JOURNAL OF VIROLOGY, July 1992, p. 3996–4002
0022-538X/92/073996-07$02.00/0

Vol. 66, No. 7

Posttranslational Side Chain Modification of a Viral Epitope Results in Diminished Recognition by Specific T Cells

JOVI K. LARSON,† LASZLO OTVOS, JR., AND HILDEGUND C. J. ERTL*
The Wistar Institute, Philadelphia, Pennsylvania 19104

Received 15 August 1991/Accepted 25 March 1992

A stretch of 16 amino acid residues within the nominal phosphoprotein of rabies virus was shown to carry an immunodominant epitope for class I- and class II-restricted T cells. The nominal phosphoprotein of rabies virus is thought to be heterogeneously phosphorylated at multiple serine and threonine residues. The synthetic peptide that expressed the T-cell epitope contained a single serine residue corresponding to position 196 of the protein. Phosphorylation of this serine within the synthetic peptide caused a significant decrease of the antigenic potency of the peptide. A similar effect was seen if the serine was replaced by an alanine or if the peptide was glycosylated at its acidic residues. These data suggest that T-cell-mediated recognition of antigen presented by major histocompatibility complex class I- or II-positive cells is impaired not only by point mutations but also by posttranslational side chain modifications of residues within viral epitopes.

Little is known about the effect of side chain modifications on recognition by T cells. Previous studies suggested that inhibition of glycosylation of viral antigens might influence T-cell recognition (8, 25). In recent years, numerous viral epitopes have been identified for both class I- and class II-restricted T cells (13, 18, 23). Although many of these viral proteins carry modified side chains under physiological conditions, a review of the literature reveals that, in general, the epitopes identified to date lack natural glycosylation or phosphorylation sites, suggesting that these sites might not be readily accessible for recognition by T cells.

The effect of side chain modification was studied by using the nominal phosphoprotein (NS protein [this protein was initially thought to be a nonstructural protein, hence the abbreviation NS]) of rabies virus, a 297-amino-acid protein that is thought to be heterogeneously phosphorylated at multiple serine and threonine residues (4, 6), as been demonstrated for vesicular stomatitis virus, the prototype virus of the *Rhabdoviridae* family, which includes rabies virus.

MATERIALS AND METHODS

Mice. Female C3H/He mice were purchased from The Jackson Laboratories, Bar Harbor, Maine, or from Harlan Sprague-Dawley, Indianapolis, Ind. They were immunized at between 8 and 12 weeks of age.

Cell lines. The murine L929 fibroblast line ($H\text{-}2^k$) was maintained in vitro in Dulbecco's minimal essential medium (MEM) supplemented with 50 μg of gentamicin sulfate per ml, 10 U of penicillin G per ml, 10 μg of streptomycin sulfate per ml, and 10% heat-inactivated fetal bovine serum (FBS). BHK-21 cells and HeLa cells (ATCC CCL-2; American Type Culture Collection, Rockville, Md.) were maintained in Eagle's MEM supplemented with 5% FBS and 50 μg of gentamycin sulfate per ml. HT-2 cells were maintained in Dulbecco's MEM supplemented with 10% FBS, 10^{-5} M 2-mercaptoethanol (2-ME), and 10% rat concanavalin A (ConA) supernatant.

Viruses. The rabies virus strain Evelyn-Rokitniki-Abelseth (ERA) was propagated on BHK-21 cells and purified as described previously (21). Purified virus was inactivated with β-propiolactone (BPL) and adjusted to 0.1 mg of protein per ml. A vaccinia rabies NS (VRNS) recombinant virus was constructed by insertion of the entire NS coding sequence from the ERA virus, including translational initiation and termination codons, into the thymidine kinase gene of vaccinia virus Copenhagen strain, using established methods as described previously (17). In the recombinant virus, the NS gene was under the control of the vaccinia virus 7.5-kDa early promoter. Expression of the NS protein in cells infected with VRNS was verified by Western immunoblot analysis (15). Both the parental vaccinia virus and the VRNS recombinant virus were grown in HeLa cells in Eagle's MEM supplemented with 5% FBS and antibiotics. Vaccinia viruses were purified over sucrose density gradients and titrated on confluent monolayers of HeLa cells (27).

Peptides. Peptides were synthesized on a Biosearch SAM 2 peptide synthesizer, using 9-fluoroenylmethoxycarbonyl amino acid chemistry (11). Peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on an Altex Ultrasphere ODS column, and their structure was verified by amino acid composition analysis, fast atom bombardment mass spectroscopy, and direct peptide sequencing. A synthetic peptide containing residues 191 to 206 of the NS protein (i.e., EKDDLSVEAEIAHQIA), termed $NS_{191-206}$, was phosphorylated (19) at Ser-196, and the presence of a single phosphate group was detected by mass spectroscopy and phosphate analysis. Peptide $NS_{191-206}$-P (the phosphorylated version) eluted earlier (0.8 min) than peptide $NS_{191-206}$ during RP-HPLC, and no overlap of the two peaks was detectable. A longer peptide corresponding to residues 191 to 211 of the NS protein (i.e., EKDDLSVEAEIAHQIAESFSK), termed $NS_{191-211}$, was phosphorylated as well and purified by HPLC. Several peaks reflecting different degrees of phosphorylation of the three serine residues were collected (19). Two peaks were analyzed by mass spectroscopy; the preparation containing the highest percentage of fully phosphorylated peptide was chosen for the experiments. In this preparation, 68% of the peptide carried three phosphate groups whereas 32% had only two phosphate groups. Acidic residues of $NS_{191-206}$ were glycosylated by carbodiimide coupling of 1-amino-*N*-

* Corresponding author.
† Present address: Laboratory of Immunology, Wadsworth Center for Laboratories and Research, Albany, NY 12201.

acetylglucosamine to the purified peptide. Circular dichroism measurements were made on a Jasco J720 Spectropolarimeter at room temperature in different ratios of H_2O or phosphate-buffered saline and trifluoroethanol (TFE). The percentage of helicity was calculated on the basis of the mean residue ellipticity at 208 nm (12).

