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Phosphorylated Peptides Can Be Transported by TAP Molecules, Presented by Class I MHC Molecules, and Recognized by Phosphopeptide-Specific CTL[1]

Mads Hald Andersen,*† Jordi Espuny Bonfill,‡ Anne Neisig,§ Gemma Arsequell,‡ Ib Søndergaard,† Jacques Neefjes,§ Jesper Zeuthen,* Tim Elliott,¶ and John S. Haurum[2]*¶

CTL recognize short peptide fragments presented by class I MHC molecules. In this study, we examined the effect of phosphorylation on TAP transport, binding to class I MHC molecules, and recognition by CTL of peptide fragments from known phosphorylated oncogene proteins or virus phosphoproteins. We show that phosphopeptides can be efficiently transported from the cytosol to the endoplasmic reticulum by the TAP. Furthermore, we show that phosphorylation can have a neutral, negative, or even a positive effect on peptide binding to class I MHC. Finally, we have generated phosphopeptide-specific CTL that discriminate between the phosphorylated and the nonphosphorylated versions of the peptide. We conclude that phosphopeptide-specific CTL responses are likely to constitute a subset of the class I MHC-restricted CTL repertoire in vivo. ***The Journal of Immunology,*** **1999, 163: 3812–3818.**

Major histocompatibility complex class I molecules present cytosolic peptide Ags for recognition by CTL. Peptide fragments are generated in the cytosol by proteasome-mediated proteolytic cleavage of normal endogenous proteins, as well as of viral proteins, and products of endogenous genes that have undergone molecular alterations during malignant transformation. Any single class I MHC allele has a peptide repertoire dominated by oligopeptides of 8–10 amino acids length, which share a sequence motif corresponding to two or more essential amino acid side chains, the so-called peptide anchor residues (1–3). The solution of x-ray crystallographic structures of MHC class I-peptide complexes (4–6) revealed that depressions or pockets in the MHC peptide-binding groove preferentially accommodate particular amino acids corresponding to the anchor residues of the peptide ligand.

Little is known about the effect on class I MHC-restricted Ag presentation of naturally occurring posttranslational modifications of peptide Ags in vivo. These modifications may affect Ag processing, MHC binding, and interaction with the TCR. Evidence that posttranslational modifications may play a role in Ag presentation and T cell recognition has been accumulating over the last few years. Recently, it has been described that class I (7–10) and class II (11) MHC-restricted T cells can recognize posttranslationally modified peptides specifically (12, 13).

Phosphorylation is a tightly regulated posttranslational cytosolic event. However, inflammation, intracellular infection, cellular activation, and malignant transformation may cause deregulation of phosphorylation, perhaps leading to generation of sufficient amounts of phosphopeptides for class I MHC-restricted presentation to occur. For example, it is known that following chromosomal translocation in leukemia, certain protooncogenes acquire novel kinase activities leading to the appearance of new transformation-specific phosphorylation (14, 15). Similarly, novel phosphopeptide Ags could be derived from phosphorylated oncogenes exhibiting deregulated phosphorylation upon malignant transformation. Such proteins are phosphorylated on tyrosine, serine, and threonine residues by different cellular kinases, and up-regulation of phosphorylation at specific sites has been associated with transformation (16–18). Finally, a large number of phosphorylated viral proteins have been characterized, which also might generate phosphopeptide fragments for MHC-restricted presentation.

In this study, we have analyzed the effect of phosphorylation on the ability of peptides to be transported by TAP, binding to class I molecules, and CTL recognition. We conclude that posttranslationally modified cytosolic phosphopeptides could be presented to the immune system by class I MHC molecules and recognized by CTL in a phosphorylation-specific manner.

Materials and Methods

Cell lines

The TAP-deficient cell lines RMA-S and T2 were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS and glutamine. T2 cells transfected with HLA-B*0702 (T2-B7) (19), HLA-B*3501 (T2-B35) (20), and HLA-B*2705 (T2-B27) (21) were kindly supplied by A. McMichael (Oxford University, Oxford, U.K.), and T2 cells transfected with H-2K^k (T2-K^k) were kindly supplied by P. Cresswell (Yale University, New Haven, CT). For the T2-B7 and T2-B27 cell lines, the medium was supplemented with 100 μg/ml hygromycin and 250 μg/ml G418, respectively.

