptides extracted from HLA-B7 were analyzed by this method, 60 phosphorylated peptides were detected (Table I). Thus, phosphorylated peptides are a significant part of the repertoire of antigens presented by this MHC class I molecule for recognition by CD8^+ T lymphocytes.

To confirm the generality of this observation, we analyzed peptides eluted from several additional human MHC class I alleles including: HLA-A1, -A*0201, -A3, -A*6802, -B8, -B27, and HLA-B35. Naturally processed phosphorylated peptides were found to be associated with all of these MHC class I molecules (Table I). Interestingly, many of the HLA-B alleles examined present a larger number of phosphorylated peptides (43–60 peptides) than do any of the HLA-A alleles analyzed (9–15 peptides [Table I]). This disparity could be due to significant differences in overall ionization efficiency, reflecting the fact that the binding motif for many of the HLA-B alleles includes positively charged residues, whereas those for the HLA-A alleles do not. To eliminate this possibility, we also determined the number of phosphorylated peptides in HLA-A2 and HLA-B7 extracts by negative ion MS using precursor scans (36, 37). In this approach, MS/MS spectra of negatively charged phosphopeptide ions were screened for the presence of a diagnostic fragment mass corresponding to PO_3^− (m/z 79 [36, 37]). When the HLA-B7 extract was analyzed by this technique, 122 phosphopeptides were detected (Table I). Only 21 phosphopeptides were detected in the HLA-A*0201 extract (Table I). Thus, the relative number of phosphopeptides observed in these two extracts is not a function of the ionization method used to detect them. We suggest that the differences in numbers of phosphopeptides presented by HLA-A and -B alleles are likely to reflect common structural features of MHC binding and phosphokinase recognition motifs.

To investigate the pathway by which phosphorylated MHC class I-associated peptides are presented, we analyzed peptides eluted from HLA-A2 and HLA-B7 molecules expressed in a cell line that is deficient for TAP expression (T2-B7). We did not detect any phosphorylated peptides associated with either HLA-A2 or HLA-B7 in the TAP-deficient cell line (Table I). However, nonphosphorylated peptides were present in these extracts (data not shown). These have been shown previously to originate from proteolysis of peptides within the lumen of the endoplasmic reticulum (22, 38). The absence of phosphorylated peptides from these extracts demonstrates that their presentation is strictly TAP dependent. This in turn suggests that the major source of these peptides is proteins that are processed in the cytosol.

15 phosphorylated peptides in the IMAC-enriched MHC class I extracts were sequenced on the ion trap mass spectrometer. An example is shown in Fig. 1. These sequences were confirmed by comparing the observed spectra with those of the corresponding synthetic peptides. 13 of these phosphorylated peptides contained pSer, whereas only two contained pThr and none contained pTyr (Table

| Cell line   | MHC class I allele | Positive ion analysis* | Negative ion analysis† |
|------------|-------------------|------------------------|------------------------|
| Ruppen     | HLA-A1            | 15                     | –                      |
| JY         | HLA-A*0201        | 11*                    | 21                     |
| T2-B7      | HLA-A*0201        | 0                    | –                      |
| ABB        | HLA-A3            | 12                     | –                      |
| VBT2       | HLA-A*6802        | 9                      | –                      |
| JY         | HLA-B7            | 60§                    | 122                    |
| T2-B7      | HLA-B7            | 0§                     | –                      |
| Ruppen     | HLA-B8            | 43                     | –                      |
| Ruppen     | HLA-B27           | 45                     | –                      |
| ABB        | HLA-B35           | 26                     | –                      |

*Enumeration of phosphorylated peptides was completed using the Neutral Loss Tool as described in Materials and Methods.
†The number of phosphorylated peptides was determined by enumerating ion species that lost m/z of 79 in negative ion mode as described in Materials and Methods.
‡For the HLA-A2.1 and HLA-B7 extracts, 1.5 × 10^9 c.eq. was utilized in order to account for the homozygosity of the alleles in the JY cell line.
§Due to the level of HLA-A2 and HLA-B7 found on the cell surface of T2-B7, 4.5 × 10^8 c.eq. was loaded for HLA-A2 and 1.3 × 10^9 c.eq. for HLA-B7.
Identification of Phosphorylated MHC Peptides

II). This finding is in accord with the relative levels of these phospho-amino acids in the cell (39). 13 of these peptides contained only a single phosphorylated residue, even though many contained additional Ser, Thr, or Tyr residues. One peptide identified in the HLA-B27 extract (RRFpSRpSPIRR) contained two pSer residues (Table II). Interestingly, this extract also contained a truncated version of this peptide (RRFpSRpSPIR), as well as a singly phosphorylated variant (RRFpSRpSPIRR [Table II]). Finally, an Arg or Lys residue is found 3–4 amino acids NH₂-terminal to the phosphorylated residue in 13 of these peptides, and a Pro residue is found 2–3 amino acids NH₂-terminal of the phosphorylated residue in 5 of these peptides. These findings suggest that phosphorylation of these peptides is sequence specific and are consistent with the motif requirements for Ser–Thr kinase recognition.