Generation and testing of rabies virus-specific CTL. Mice were immunized with 10^6 PFU of ERA virus injected intramuscularly into each hind leg. Single cell suspensions were prepared from draining lymph nodes (i.e., popliteal and periaortal) 7 days later. Lymphocytes were cultured in 24-well plates for 5 days in Dulbecco's MEM supplemented with 2% FBS and 10^{-5} M 2-ME at 5×10^6 cells in a volume of 1.6 ml with 0.25 μg of BPL-inactivated ERA virus (ERA-BPL) per well. The presence of cytotoxic T cells (CTL) was determined with a standard 5- to 6-h ^{51}Cr release assay, using infected or peptide-treated ^{51}Cr-labeled L929 target cells at different effector-to-target cell ratios. Target cells were infected with 10 PFU of either vaccinia virus or the VRNS recombinant virus per cell 2 h prior to ^{51}Cr labeling. Control cells were mock infected. Target cells presenting peptides were suspended at 10^6 cells per ml in Dulbecco's MEM containing 10% FBS and the indicated concentration of peptide for 12 h at room temperature prior to labeling. Labeled target cells were added at 5×10^3 cells per 50 μl to V-bottom microtiter plate wells. Effector cells were added in a volume of 100 μl at different concentrations. To assess spontaneous release, medium was added to the target cells, and to determine maximal release, 1% sodium dodecyl sulfate was added. Microtiter plates were centrifuged for 3 min at $1,000 \times g$ and then incubated for 5 to 6 h in a humidified 10% CO_2 incubator. Supernatants were subsequently harvested (75 μl per well) and counted in a γ counter. Percent specific lysis was calculated by using the formula $100 \times$ [(^{51}Cr release in the presence of lymphocytes − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)].

Generation of NS protein-specific T-cell lines. Female C3H/He mice were immunized intraperitoneally with 10^7 PFU of VRNS recombinant virus. Seven days later, graded numbers (1×10^1 to 8×10^3) of splenic lymphocytes were added to individual round-bottom wells (48 wells per dilution) of 96-well microtiter plates containing 5×10^5 γ-irradiated (2,000 rads) syngeneic naive splenocytes, one-tenth of which had been pretreated with 0.5 μg of ERA-BPL per 10^8 splenocytes, and 25% rat ConA supernatant in 200 μl of Dulbecco's MEM supplemented with 10% FBS and 10^{-5} M 2-ME. Cells of wells that showed good growth after 10 days were transferred into individual wells of a 24-well Costar plate with 6×10^6 irradiated ERA-BPL-pretreated splenocytes and 10% rat ConA supernatant in 1.6 ml of Dulbecco's MEM supplemented with 2% FBS. Cells that continued to grow were subcultured routinely once a week. They were eventually tested for proliferation to antigen and subcloned at 0.2 cells per well (10).

Release of lymphokines by and proliferation of T-cell lines in response to antigen. T cells were tested for proliferation or release of lymphokines in response to antigen as described previously (10). Briefly, 5×10^5 irradiated syngeneic splenocytes in 100 μl of medium were incubated in 96-well round-bottom microtiter plate wells with 25 μl of antigen. After 30 min of incubation at 37°C, 2×10^4 T cells in 50 μl were added. For proliferation assays, the original plates containing the rabies virus-specific T cells were pulsed 40 to 44 h later for 6 to 8 h with 0.6 μCi of [^{3}H]thymidine per well. For lymphokine release assays, plates were centrifuged for 3 min at $2,000 \times g$; 20 h later, 50 μl of cell-free supernatants were transferred onto 2×10^3 HT-2 cells in 100 μl of Dulbecco's MEM supplemented with 15% FBS and 2-ME in round-bottom microtiter plate wells. Cells were pulsed 24 to 36 h later for 6 h with 0.5 μCi of [^{3}H]thymidine per well.

For the competition assay, an excess of varied concentrations of the competing peptide, i.e., a 10-amino-acid long hen egg lysozyme (HEL) peptide (HEL_{51-61} [2]) in 25 μl of medium was cocultured with irradiated splenocytes diluted to 5×10^5/75 μl in microtiter plate wells for 60 min at 37°C. The antigenic peptide was subsequently added, and a lymphokine release assay was conducted from thereon as described above.

To test the kinetic of association of peptides with major histocompatibility complex (MHC) class II determinants, 25 μl of peptide $NS_{191-206}$ or $NS_{191-206}$-P was added in microtiter plate wells to 5×10^5 irradiated splenocytes for varied periods of time. Cells were subsequently washed twice with 200 μl of serum-free medium, resuspended in 100 μl of culture medium, and cocultured with T cells. In this assay, data reflect the proliferative response of an NS protein-specific T-cell line.

Paraformaldehyde fixation of antigen-presenting cells (APC). Splenocytes from naive C3H/He mice were separated over a Ficoll gradient. Cells were washed in phosphate-buffered saline and treated with 0.5% paraformaldehyde as described previously (3).

Elution of peptides from APC. A modification of a previously described method was used (26). Ficoll-purified C3H/He splenocytes (10^8 per group) in 1 ml of Dulbecco's MEM were incubated with 0.5 mM of peptide for 3 h at 37°C. Cells were subsequently washed three times with phosphate-buffered saline, and 0.5 ml of 2.5 M acidic acid was added to the cell pellet for 30 min at 37°C. Cells were pelleted by a 10-min centrifugation at $5,000 \times g$. Supernatants were separated by RP-HPLC. Fractions were lyophilized and reconstituted with 100 μl of Dulbecco's MEM supplemented with 2% FBS and 2-ME prior to testing.