EBV-transformed B lymphoblastoid cell lines (BCL)[3] were established by coculturing PBMC with supernatant from the EBV-infected marmoset

*Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark; †Department of Biochemistry and Nutrition, Technical University, Lyngby, Denmark; ‡Unit for Glycoconjugate Chemistry, CID-Consejo Superior de Investigaciones Cientificas, Barcelona, Spain; §The Netherlands Cancer Institute, Amsterdam, The Netherlands; and ¶Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom

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[2] Address correspondence and reprint requests to Dr. John S. Haurum, Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, 2100 Copenhagen OE, Denmark. E-mail address: haurum@cancer.dk

[3] Abbreviations used in this paper: BCL, B lymphoblastoid cell line; BCR, breakpoint cluster region; ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; LCL, lymphoblastoid cell line; NP, nucleoprotein.

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cell line B95.8 in the presence of cyclosporin A. Approximately 3×10^7 PBMC were resuspended in 5 ml R10 and 5 ml B95.8 supernatant and incubated for 4 h at 37°C. The cells were then resuspended in fresh R10 (8 ml), incubated at 37°C, and the following day cyclosporin A was added to the culture (1 μg/ml). Two EBV-transformed BCL lines were established: JS-BCL from the donor JS expressing HLA-A2, -A3, -B7, -B60, and -Cw3, and KIG-BCL from the donor KIG expressing HLA-A2, -A3, -B7, and -B15(62).

Synthetic peptides

The nonphosphorylated peptides were from Research Genetics (Huntsville, AL) and provided at >90% purity, as analyzed by HPLC and mass spectroscopy analysis.

Phosphopeptides containing phosphoserine or phosphotyrosine residues were synthesized using a phosphorylated Fmoc-serine or Fmoc-tyrosine building blocks (FmocSer(PO(Obzl)OH)OH or Fmoc-Tyr(PO(Obzl)OH)OH); Calbiochem, Lisztweg, Germany) in the manual solid-phase Fmoc synthesis of phosphopeptides. All phosphopeptides were HPLC purified and characterized by mass spectroscopy and nuclear magnetic resonance. In addition to oncogene- or virus protein-derived phosphopeptides, the following peptides were synthesized for the TAP assays: 417 [TVNKTERAY], and 417-S [TVNKTESAY], as well as the phosphopeptide 417-P [TVNKTES($-PO_3^{2-}$)AY]. Before use in the TAP assay, some peptides were radiolabeled with $Na^{125}I$ catalyzed by chloramine-T.

Assembly assay for peptide binding to class I MHC molecules

Assembly assays for binding of the synthetic peptides to class I MHC molecules metabolically labeled with [^{35}S]methionine were conducted essentially as described (22, 23). This assay is based on the peptide-dependent stabilization of class I molecules after lysis of peptide transporter-deficient cell lines (T2 or RMA-S) and the subsequent detection of correctly folded, stable MHC heavy chains by immunoprecipitation with conformation-dependent Abs. To prevent dephosphorylation of peptides during the assembly assay, phosphatase inhibitors were added (1 mM Na_3VO_4 and 5 mM NaF). After electrophoresis, gels were exposed to PhosphorImager screens, and peptide binding was quantitated using the Imagequant PhosphorImager program (Molecular Dynamics, Sunnyvale, CA).

TAP-mediated transport of phosphopeptides across the ER membrane

Assays for the TAP-mediated translocation across the ER membrane of the radiolabeled peptide 417, the serine-substituted peptide 417-S, as well as a phosphorylated (417-P) peptide analogue in LCL721 cells (LCL; human) or RMA cells (murine) were performed as described (24, 25). T2 cells or RMA-S cells were used to demonstrate the TAP dependence of transport. Briefly, 5×10^6 cells per sample were washed in incubation buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.3) before permeabilization with 2.5 IU/ml streptolysin O (Wellcome Diagnostics, Dartford, U.K.). The cell suspension was then added to 0.1 μg of iodinated peptide (sp. act., 30 μCi/μg), with or without ATP (Boehringer Mannheim, Mannheim, Germany) in a final volume of 100 μl. Translocation was performed at 37°C over 5 min before ice-cold lysis buffer (1% Triton X-100, 500 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) was added to each tube. After 30 min of lysis (4°C), the nuclei were removed by centrifugation (12,000 rpm, 10 min). Then the *N*-glycosylated peptides were precipitated by the addition of Con A-Sepharose beads (Pharmacia, Piscataway, NJ) and washed five times with ice-cold lysis buffer, and the counts were associated with the Con A-Sepharose pellet quantitated by gamma counting. For experiments with phosphorylated peptides, the phosphatase inhibitor NaF (10 mM) was included in all buffers after having established that this did not affect translocation of peptide 417.