All sequences in Table II (with the exception of the RRFpSRpSPIRR epitope) matched to unique source proteins in the nonredundant and OWL (available at http://prospector.ucsf.edu/ucsfhtml3.4/mstagfd.htm) protein databases. Three of the source proteins are known protein kinases, two (insulin receptor substrate 2 and myristoylated Ala–rich protein kinase C substrate [MARCKS]) are known kinase substrates, and two others are known phosphoproteins (MADS box transcription enhancer factor and MHC–binding protein 2). However, only the HLA-B27–restricted peptide derived from the MARCKS protein contains a previously identified phosphorylation site (40). Two of the source proteins (MUM2 and MHC class III protein g18.1b) have not been previously shown to be phosphorylated, and their functions are unknown. These sequences illustrate the diversity of phosphorylated cellular proteins that are processed and presented by MHC class I molecules, and illustrate the power of MS to identify new members of phosphorylation cascades.

We used an HLA-A*0201–restricted phosphorylated peptide derived from the MUM2 protein with the sequence RLDpSYVRS.
quence RLDpSYVRSL as a model to assess whether this posttranslational modification could be recognized by the immune system. AAD transgenic mice were primed intravenously with phosphopeptide-pulsed DCs, and the splenic T cell effector function was assessed after restimulation with the same peptide in vitro. Phosphopeptide-specific cell division and growth in a population of CD8+ T cells in these cultures were detected (data not shown). However, in three independent experiments, we could not detect specific cytolytic activity or IFN-γ production in response to either the phosphorylated or unphosphorylated forms of this peptide (Fig. 2A, and data not shown). Therefore, we also evaluated responses induced by a homologous MUM2-derived peptide with the sequence GLDpSYVRSL. This peptide contains an R to G mutation, at position 1, previously identified by Chiari et al. (41) as a tumor antigen. CD8+ T cells from mice primed and cultured in vitro with GLDpSYVRSL produced IFN-γ after a 5-h incubation with phosphopeptide-pulsed cells (Fig. 2A). They also killed targets pulsed with this same peptide (Fig. 2B). These CD8+ CTLs were specific for the phosphopeptide and did not recognize targets pulsed with the nonphosphorylated GLDSYVRSL (Fig. 2B). These findings established that phosphorylated peptides can stimulate specific CTL responses after in vivo immunization and in vitro culture.

To further evaluate the specificity of these CTLs, we tested their ability to recognize target cells pulsed with other phosphorylated peptides. Importantly, the CTLs elicited with the GLDpSYVRSL peptide from the mutated form of MUM2 cross-recognized the phosphorylated peptide, RLDpSYVRSL, derived from wild-type MUM2 (Fig. 2A and B). As expected, they failed to recognize the unphosphorylated form of this peptide (data not shown). In addition, as shown in Fig. 2, the CTLs elicited with GLDpSYVRSL did not recognize targets pulsed with a distinct HLA-A2.1–binding phosphopeptide (RVApSPTSGV). Because this peptide also contains pSer at position 4, this result indicates that the CTLs not only recognized the pSer, but also the specific peptide context of this residue. Finally, we evaluated the effect of substituting pThr or pTyr for pSer in the GLDpSYVRSL sequence. We found that the CTLs recognized the pThr and pSer containing peptides similarly, whereas they failed to recognize the pTyr peptide at any concentration tested (Fig. 2B). Though the pTyr substitution diminished binding to HLA-A*0201 by approximately fourfold relative to the other two (Table III), this does not account for the difference in CTL recognition. Thus, CTLs elicited with a phosphopeptide strongly discriminate between phosphorylated and nonphosphorylated peptides. However, these CTLs are not simply phosphate specific; they also discriminate the context of the phosphate within a specific peptide sequence.

**Table III. Relative Binding Affinities of MUM2-derived Peptides for HLA-A*0201**

| Sequence       | IC50 nM |
|----------------|---------|
| RLDpSYVRSL     | 78      |
| GLDpSYVRSL     | 58      |
| GLDpYYVRSL     | 211     |
| GLDpTYVRSL     | 67      |

**Discussion**

The phosphorylation of cellular proteins in transformed cells is increased compared with normal cells (42, 43) and offers an attractive target for immunotherapeutic intervention. Consequently, we have investigated whether phosphorylated peptides are presented on the cell surface by MHC molecules for recognition by T lymphocytes. Naturally processed phosphorylated peptides were presented by
all MHC class I alleles evaluated in this study, including HLA-A1, -A201, -A3, -A6802, -B7, -B8, -B27, and HLA-B35. The presence of phosphorylated peptides in extracts from these molecules was established by their binding to a phosphate-specific metal ion affinity column and the loss of either H3PO4 or PO3\(^-\) during MS/MS (29, 36, 37). De novo sequencing confirmed that these species were phosphorylated peptides.