RESULTS AND DISCUSSION

We previously identified an immunodominant CTL epitope in *H-2*^{k} mice between residues 191 and 206 of the NS protein of the rabies virus strain ERA (16). The synthetic peptide ($NS_{191-206}$) delineated from this sequence (14) was shown to be recognized by CTL derived from ERA virus-infected mice in association with K^k determinants of the MHC (16). The $NS_{191-206}$ peptide contains a single serine residue corresponding to position 196; a longer peptide ($NS_{191-211}$) spanning residues 191 to 211 contains two additional serine residues in positions 208 and 210 of the NS protein which within the rabies virus particle or rabies virus-infected cells is thought to be heterogeneously phosphorylated at its multiple serine and threonine residues. To study the effect of a physiological posttranslational side chain modification on the antigenic potency of a T-cell epitope, the one serine residue of peptide $NS_{191-206}$ and the three serine residues of peptide $NS_{191-211}$ were phosphorylated. The peptides were purified by RP-HPLC, which separated peptides $NS_{191-206}$-P and $NS_{191-206}$ into two nonoverlapping peaks. $NS_{191-211}$ separated clearly from several peaks of $NS_{191-211}$-P. These peaks reflected varied degrees of phosphorylation of the three serine residues. An early HPLC peak that was used for the experiments contained 68% of fully phosphorylated peptide and 32% of peptide that carried only two phosphate groups. The anti-

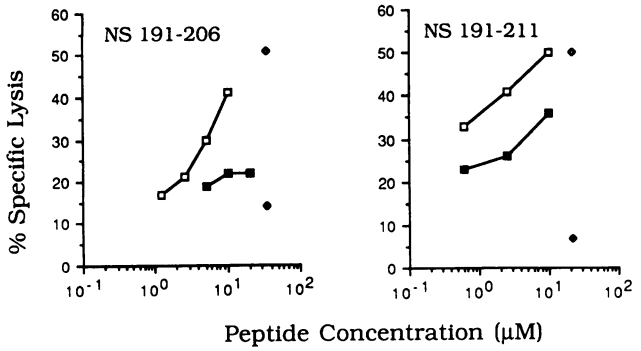

FIG. 1. Evidence that phosphorylation of $NS_{191-206}$ decreases its antigenic potency for CTL. The graphs represent two separate experiments. C3H/He mice were immunized with 10^6 PFU of rabies virus. One week later, draining lymph node lymphocytes were cultured in vitro at 5×10^6 cells per well in 24-well Costar plates in the presence of 0.25 μg of purified ERA-BPL virus. After 5 days in culture, effector cell activity was tested in a 5- to 6-h ^{51}Cr release assay on L929 target cells pretreated with nonphosphorylated (□) or phosphorylated (■) peptide at an effector/target cell ratio of 50:1 ($NS_{191-211}$) or 100:1 ($NS_{191-206}$). The L929 cells had been treated with peptides for 12 h at room temperature. Other L929 cells were infected with either vaccinia virus Copenhagen strain (◆) or VRNS (◇) at 10 PFU per cell for 2 h. L929 cells were subsequently labeled for 1 to 2 h at 37°C with ^{51}Cr and then cocultured with effector cells. Data are expressed as percent specific lysis of triplicate determinations. Standard errors were <10% of the mean.

genic potency of the phosphorylated peptide was initially compared with that of nonphosphorylated peptide by a CTL assay, testing target cells pretreated with either peptide for lysis by ERA virus-induced T cells. As shown in Fig. 1, target cells pretreated with 10 μM peptide $NS_{191-211}$ were as susceptible to lysis as were target cells that expressed the entire NS protein upon infection with a VRNS recombinant virus. Lower concentrations of peptides resulted in proportionally reduced lysis. Target cells pretreated with the $NS_{191-211}$-P peptide showed equivalent lysis if the phosphorylated peptide was added at 10-times-higher concentrations than was the nonphosphorylated peptide. Target cells pretreated with 10 μM peptide $NS_{191-206}$ were less susceptible to lysis than were VRNS virus-infected target cells, but again a clear dilution effect could be observed upon reduction of the dose of peptide (Fig. 1). Although upon intracellular processing of proteins, peptides of a length of 9 amino acids have been shown to be presented in association with MHC class I determinants (20), an increase in antigenic potency directly correlated with the length of the peptide used to coat stimulator cells has also been observed previously (10). Longer peptides show in general a reduced degree of flexibility in solution and might thus, depending on the residues flanking the epitope, stabilize the conformation that allows binding to MHC determinants. Target cells pretreated with varied concentrations of the $NS_{191-206}$-P peptide showed marginal lysis above the level for the control target cells, suggesting that the phosphorylated peptide at the concentrations used was either unable to bind to K^k, the restricting element or, alternatively, failed to be recognized by the T-cell receptor (TCR). Lysis observed upon pretreatment with $NS_{191-211}$-P that contains a subpopulation (32%) of incompletely phosphorylated peptides might, under the assumption that phosphorylation of two of the three serine residues was random, reflect recognition of the ~10% of peptide lacking phosphorylation of the crucial serine in position 196. The observed shift (i.e., 1 log unit; Fig. 1) in the dose-response curve supports this assumption.