Induction of murine CTL in vivo

Peptide-specific CTL lines were generated by immunization of C57BL/6 mice, as described (26). Briefly, RMA-S cells (10^8) were pulsed with the phosphopeptide K3-P (10 μM) at 26°C for 18 h. The cells were irradiated (60 Gy) and inoculated i.p. into C57BL/6 mice. Mice were sacrificed 3 wk later and the spleen cells were restimulated in vitro with syngeneic spleen cells (4×10^6) pulsed with K3-P for 5 days in RPMI 1640 supplemented with 10% FCS.

Generation of human CTL lines as primary responses in vitro

Peptide-specific CTL were established in vitro as primary responses using autologous dendritic cells (DC) as APC. DC were developed from PBMC from healthy donors, as described previously (27). Briefly, PBMC were resuspended at 4×10^6 cells/ml in R10 medium in six-well plates (Nunc, Naperville, IL) at 3 ml/well. After 2 h of incubation at 37°C, the nonadherent cells were washed away, and 3 ml R10 medium supplemented with 500 U/ml GM-CSF (Leucomax, Sandoz, Germany) and 25 U/ml IL-4 (PeproTec, Boston, MA) was added to each well. Every third day, 0.5 ml medium was replaced with fresh R10 medium containing GM-CSF and IL-4 to a final concentration of 500 U/ml and 25 U/ml, respectively. After 9 days, 50 U/ml IL-1α was added to the culture, and DC were harvested the next day. DC were resuspended at 5×10^6 cells/ml in RPMI without serum containing 40 μg/ml peptide and 3 μg/ml human β_2-microglobulin for 4 h and subsequently irradiated (30 Gy). The peptide-pulsed, irradiated DC (2×10^5/well) were used to stimulate fresh lymphocytes (5×10^6/well) in 24-well plates (Costar) in 2 ml RPMI medium with 20% AIM V medium (Life Technologies) and 5% human serum supplemented with 5 ng/ml IL-7 (PeproTech). On day 10, and weekly thereafter, the responder population was restimulated. Irradiated, peptide-pulsed autologous PBMC were used to restimulate the T cell line at a stimulator:responder ratio of 1:5, with addition of IL-2 (40 U/ml; PeproTech) on the following day. CTL clones were established from bulk cultures by limiting dilution in 96-well plates using peptide-pulsed, irradiated PBMC as feeder cells in the presence of 40 U/ml IL-2 and 1 μg/ml PHA.

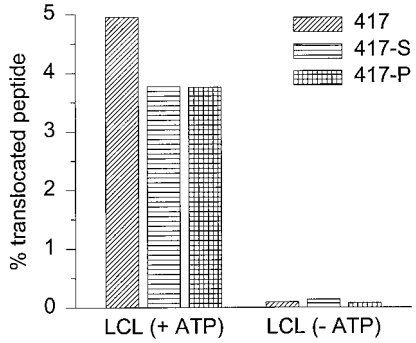

FIGURE 1. Translocation of phosphopeptide by human TAP. The index peptide (417), a serine-substituted analogue (417-S), as well as a phosphorylated (417-P) version thereof, all containing an acceptor site for ER-type *N*-linked glycosylation, were iodinated and added to streptolysin *O*-permeabilized human LCL cells in the presence or absence of ATP. Peptides transported into the ER were recovered with Con A-Sepharose from detergent lysates and quantified by gamma counting. The results indicated are the amounts of translocated peptide recovered, expressed as a fraction of the total input peptide.

CTL cytotoxicity assay

Conventional ^{51}Cr release assays for CTL-mediated cytotoxicity were used to test the specificity of CTL lines against peptide-pulsed target cells. Target cells were autologous EBV-transformed B cell lines (assays with human cells) or T2 cells transfected with H-2K^b or H-2D^b (murine assays), as described (7). For a typical CTL assay, 10^6 target cells in 50 μl R10 were labeled with ^{51}Cr (100 μCi; Amersham, Arlington Heights, IL) and in some cases pulsed with peptide (4 μg) in a round-bottom well of a 96-well plate at 37°C for 60 min. The washed target cells were plated out in 96-well plates with CTL at various E:T ratios and incubated at 37°C for 4 h, before 100 μl of medium was aspirated and ^{51}Cr release counted in a gamma counter (Cobra 5005; Packard Instruments, Meriden, CT). The maximum ^{51}Cr release was determined in separate wells by addition of 100 μl 10% Triton X-100, and spontaneous release was determined by the addition of 100 μl R10 only to target cells. Specific lysis was calculated using the following formula: ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100.