A comparison of the number of phosphorylated species demonstrated that the HLA-B alleles evaluated presented a greater number of phosphorylated peptides than did the HLA-A alleles. We hypothesize that this is due to the enhanced presentation of phosphorylated peptides by class I MHC molecules whose binding motifs consist of amino acids that are also components of kinase motifs. In particular, the consensus binding motifs for HLA-B7, -B8, and -B27 contained Arg or Lys at positions 1, 2, or 3, while motifs for HLA-B7 and HLA-B35 contain Pro at position 2 (3, 44, 45). Several kinases phosphorylate Ser or Thr residues that are three to four amino acids COOH terminal of an Arg or Lys, or two to three amino acids COOH terminal of a Pro (46). Thus, it would be expected that these HLA-B alleles could present peptides that are phosphorylated at positions 4–7. Of the 12 complete and 4 partial peptide sequences obtained from HLA-B7 and -B27 alleles, 14 are phosphorylated at position 4 and have an Arg or Lys at position 1 or 2. Similarly, of the six peptides associated with HLA-B7, all but one are phosphorylated two to three amino acids COOH terminal of the Pro at position 2. It is interesting that HLA-B7, which contains both an Arg and Pro within its binding motif, displays a larger number of phosphorylated peptides than the other HLA-B alleles analyzed. In contrast, the HLA-A alleles we have analyzed do not require an Arg, Lys, or Pro within their consensus binding motif. This does not preclude presentation of phosphorylated peptides containing kinase motifs: the two phosphopeptide sequences determined for HLA-A*0201 and -A*6802 also contain an Arg at position 1 and a pSer at position 4. However, the presentation of peptides containing consensus kinase motifs will not be selected for, as it is the case for many HLA-B alleles.

In this study, we also evaluated whether MHC class II molecules presented phosphorylated peptides, and failed to detect any phosphorylated peptides in extracts from two different B-LCLs that expressed four different HLA-DR alleles (data not shown). It remains to be determined whether this deficiency extends to HLA-DQ and -DP. However, this result suggests that phosphorylated protein segments are not generated or do not persist in the endocytic environment from which the processed peptides displayed by class II MHC molecules originate. In contrast, the pSer and pThr containing phosphopeptides displayed by class I MHC molecules appear to be processed via the classical class I antigen-presentation pathway involving the TAP protein. The involvement of TAP demonstrates that the main source of these peptides is phosphorylated proteins that are processed in the cytosol, although our studies do not address the proteases responsible. Our results extend those of Anderson et al. (19), whose study demonstrated that synthetic peptides containing pSer could be transported by TAP.

Several lines of evidence suggest that the phosphopeptides displayed by class I MHC molecules are the products of normal protein phosphorylation pathways in cells rather than adventitious or artifactual phosphorylation of the peptides themselves. As previously mentioned, most of the phosphopeptide sequences determined are consistent with known Ser-Thr kinase motifs, and these peptides originate from the cytosol, where the majority of such kinases are expected to operate. In addition, the relative representation of pSer, pThr, and pTyr among these sequences is consistent with the relative quantities of these phospho-amino acids in cells (39). In particular, pTyr represents only 0.1% of the phospho-amino acid content of the cell, and therefore may not make a significant contribution to the number of the phosphopeptides on the cell surface associated with MHC class I molecules. Finally, many of the sequences we have identified are from known phosphoproteins, kinases, or kinase substrates. Whereas the exact phosphorylation sites on most of these proteins have not been previously determined, a peptide derived from the MARCKS protein did contain a known site (40). Importantly, the identification of MHC-presented phosphopeptides can provide insight into known phosphorylation pathways and also offers the possibility to identify new ones.

In this study, we demonstrated that phosphopeptides could be used to elicit CTLs, and that these cells could discriminate between phosphorylated and nonphosphorylated peptides. Similar findings have been reported by Andersen et al. (19). Here we have also shown that the CTLs elicited with the GLDpSYVRSL peptide failed to recognize other peptides that carried a pSer at position 4. These CTLs could also discriminate between different phosphorylated amino acids even when they were presented within the same peptide context. Thus, these CTLs are not phosphate specific, but rather recognize this moiety in the context of a specific peptide sequence. It should be noted that the ability to generate phosphopeptide-specific CTLs indicates that phosphopeptides presented by MHC molecules are protected from the action of phosphatases both in vivo and in vitro. This augers well for the use of such peptides both as vaccines and to develop T cell reagents for use in adoptive immunotherapy.

The identification of peptide epitopes expressed on tumors has led to the development of novel vaccines for cancer immunotherapy (47). However, the number of identified targets is still small, and these tumor antigens are often expressed on normal cells and on the tumors (48, 49). Because proteins from cancer cells have been shown to be differentially phosphorylated in contrast to normal cellular proteins due to increased deregulation of phosphorylation (43, 50), we are isolating phosphorylated peptides found on different carcinoma cell lines to identify tumor-specific antigens. Identification of these phosphorylated epitopes will provide a new set of tumor-specific antigens for use in immunotherapy.
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