The epitope expressed by peptide $NS_{191-206}$ that was shown to be immunodominant for *H-2*^{k}-restricted CTL was also recognized by T helper (T_H) cells of *H-2*^{k} origin (16). Stable NS protein-specific $CD4^+$ T cells could not be generated from rabies virus-immune C3H/He mice, which presumably reflects the low frequency of NS protein-specific T_H cells within a mixture of ERA virus-specific T cells. Upon immunization of C3H/He mice with VRNS recombinant virus and selection for NS protein-specific T cells by in vitro stimulation with ERA-BPL virus, several stable NS protein-specific T-cell lines were obtained. All of these T-cell lines, as well as subclones thereof, were phenotypically Thy-1.2^+, $CD4^+$, and $CD8^-$, as shown by indirect immunofluorescence and subsequent analysis by a fluorescence-activated cell sorter (data not shown). The T cells proliferated and released lymphokine in response to ERA virus, the VRNS recombinant virus, and the $NS_{191-206}$ peptide presented by IA^k-compatible APC (16).

Peptide $NS_{191-206}$ had a higher antigenic potency for T_H cells than for CTL; while recognition of peptide in association with MHC class I determinants required peptide concentration of >1 μM, T_H cells proliferated or released lymphokines in response to nanomolar amounts (<10 nm) of peptide (Fig. 2). Phosphorylation of the serine residue of peptide $NS_{191-206}$ impaired recognition by $CD4^+$ T-cell lines, as measured by lymphokine release (or proliferation; data not shown) in response to syngeneic stimulator cells presenting peptide (Fig. 2). The difference in antigenic potency between the nonphosphorylated and the phosphorylated peptide varied between 5- and 50-fold in different experiments. These results clearly show that a physiological modification of an amino acid residue can alter the recognition by both class I- and class II-restricted T effector cells.

The residual T_H cell response observed to the phosphorylated peptide might reflect a response to nonphosphorylated peptide either contaminating the preparation or produced by intracellular or extracellular removal of the phosphate group. As nonphosphorylated peptide could not be detected by chromatography, a method that would detect a minimal contamination of 5%, and reduction of the response ranged from 80 to 98% for class II-restricted T cells, it seems unlikely that the response was caused by residual nonphosphorylated peptide present in the phosphorylated preparation. Neither could intracellular removal of the phosphate group account for the response, as the difference in antigenic potency of the nonphosphorylated and the phosphorylated peptide was essentially equivalent in nonfixed and paraformaldehyde-fixed stimulator cells (3) (Fig. 3), with the latter unable to present purified rabies virus, indicating that the antigen processing pathway had been inactivated by the paraformaldehyde treatment. Extracellular removal of the phosphate group prior to or upon binding of the peptide to cell surface determinants could not be demonstrated either, as both phosphorylated and nonphosphorylated peptide, when bound and subsequently acid eluted from *H-2*^{k} splenocytes, separated upon RP-HPLC in the same individual but separate fractions, as was shown before binding to the splenocytes (Fig. 4). Although the experiment used intact cells rather than purified K^k molecules and thus does not allow us to distinguish whether the phosphorylated peptide bound to the MHC determinant or some other cell surface molecules, the result demonstrates that a global removal of the phosphate group due to extracellular enzymatic activities did not occur.