Results

Phosphorylated peptides are efficiently transported by TAP

Assays for direct translocation of phosphopeptides by TAP were conducted as described (24, 25) by adding radiolabeled peptides and phosphopeptides, which contain an *N*-linked glycosylation sequon to streptolysin O-permeabilized cells. Fig. 1 shows the result

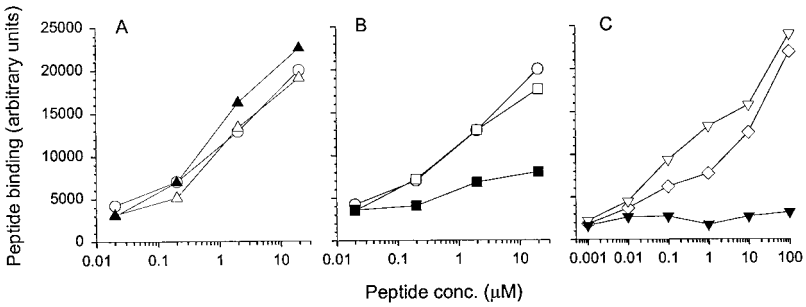

FIGURE 2. Phosphopeptide binding to HLA-A*0201 (*A* and *B*) or HLA-B*2705 (*C*). The binding of the phosphorylated $CRKL_{207–386}$ peptide OP42-P (Y($-PO_3^{2-}$)AQPQTTTPL) (▲) was compared with the nonphosphorylated OP42 (YAQPQTTTPL) (△) and the positive control peptide C1 (○) (*A*). The binding of the phosphorylated c-$Jun_{67–76}$ peptide OP27-P (GLLKLAS($-PO_3^{2-}$)PEL) (■) was compared with the nonphosphorylated OP27 (GLLKLASPEL) (□) and the positive control peptide C1 (○) (*B*). The binding of the phosphorylated $p53_{378–386}$ peptide OP39-P (S($-PO_3^{2-}$)RHKKLMFK) (▼) was compared with the nonphosphorylated OP39 (SRHKKLMFK) (▽) and the positive control peptide C4 (◇) (*C*).

of an assay for translocation of the peptides 417, the serine substituted 417-S, and the phosphorylated 417-P using the human LCL cells in the presence or absence of ATP for each peptide. All peptides are transported across the ER membrane in a strictly ATP-dependent manner by both the human (Fig. 1) and murine (results not shown) TAP, as determined by the resulting *N*-glycosylation and recovery of the iodinated peptides with Con A-Sepharose. Peptide 417 as well as the serine-substituted 417-S were very efficiently translocated with recoveries of 4–5% for LCL, consistent with previously published data (28). Phosphorylation of 417-S (giving 417-P) resulted in translocation of similar high efficiency in LCL. These data show that phosphorylated peptides can be efficiently transported from the cytosol into the ER by TAP.

Pleiotropic effect of peptide phosphorylation on binding to class I MHC

Based on an extensive binding study[4] with 73 potential MHC-binding peptides all containing a natural phosphorylation site from viral phosphoproteins as well as from phosphorylated oncogene products that display altered phosphorylation upon malignant transformation, nine peptides that showed strong binding were selected for phosphorylation. Four peptides were derived from human oncogene phosphoproteins and four peptides were from viral phosphoproteins. In addition, the model peptide K3 (FAPSNYPAL) (7) was included in the study. K3 is an analogue of the H-$2K^b$-restricted immunodominant Sendai virus $NP_{324–332}$ epitope FAPGNYPAL.

The tyrosine-phosphorylated version of the CRKL-derived peptide OP42-P (YAQPQTTTPL) bound strongly to HLA-A*0201 (Fig. 2*A*), illustrating that phosphorylation did not prevent the efficient binding of this peptide to class I MHC. However, we also observed several instances in which the phosphorylation of non-anchor tyrosine, serine, or threonine residues had either an intermediate or a severe negative effect on peptide binding to class I .MHC alleles. Thus, the phosphorylated c-Jun peptide OP27-P (GLLKLASPEL) only bound weakly to HLA-A*0201 compared with the nonphosphorylated peptide (Fig. 2*B*), and the phosphorylated version of the $p53_{378–386}$ peptide OP39-P did not show any binding to HLA-B*2705, whereas OP39 (SRHKKLMFK) bound better than the positive control epitope (KRWIIMGLNK) (29).

We then tested the binding of nine different phosphopeptide versions of class I MHC-binding peptides, in which modifications lay outside the primary anchor residues, for their ability to bind to seven different class I MHC molecules. The binding of each peptide was compared with a positive control peptide (Table I), and a binding score was assigned to the different peptides.

Thus, peptides binding with the same high affinity as the positive control peptides were assigned the score +++, whereas peptides without measurable binding were assigned the value −. In general, phosphopeptides could be divided into three groups in which phosphorylation had no effect, an intermediate negative effect, or a strong negative effect on binding to class I MHC. The following three phosphopeptides belonged to the group of peptides that maintained efficient class I MHC binding upon phosphorylation: OP42-P ($CRKL_{207–216}$, YAQPQTTTPL) bound to HLA-A*0201, OP46-P ($BCR_{174–182}$, KPFYVNVEF) bound to both HLA-B*0701 and HLA-B*3501, and the Sendai virus peptide analogue K3-P (FAPSNYPAL) bound to both H-$2D^b$ and H-$2K^b$ with high affinity (Table II). Of the peptides whose binding is blocked by phosphorylation, the influenza $M2_{87–94}$ peptide P49 (DDSHFVSI) is the only one that is phosphorylated on two sites in vivo (41). However, neither the diphosphorylated version nor any of the two monophosphorylated versions of the peptide showed any measurable binding to H-$2K^k$. The fact that phosphorylation

[4] M. H. Andersen, L. Tan, I. Søndergaard, J. Zeuthen, T. Elliott, and J. S. Haurum. Poor correspondence between predicted and experimental binding of peptides to class I MHC molecules. *Submitted for publication*

Table I. *Positive control peptides*

MHC Allele	Name	Sequence	Protein[a]	Score	C_{50} (μM)[b]	Ref.
A*0201	C1	ILKEPVHGV	HIV-1 $pol_{476–484}$	+++	1 μM	30
B*0702	C2	QPRAPIRPI	EBV EBNA $3C_{881–889}$	+++	0.8 μM	31
B*3501	C3	MPLETQLAI	Malaria LFA-$1_{77–85}$	+++	0.9 μM	32
B*2705	C4	KRWIIMGLNK	HIV-1 $gag_{263–272}$	+++	8 μM	29, 33
H-$2K^b$	C5	FAPGNYPAL	Sendai virus $NP_{324–332}$	+++	0.05 μM	34
H-$2D^b$	C5	FAPGNYPAL	Sendai virus $NP_{324–332}$	+++	0.005 μM	34
H-$2K^k$	C6	SDYEGRLI	Influenza virus $NP_{50–57}$	+	>20 μM	35

[a] The value range listed in subscript indicate the position of the peptide in the sequence.
[b] The C_{50} value is the concentration of the peptide required for half maximal binding.

Table II. *Binding of phosphopeptides to class I MHC molecules*

Peptide Name	Sequence[a]	Protein	Score	C_{50} (μM)	Ref.[b]
HLA-A*0201					
OP42	YAQPQTTTPL	$CRKL_{207–216}$	+++	1	36
OP42-P	YAQPQTTTPL		++++	0.5	
OP27	GLLKLASPEL	$c\text{-}JUN_{67–76}$	+++	1	37
OP27-P	GLLKLASPEL		+	>20	
HLA-B*0702					
OP42	YAQPQTTTPL	$CRKL_{207–216}$	+	>20	36
OP42-P	YAQPQTTTPL		+	>20	
OP46	KPFYVNVEF	$BCR_{174–182}$	+++	0.8	38
OP46-P	KPFYVNVEF		++	3	
P26	SPIVPSFDM	Influenza virus $NP_{473–481}$	++	3	39
P26-P	SPIVPSFDM		–		
HLA-B*3501					
OP42	YAQPQTTTPL	$CRKL_{207–216}$	+++	0.9	36
OP42-P	YAQPQTTTPL		+	>20	
OP46	KPFYVNVEF	$BCR_{174–182}$	+++	1.6	38
OP46-P	KPFYVNVEF		++	8	
P26	SPIVPSFDM	Influenza virus $NP_{473–481}$	+++	0.5	39
P26-P	SPIVPSFDM		–		
HLA-B*2705					
OP39	SRHKKLMFK	$p53_{378–386}$	++++	0.5	40
OP39-P	SRHKKLMFK		–	–	
H-2K^b					
K3	FAPSNYPAL	Sendai virus $NP_{324–332}$	+++	0.2	7
K3-P	FAPSNYPAL	(analogue)	+++	0.2	
H-2D^b					
K3	FAPSNYPAL	Sendai virus $NP_{324–332}$	+++	0.01	7
K3-P	FAPSNYPAL	(analogue)	+++	0.01	
H-2K^k					
P9	SDEKAASPI	Influenza virus $NP_{467–475}$	++	>20	39
P9-P	SDEKAASPI		+	>20	
P10	IDEKLSEIL	RSV RNA $pol_{138–146}$	+++	0.6	16
P10-P	IDEKLSEIL		–	–	
P49	DDSHFVSI	Influenza virus $M2_{87–94}$	+++	1	41
P49-PP	DDSHFVSI		–		
P49-P1	DDSHFVSI		–		
P49-P2	DDSHFVSI		–		