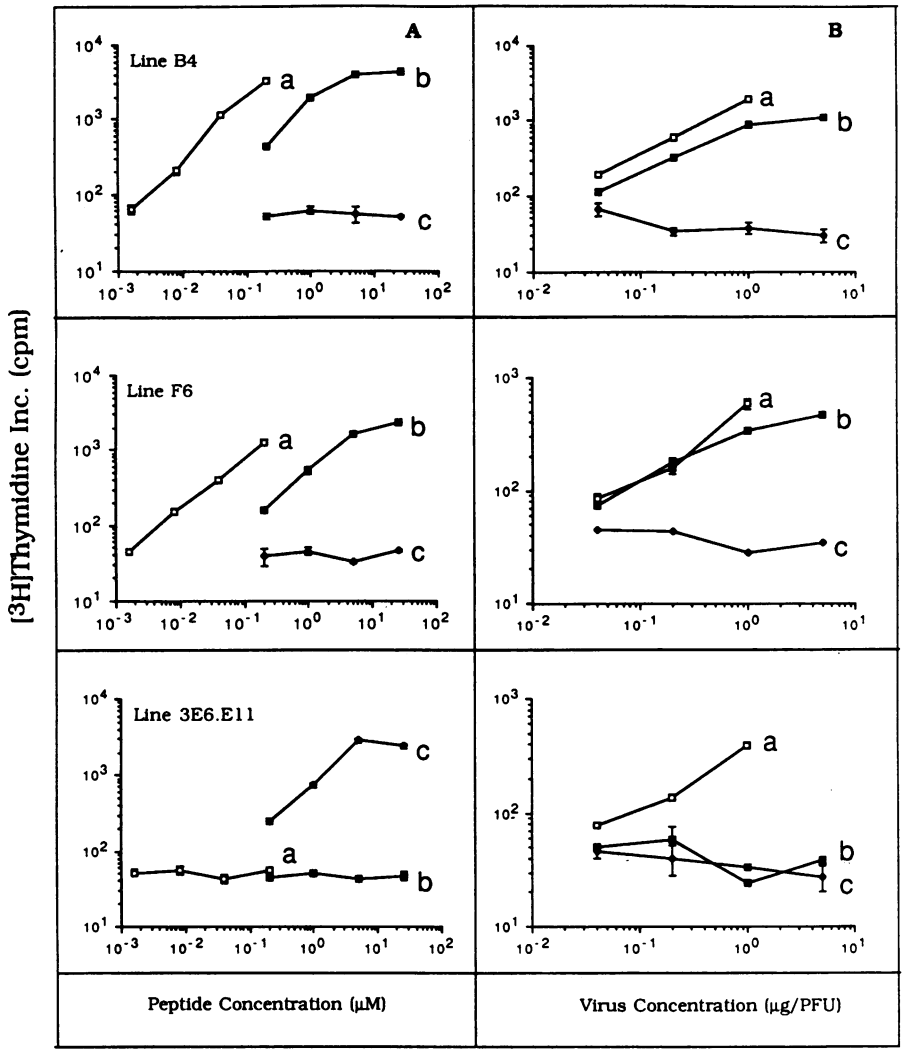

FIG. 2. Evidence that phosphorylation of $NS_{191-206}$ decreases its antigenic potency for lymphokine-secreting T cells. Lymphokine release from long-term T-cell lines was measured by proliferation of HT-2 cells in response to supernatants from these T-cell lines. (A) Response to peptides $NS_{191-206}$ (a [□]), $NS_{191-206}$-P (b [■]), and 31D (c [◆]), which carries an immunodominant T_H-cell epitope of the rabies virus nucleoprotein (9). (B) Response to ERA-BPL (micrograms per milliliter; a [□]), VRNS (b [■]), and vaccinia virus Copenhagen strain (c [◆]) (PFU per milliliter). Lines B4 and F6 were induced in C3H/He mice by immunization with VRNS. They were subsequently subcloned and maintained in vitro in the presence of lymphokines and ERA-BPL-presenting stimulator cells. Line 3E6.E11 is specific for the nucleoprotein and has been described in detail elsewhere (10). Assays were performed by coculturing 2×10^4 T cells with 5×10^5 γ-irradiated (2,000 rads) C3H/He splenocytes and 25 μl of antigen at the concentration indicated on the x axis in a total volume of 175 μl in round-bottom microtiter plate wells. Lymphokine release was measured as described previously (10). In control experiments (not shown), lines B4 and F6 failed to respond to peptide $NS_{201-211}$ but did respond to peptide $NS_{191-211}$. A third line, termed F1, and subclones derived from all three lines exhibited the same pattern of recognition (data not shown).

To test whether the reduction in antigenic potency upon phosphorylation of the serine[196] residue reflected a decrease in binding of the peptide to IA^k or an impaired recognition at the level of the TCR, competition studies were performed with an unrelated peptide derived from residues 51 to 61 of HEL (TDYGILQINS [sequence in single-letter code]), termed HEL_{51-61}, that has previously been described to bind to IA^k (2). As shown in Fig. 5, high concentrations (500 μM) of the HEL_{51-61} peptide interfered with functional binding of the $NS_{191-206}$ peptide, as measured by the ability of peptide-coated APC to stimulate NS protein-specific T_H cells to release lymphokines. The degree of inhibition was directly dose dependent on the concentration of inhibitory peptide as well as on the concentration of the antigenic peptide (i.e., 50% inhibition of 1 μM $NS_{191-206}$, compared with 70% inhibition of 0.2 μM $NS_{191-206}$ in the presence of 500 μM peptide HEL_{51-61}). The $NS_{191-206}$-P peptide used at 1 and 5 μM could not be inhibited by excess amounts of the HEL_{51-61} peptide, suggesting that either the phosphorylated peptide had a higher affinity to MHC than did the unphosphorylated peptide or that the decrease in antigenic potency reflects a reduced recognition by the TCR. Alternatively, the phosphorylated peptide might bind to different residues of the IA^k molecule compared with the $NS_{191-206}$ or HEL_{51-61} peptide.

As the competition studies that take primarily the thermo-

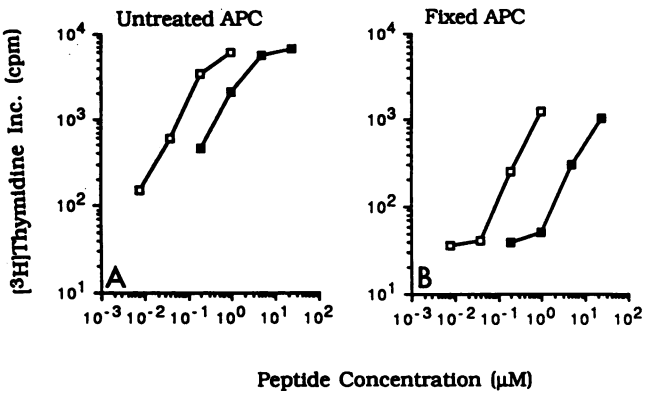

FIG. 3. Presentation of peptides by paraformaldehyde-fixed APC. The assay was performed with line B4. Splenocytes were Ficoll purified, and part of them were pretreated with 0.5% paraformaldehyde (A). The responses (counts per minute) to medium and 1 μg of ERA-BPL per ml were as follows: (A) medium = 57 cpm ± 13; ERA-BPL = 3,154 cpm ± 415; (B) medium = 27 cpm ± 17, ERA-BPL = 41 cpm ± 11. □, $NS_{191-206}$; ■, $NS_{191-206}$-P.

dynamic interactions between peptide and MHC determinants into account could be interpreted as a sole effect of diminished TCR recognition of the phosphorylated peptide-MHC complex, the kinetic of association of peptide with the restricting element was determined next by preincubating peptides for varied lengths of time with APC and subsequently removing peptides remaining in solution. Although

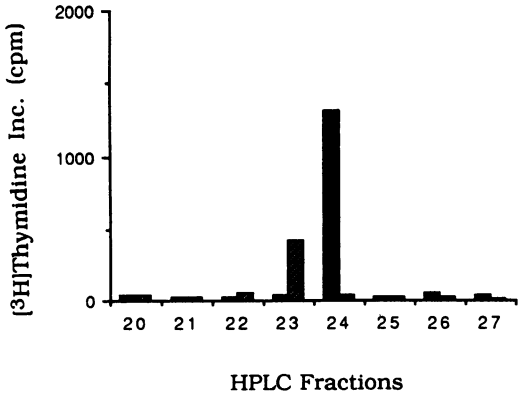

FIG. 4. Binding of both phosphorylated and nonphosphorylated peptide to MHC determinants. Ficoll-purified C3H/He splenocytes (10^8 per group) in 1 ml of medium were incubated with 0.5 mM $NS_{191-206}$ (■) or $NS_{191-206}$-P (□) for 3 h at 37°C (26). Supernatants were then separated by HPLC into 32 fractions. Fractions were lyophilized and reconstituted with 100 μl of medium. Twenty-five microliters of this solution was cocultured with 5×10^5 irradiated C3H/He splenocytes in 100 μl of medium and 2×10^4 T cells of the F1 subclones in 50 μl of medium in round-bottom microtiter plate wells. Plates were pulsed 48 h with 1 μCi of [3H]thymidine. Data are shown for fractions 20 to 27; all other fractions were negative, as were fractions derived from the RP-HPLC-separated acetic acid eluate of untreated C3H/He splenocytes. The cells were also tested for proliferation to medium (31 ± 5 cpm), 1 μg of ERA-BPL per ml (2,198 cpm ± 882), 0.2 μM $NS_{191-206}$ (1,505 cpm ± 568), and 5 μM $NS_{191-206}$-P (2,004 cpm ± 229). In a separate experiment, the acetic acid eluates of $NS_{191-206}$ and $NS_{191-206}$-P-treated splenocytes were mixed before separation by HPLC. Activity was recovered in the same fractions as shown in this experiment (data not shown).

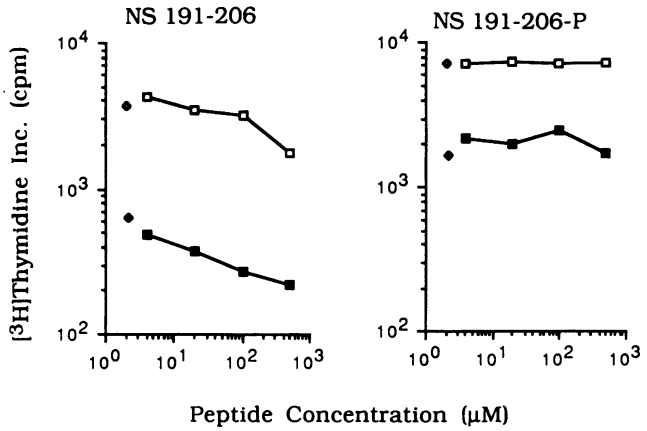

FIG. 5. Inhibition of binding of the NS peptides to the MHC determinant by an unrelated peptide with known specificity for the same restriction element. Irradiated C3H/He splenocytes were preincubated with varied concentrations of peptide HEL_{51-61}. Peptides $NS_{191-206}$ and $NS_{191-206}$-P, as indicated, were then added to a final concentration of 5 (□) or 1 (■) μM. Lymphokine release by a subclone of the B4 T cell line, termed B4.C2, was determined as described in Materials and Methods. Indicator HT-2 cells incorporated between 56 and 83 cpm of [3H]thymidine upon coculture of T cells with the different concentrations of the HEP_{51-61} peptide or medium as an additional negative control. The graphs show the response to $NS_{191-206}$ (◇) or $NS_{191-206}$-P (◆) in the absence of competing peptide.

this type of assay, which measures the speed of peptide-MHC association, does not reflect the final affinity of the interaction, it does give an indication on the tendency of the peptide to assume the conformation needed for association with the restricting element. In other words, the assay measures not only the thermodynamic control of the interaction that is governed by affinity but also the kinetic of the interaction that is controlled by conformation, which in the case of small peptides tends to exhibit a high degree of flexibility. A biological assay, i.e., proliferation of one of the NS protein-specific T_H-cell lines to peptide-coated APC, was used as a read-out for peptide-IA^k association. The results (Fig. 6) clearly show that at a high concentration (i.e., 5 μM), the $NS_{191-206}$ peptide saturated the functional binding sites on the APC within 30 min, while at a lower dose, peptide-MHC complex formation was delayed. With the $NS_{191-206}$-P peptide at 5 μM, functional association with the restricting element was substantially delayed compared with that of the $NS_{191-206}$ peptide, suggesting that phosphorylation impaired the tendency of the peptide to assume the conformation that promotes binding to the MHC class II determinant. Alternatively, the phosphorylated peptide might be more susceptible to further modification by serum- or cell-derived peptidases, resulting in loss of antigenic potency.

The reduction of either association (i.e., MHC versus TCR) could be caused by a modification in the secondary structure of the peptide, by steric hindrance of an essential residue, or by both. On the basis of the secondary-structure prediction algorithm of Chou and Fasman (5), peptide $NS_{191-206}$ has a strong propensity to assume an α-helical structure with partial amphipathicity in this conformation. A direct analysis of the potential secondary structure of each peptide by circular dichroism spectroscopy of peptides solubilized in different ratios of H_2O and TFE, a solvent that