[a] Phosphorylated residues are underlined, and phosphopeptides are designated with "–P."
[b] All peptides contain a natural phosphorylation site.

completely abrogates class I MHC binding of some peptides examined is a strong indication that dephosphorylation does not occur during the peptide-binding assay.

It is interesting to note that phosphorylation of peptide residue 1 in P26 blocks peptide binding to HLA-B*0702 and HLA-B*3501, and similarly phosphorylation of residue 1 in peptide OP39 blocks binding to HLA-B*2705. The side chain of the N-terminal peptide residue does not usually interact directly with the class I MHC molecule. However, charge-pairing effects between the α-amino terminus of the peptide and the phosphate group may prevent peptide binding.

Phosphopeptide-specific CTL responses

Both the nonphosphorylated and the phosphorylated version of the synthetic peptide K3 (FAPSNYPAL) bound with high affinity to both H-2D^b and H-2K^b. Spleen cells from C57BL/6 mice immunized with phosphopeptide K3-P-pulsed RMA-S cells were harvested after 3 wk later and tested for lytic activity against peptide-pulsed T2-K^b and T2-D^b cells. As seen in Fig. 3*A*, the spleen cell-derived CTL showed a high degree of specificity against the phosphopeptide. However, some CTL activity against the nonphosphorylated peptide was also observed, either due to the coexistence of nonphosphorylated peptide-specific CTL or due to cross-reacting CTL. Furthermore, the CTL response against the phosphopeptide was H-2K^b restricted, because only T2-K^b but not T2-D^b cells pulsed with K3-P were lysed.

Having confirmed the efficient binding to HLA-A2 (see Fig. 2*A*), the CRKL-derived phosphopeptide OP42-P peptide was also used to generate human CTL in vitro primary responses. Autologous DC were generated from HLA-A2-positive PBMC in the presence of IL-4 and GM-CSF. Next CTL were stimulated in vitro using irradiated OP42-P-pulsed dendritic cells as APC in the presence of IL-2 and IL-7. After five in vitro restimulations, the peptide specificity was tested in standard ^{51}Cr release assays using peptide-pulsed autologous EBV-transformed KIG-BCL as target

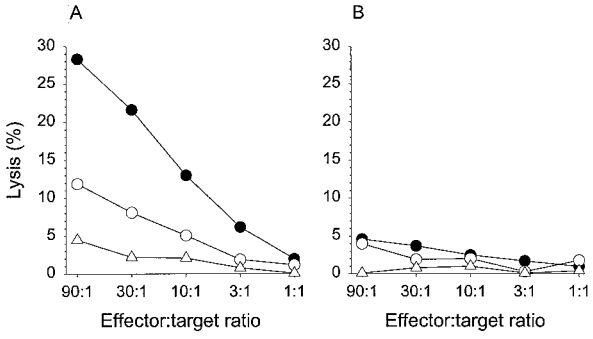

FIGURE 3. Peptide recognition by murine CTL raised against K3-P. Splenocyte-derived CTL from C57BL/6 mice immunized with K3-P-pulsed RMA-S cells were used as effectors against T2-K^b (*A*) or T2-D^b (*B*) target cells pulsed with K3-P (●), K3 (○), or without peptide (△).