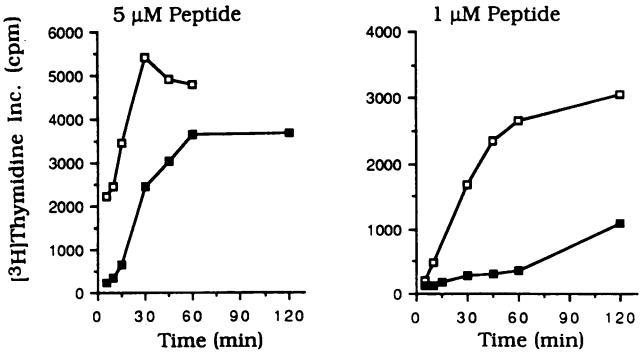

FIG. 6. Association rate of phosphorylated versus nonphosphorylated peptide to MHC determinants, as determined by proliferation of an NS protein-specific T_H-cell clone. Peptides $NS_{191-206}$ (□) and $NS_{191-206}$-P (■) were added to irradiated splenocytes for varied lengths of time and then washed off. Proliferation of the B4.C2 T cell line was determined. In addition, proliferation of the T-cell line was determined without removal of the peptides. Proliferation to the nonphosphorylated peptide was reduced in continued presence of the peptide (1,852 cpm at 5 μM; 2,968 cpm at 1 μM [a common observation that a supraoptimal concentration of peptide reduces the proliferative response without negatively affecting the magnitude of lymphokine release]), while the T-cell response benefited by the continuous presence of $NS_{191-206}$-P (7,096 cpm at 5 μM; 4,300 cpm at 1 μM). Cells in absence of antigen incorporated 160 cpm of [^{3}H]thymidine.

stabilizes nascent structures (7) and is assumed to mimic the environment of membrane-bound proteins (1), showed that although peptides $NS_{191-206}$ and $NS_{191-206}$-P exhibit similar spectra in water, the nonphosphorylated peptide displayed a much stronger propensity for helicity in 25 to 75% of TFE, in contrast to the phosphorylated peptide (92% helicity in 50% TFE for peptide $NS_{191-206}$ compared with 56% helicity for peptide $NS_{191-206}$-P). With use of a different side chain modification, i.e., glycosylation of the acidic residues (i.e., glutamic and aspartic acid; $NS_{191-206}$-GlcNAc) of peptide $NS_{191-206}$, a similar reduction of both helicity (58% helicity in 50% TFE) and antigenic potency of the modified peptide was observed (Fig. 7). These results might suggest a correlation between secondary structure and antigenic potency, but an alternative explanation might be that recognition of the serine residue is essential and that modification of the side chain impairs binding to either MHC determinants or the TCR. A peptide containing an alanine substitution for the original serine ($NS_{191-206}$-Ala; EKDDLAVEAEIAHQIA), which shows a propensity for helicity in TFE-water mixtures similar to that of the original peptide (i.e., 83% helicity in 50% TFE), was not recognized by $NS_{191-206}$-specific CD4$^+$ T cells (Fig. 7), indicating that phosphorylation might reduce recognition by steric hindrance of an amino acid involved in direct binding or by elimination of the hydroxyl side chain as a functional group within the trimolecular TCR-MHC-epitope complex.

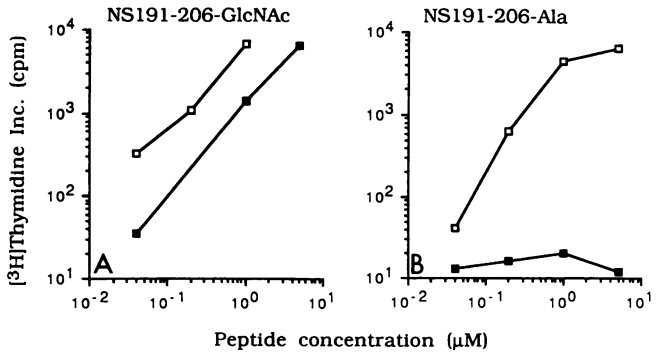

FIG. 7. Evidence that glycosylation of $NS_{191-206}$ and substitution of the serine residue reduce antigenic potency of the peptide. The graphs represent two separate experiments, both using a subclone of line F1. The responses (counts per minute) to medium and 1 μg of ERA-BPL per ml were as follows: (A) medium = 46 ± 16, ERA-BPL = 328 ± 94; (B) medium = 51 ± 6, ERA-BPL = 378 ± 88. (A) □, $NS_{191-206}$; ■, $NS_{191-206}$-GlcNAc; (B) □, $NS_{191-206}$; ■, $NS_{191-206}$-Ala.

Several T-cell epitopes identified on pathogens to date were shown to be located within hypervariable regions of antigens, suggesting that not only antibodies but also T cells might contribute to the selection of antigenic variants (9, 24). Amino acid substitutions in the antigenic regions of viral antigens are known to enable viruses to evade recognition by antibodies and T cells. Some of the amino acid substitutions introduce oligosaccharide attachment sites that alter the secondary structure of epitopes or cause steric hindrance of binding to immune receptors (22). Alternatively, in the case of some viral proteins such as the NS protein of rhabdoviruses, heterogeneous phosphorylation might provide the virus with a mechanism to weaken recognition by T cells. This might explain why the NS protein as expressed by live or inactivated rabies virus induces only a marginal T_H-cell response as opposed to the viral nucleoprotein, although the $NS_{191-206}$ peptide that expresses the immunodominant T_H-cell epitope of the NS protein has, at least in vitro, an antigenic potency more than 10 fold higher than that of peptide 31D (Fig. 1), which carries the immunodominant T_H-cell epitope of the nucleoprotein in *H-2^k* mice which clearly dominates the T_H-cell response upon immunization with rabies virus. Nevertheless, the NS protein as presented by live ERA virus is the dominant protein in inducing (at least in mice of the *H-2^k* haplotype) CTL, which in return are unable to recognize the phosphorylated version of peptide $NS_{191-206}$ in vitro. Whether this distinction in the induction of CD4$^+$ versus CD8$^+$ T cells (by the epitope identified by peptide $NS_{191-206}$) reflects differences in antigen processing (by the lysosomal as compared with the cytosolic pathway), which might influence the fate of posttranslational side chain modifications, remains to be investigated.