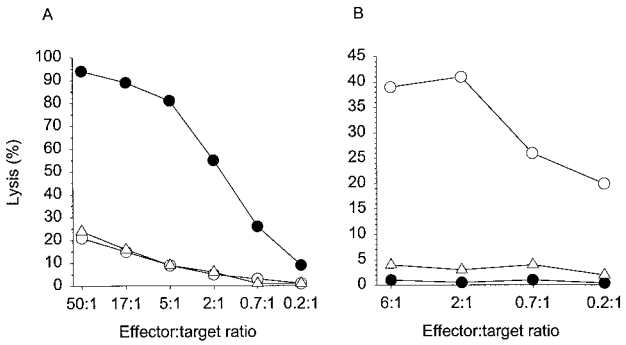

FIGURE 4. Peptide recognition by human CTL raised against OP42-P (*A*) or against nonphosphorylated OP42 (*B*). HLA-A2-restricted human in vitro primary CTL raised against the phosphopeptide OP42-P (Y(−PO_3^{2-})AQPQTTTPL) (*A*) or the nonphosphorylated peptide OP42 (*B*) were tested in a ^{51}Cr release assay using autologous EBV-transformed BCL pulsed with OP42 (○), OP42-P (●), or without peptide (△).

cells. Target cells pulsed with OP42-P were lysed (Fig. 4*A*), whereas target cells pulsed with OP42 or without peptide were not recognized. Thus, the resulting CTL line was specific for the phosphopeptide OP42-P.

CTL were also raised in vitro against the nonphosphorylated version of the peptide OP42, which binds with high affinity to HLA-A2, using a HLA-A2-positive donor. This CTL cell line was cloned, and the specificity of the resulting clones was examined. From Fig. 4*B* it is seen that a CTL clone raised against OP42-lysed target cells pulsed with OP42, and that it did not recognize target cells pulsed with phosphorylated OP42-P or without peptide. Thus, a peptide-specific CTL clone was established that did not cross-react with the phosphorylated peptide. Furthermore, these data indicate that no dephosphorylation of peptide occurred during the CTL assay.

These results clearly demonstrate the ability to generate phosphopeptide-specific class I MHC-restricted CTL responses, and suggest that the phosphate group is involved in a specific contact with TCR.

Discussion

In this study, we demonstrate that phosphopeptides can be transported from the cytosol into the ER by TAP, and that a class I MHC-binding peptide can be phosphorylated without affecting its binding to MHC class I. Thus, it is likely that phosphopeptides are presented by class I MHC molecules in vivo. Furthermore, we demonstrate that at the cell surface phosphopeptides presented by class I MHC molecules can elicit phosphopeptide-specific CTL. Also, CTL raised against a nonphosphorylated peptide recognize target cells pulsed with the nonphosphorylated peptide, without recognizing target cells pulsed with the phosphorylated peptide. Thus, T lymphocytes discriminate between phosphorylated and nonphosphorylated peptides. In future studies, we will address whether class I MHC-restricted T cell responses against endogenously expressed phosphopeptides are part of the natural T cell repertoire in vivo. Also, it cannot be ruled out that proteasome degradation or cytosolic phosphatases prevent the presentation of phosphopeptides in vivo.

We have investigated CTL responses against phosphopeptides in two model systems. We observed a HLA-A2-restricted phosphopeptide-specific primary response against OP42-P in vitro. We also immunized mice with the phosphopeptide K3-P analogue of the dominant K^b-restricted epitope from Sendai virus NP. Even though this phosphopeptide binds with high affinity to H-2K^b or H-2D^b, we only observed a K^b-restricted CTL response against the peptide, thus resembling the natural H-2K^b-restricted response in C57BL/6 mice against Sendai virus. Our group has previously described that glycosylation of K3 peptide on the same serine residue at position 4 with *N*-acetylglucosamine (GlcNAc) does not effect peptide binding to H-2K^b or H-2D^b, and that it is possible to elicit both H-2K^b- and H-2D^b-restricted glycopeptide-specific CTL response (7). However, in our previous study, CTL were restimulated several times in vitro, which may have induced the D^b-restricted response. Recently, we have solved the crystal structure of a glycosylated version of K3 (FAPS(O-β-GlcNAc)NYPAL) in complex with H-2D^b, and it was revealed that the carbohydrate residue is pointing out of the groove (42).

Four of the phosphopeptides examined did not show any binding to MHC when phosphorylated. The molecular basis for this inhibition is unclear because all modifications were made at positions outside the primary anchor residues. Two of the four affected peptides were phosphorylated at position 1, which could possibly affect the formation of the conserved bonding in the A pocket of the class I MHC-binding groove. The other two affected peptides were H-2Kk restricted, and binding to this allele, which requires a negatively charged position 2 anchor residue, may be adversely affected by the addition of a further highly negatively charged group. The negative influence of phosphorylation on class I MHC binding is of immunological interest, because it provides a novel potential immune escape mechanism. For example, the influenza $NP_{473-481}$-derived phosphopeptide P26 did not show any appreciable binding to HLA-B*0702 and HLA-B*3501, whereas the nonphosphorylated version of the same peptide bound with high affinity to both alleles. Thus, if the source protein is phosphorylated in an infected cell, resulting in the generation of the phosphorylated peptide by the Ag-processing apparatus, it cannot be presented at the cell surface by the MHC molecule, thus allowing the virus to avoid immune elimination.

To date, only a few examples of posttranslationally modified natural class I MHC-restricted epitopes have been described. Skipper et al. identified a HLA-A*0201-restricted tyrosinase-derived melanoma peptide, in which the asparagine in position 3 was posttranslationally modified to aspartic acid (9). Meadows et al. identified a modified HLA-A*0201-restricted peptide epitope from the human minor histocompatibility Ag H-Y, describing T cells that only recognize posttranslationally altered forms of this peptide (10). One of these modifications involves attachment to the peptide of a second cysteine residue via a disulfide bond.

Our group has previously shown that class I MHC molecules can bind synthetic glycopeptides and that the glycopeptides elicit a strong CTL response that is glycopeptide specific (7). Furthermore, we have recently shown that glycopeptides serve as natural substrates for the TAP molecule, and that class I MHC molecules present a small amount of glycosylated peptides in vivo (43). Van Stipdonk et al. have recently described that murine class II MHC-restricted T cells raised against differentially phosphorylated forms of αB-crystallin discriminate between the phosphorylated and the nonphosphorylated peptides (44).

In this study, we have investigated the effect of phosphorylation on peptide binding to class I MHC molecules and the recognition of CTL. We have examined three different kinds of phosphoproteins as sources for the cellular production of phosphopeptides. First, because known phosphorylation sites in some proteins become constitutively phosphorylated during oncogene activation, the ensuing phosphopeptide fragments offer themselves as candidates for T cell Ags in line with overexpressed, nonmutated tumor

Ags. Examples of such proteins include c-Jun and the tumor suppressor gene product p53. c-Jun becomes activated by phosphorylation of the two Ser residues 63 and 73, and has been reported to be phosphorylated in both leukemia and lung cancer cells (37). Phosphorylation of the p53 tumor suppressor gene product has been described for a number of serine residues, among these Ser^{9}, Ser^{15}, Ser^{37}, Ser^{315}, Ser^{392}, and Ser^{378} (40, 45–47).

Second, upon the critical event leading to activation of the transforming phenotype, some protooncogenes characteristically acquire novel kinase activities leading to the appearance of novel phosphorylation sites. Thus, as a consequence of the (9, 22) translocation (the Philadelphia chromosome) that is associated with all chronic myeloid leukemia cases and 20% of adult acute lymphoblastic leukemia, the c-*abl* protooncogene becomes activated by its translocation to chromosome 22 giving rise to a BCR-ABL fusion protein (48, 49). A crucial difference between the fusion protein and the normal c-*abl* is that only the chimeric protein exhibits tyrosine kinase activity (14), resulting in autophosphorylation of a number of tyrosine residues (including Tyr^{177}) of the BCR-ABL fusion protein (50, 51), as well as causing a constitutive increase in tyrosine phosphorylation of other cellular proteins. One of the best-described proteins is the CRKL, which is overexpressed and hyperphosphorylated on Tyr^{207} in Philadelphia chromosome-positive chronic myeloid leukemia cells (36, 52). Each of these new sites of phosphorylation could give rise to tumor-specific phosphopeptide neo-Ags. Furthermore, it is conceivable that transformation-associated posttranslational modification of a cellular protein could alter the pattern of peptide fragments that are generated from it.

Finally, viral phosphoproteins with known phosphorylation sites have also been included in the study. For example, the EBV protein EBNA-2 is phosphorylated at Ser^{469} and Ser^{470} (53), EBV LMP-1 at Ser^{313} and Thr^{324} (54), and EBV BZLF1 at Ser^{173} (55). Other viruses with multiple known phosphorylation sites include adenovirus, HIV, and influenza virus.

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