ACKNOWLEDGMENTS

We thank G. Krivulka for excellent technical assistance and E. Heber-Katz and V. Lee for helpful discussion.

This work was supported by Public Service grants AI 23503 and AI 27434.

REFERENCES

1. **Abergel, C., E. Loret, and J.-M. Claverie.** 1989. Conformational analysis of T immunogenic peptides by circular dichroism spectroscopy. Eur. J. Immunol. **19:**1969–1972.
2. **Allan, P. M., G. P. Mutsueda, E. Haber, and E. R. Unanue.** 1985. Specificity of the T cell receptor: two different determinants are generated by the same peptide and the IA^k molecule. J. Immunol. **135:**368–373.
3. **Allan, P. M., and E. R. Unanue.** 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. J. Immunol. **132:**1077–1079.
4. **Bell, J. C., and L. Prevec.** 1985. Phosphorylation sites on phophoprotein NS of vesicular stomatitis virus. J. Virol. **54:** 697–702.

5. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45–148.
6. **Clinton, G. M., and A. S. Huang.** 1981. Distribution of phosphoserine, phosphothreonine and phosphotyrosine in proteins of vesicular stomatitis virus. Virology **108**:510–514.
7. **Dyson, H. J., M. Rance, R. A. Houghton, P. E. Wright, and R. A. Lerner.** 1988. Folding of immunogenic peptide fragments of proteins in water solution. II. The nascent helix. J. Mol. Biol. **201**:201–217.
8. **Ertl, H. C. J., and G. L. Ada.** 1981. Role of viral infectivity and glycosylation of influenza A virus for T cell target cell formation. Immunobiology **158**:239–246.
9. **Ertl, H. C. J., B. Dietzschold, M. Gore, L. Otvos, Jr., J. K. Larson, W. Wunner, and H. Koprowski.** 1989. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol. **63**:2885–2892.
10. **Ertl, H. C. J., B. Dietzschold, and L. Otvos, Jr.** 1991. T helper cell epitope of rabies virus nucleoprotein defined by tri- and tetrapeptides. Eur. J. Immunol. **21**:1–10.
11. **Fields, G. B., and R. L. Noble.** 1990. Solid-phase peptide synthesis utilizing 9-fluoroenylmethoxycarbonyl amino acids. Int. J. Protein Res. **35**:161–214.
12. **Greenfield, N., and G. D. Fasman.** 1969. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry **8**:4108–4116.
13. **Lamb, J., D. D. Eckels, P. Lake, J. N. Woody, and N. Green.** 1982. Human T cell clones recognize chemically synthesized peptides of influenza hemagglutinin. Nature (London) **300**:66–69.
14. **Larson, J. K., and W. H. Wunner.** 1990. Nucleotide and deduced amino acid sequences of the nominal phosphoprotein of the ERA, PM and CVS-11 strains of rabies virus. Nucleic Acids Res. **18**:7172.
15. **Larson, J. K., W. H. Wunner, and H. C. J. Ertl.** Immune response to the nominal phosphoprotein of rabies virus. Virus Res., in press.
16. **Larson, J. K., W. H. Wunner, L. Otvos, Jr., and H. C. J. Ertl.** 1991. Identification of an immunodominant epitope within the phosphoprotein of rabies virus that is recognized by both class I- and class II-restricted T cells. J. Virol. **65**:5673–5679.
17. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
18. **Milich, D. R.** 1990. Probing T cell antigen recognition. Peptide Res. **3**:85–96.
19. **Otvos, L., Jr., I. A. Tangoren, K. Wroblewski, M. Hollosi, and V. M.-Y. Lee.** 1990. Reverse phase high performance liquid chromatographic separation of synthetic phosphopeptide isomers. J. Chromatogr. **512**:265–272.
20. **Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee.** 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature (London) **348**:252–254.
21. **Schneider, L. G., B. Dietzschold, R. E. Dierks, W. Mattheusen, P. J. Enzmann, and K. J. Strohmaier.** 1973. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. Virology **11**:748–755.
22. **Skehel, J. J., D. J. Stevens, R. S. Daniels, A. R. Douglas, M. Knossow, and I. A. Wilson.** 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA **81**:1779–1783.
23. **Takahashi, H., J. Cohen, A. Hosmalin, K. B. Cease, R. Houghten, J. L. Cornette, C. DeLisi, B. Moss, R. N. Germain, and J. A. Berzofsky.** 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA **85**:3105–3109.
24. **Takahishi, H., S. Merli, S. P. Putney, R. Houghten, B. Moss, R. N. Germain, and J. A. Berzofsky.** 1989. A single amino acid interchange yields reciprocal CTL specificity for HIV-1 gp160. Science **246**:118.
25. **Thomas, D. B., J. Hodgson, P. F. Riska, and C. M. Graham.** 1991. The role of the endoplasmatic reticulum in antigen processing. N-glycosylation of influenza hemagglutinin abrogates $CD4^+$ cytotoxic T cell recognition of endogenously processed antigen. J. Immunol. **144**:2789–2794.
26. **Van Bleek, G. M., and S. G. Nathenson.** 1990. Isolation of an endogenously processed immunodominant peptide from the class I $H\text{-}2K^b$ molecule. Nature (London) **348**:213–216.
27. **Wiktor, T. J., R. I. Macfarlane, K. J. Reagan, B. Dietzschold, P. J. Curtis, W. H. Wunner, M.-P. Kieny, R. Lathe, J. P. Lecocq, M. Mackett, B. Moss, and H. Koprowski.** 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. Proc. Natl. Acad. Sci. USA **81**:7194–7198.