Adenovirus Infection Inhibits the Phosphorylation of Major Histocompatibility Complex Class I Proteins

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Summary

Major histocompatibility complex (MHC) class I molecules act as peptide receptors to direct the recognition of foreign antigens by cytolytic T cells. The cell surface expression and trafficking of these peptide receptors is thought to be controlled by the conformation of the MHC molecule and possibly by the phosphorylation of the cytoplasmic portion of the heavy chain protein. It is of some interest that adenoviruses (Ads) have evolved proteins that interfere with the expression of MHC molecules. One of these proteins, called E3/19k, binds to newly synthesized MHC molecules in the rough endoplasmic reticulum (RER) and inhibits their trafficking to the cell surface. Here we show that during the infection of a human cell line with Ad2, the phosphorylation of the endogenous MHC molecules is inhibited. We also observe that the phosphorylation of the endogenous HLA molecules is grossly impaired in a human cell line transfected with the Ad2 EcoRI D fragment containing the E3/19k gene. We conclude that the E3/19k protein inhibits the phosphorylation of the MHC heavy chains and that this may be one of the important functions of this protein in infected cells. In addition, we show that a mutant of the E3/19k protein, which lacks an RER retention signal but which retains its ability to bind to HLA molecules, does not inhibit the phosphorylation of HLA molecules and that phosphorylated molecules are not Endo H sensitive. This suggests that HLA molecules are phosphorylated after leaving the medial-Golgi compartment, thus providing the most compelling evidence yet that HLA molecules are phosphorylated at or near the cell surface. Finally, to our knowledge, this is the first study under which the phosphorylation of MHC molecules is shown to be altered and may have some relevance for other pathogenic conditions.

The mammalian class I antigens of the MHC are highly polymorphic cell surface glycoproteins. One of their major functions is to bind and then present viral peptides to CTL after viral infection (1, 2). Though each individual MHC class I protein may bind only to a small set of peptides during viral infections (3–5), the extensive polymorphism between different MHC alleles and genes is believed to increase the capacity of this family of proteins to present a large variety of foreign viral antigens or peptides to virus-specific CTL. Thus, the extensive polymorphism may function on a population level to allow a species to withstand viral infections.

With such a great deal of polymorphism between MHC class I molecules, it is perhaps worth considering the common features of these molecules. They are composed of a heavy chain (mol wt 45,000) and a noncovalently associated light chain (mol wt 12,000) called β_2-microglobulin (6, 7). The heavy chain has three extracellular domains, α_1, α_2, and α_3 (8), each composed of ~90 amino acids, a transmembrane domain of ~30 amino acids, and a cytoplasmic region of ~30–40 residues (8). The polymorphism resides primarily in the α_1 and α_2 domains, whereas the α_3 domain and β_2-microglobulin are relatively conserved (8). The protein domain structure of the MHC class I antigens is consistent with the exon-intron organization of the corresponding genes (9). Thus, separate exons encode the signal sequence, the α_1, α_2, and α_3 domains, as well as the transmembrane segment. Surprisingly, the cytoplasmic region is encoded by three mini-exons, each of them containing the information for ~10 amino acids (protein domains I_1, I_2, and I_3), and this region is a site of phosphorylation (10–14). The crystal structure of the human class I proteins, HLA-A2 (15, 16) and HLA-Aw68 (17) confirms the domain structure and reveals that the α_1 and α_2 domains form a superdomain that is involved in peptide binding. This structure is stabilized by the α_3 and β_2-microglobulin domains, which lie proximal to the plasma membrane (15, 16).

In addition to polymorphism arising from amino acid sub-

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stitution between MHC class I molecules, other mechanisms exist that increase their structural complexity. Alternative processing of MHC class I mRNA can result in modified transcripts thereby encoding several different proteins from a single gene (18, 19). For example, exon VII, which encodes part of the intracytoplasmic region of MHC class I heavy chains (I_2), is a site of alternative splicing at the mRNA level (14, 18, 19). Splicing of the VII exon can result in the omission of 13 amino acids in the MHC heavy chain protein (14). The I_2 domain contains one site of serine phosphorylation at position 335 and possibly an additional site at position 332 (12–14). Thus, alternative splicing of the immature mRNA may result in the removal of a peptide structure containing a phosphorylation site(s). It is tempting to speculate that this mechanism has some functional significance, and that coordinate expression of alternatively spliced forms results in functional modification of MHC molecules, but at present, the data are incomplete. However, the phosphorylation of MHC class I molecules has been implicated in determining endocytic uptake from the cell surface and may function in down-regulating surface expression of MHC class I molecules (10, 11). The functional ramifications of this mechanism of down-regulation are not clear.

The E3/19k protein of adenovirus 2 (Ad2)[2] is a rough endoplasmic reticulum (RER) protein that spans the RER membrane (20, 21). It is thought to be a prime mediator of viral persistence (22). It binds to MHC class I proteins in the RER, inhibiting their passage to the cell surface (20, 21), and thereby reducing the recognition of infected cells by virus specific T cells (23–26). This mechanism is thought to allow the virus to evade the host immune response (22). We originally designed experiments to test whether the E3/19k protein is phosphorylated. This approach was chosen because we wanted to test whether ADP-ribosylation is a common signal for RER retention shared between luminal RER proteins such as BIP (27) and integral RER membrane proteins like E3/19k. We found that the E3/19k is not phosphorylated. Conversely, MHC molecules that are normally constitutively phosphorylated are nonphosphorylated in cells expressing the E3/19k protein.

Materials and Methods

Cell Culture, Cell Labeling, Immunoprecipitation, and SDS-PAGE. Cell lines were derived and propagated as previously described (28, 29). Cells were maintained in DMEM or αMEM containing 10% FCS. The 621.13 cell line was the kind gift of Dr. Svne Kvist (Ludwig Institute, Stockholm, Sweden).

Metabolic labeling experiments were carried out essentially as described previously (28, 29) with the following modifications. Cells to be metabolically labeled were washed twice in methionine-free or phosphate-free media and incubated in the same media for 1 h at 37°C before being incubated at 37°C with [^{35}S]methionine (1,200 Ci/mmol, Amersham Corp., England) at a concentration of 100 μCi/ml for 15 min or 250 μCi/ml of ^{32}P-orthophosphate (9,000 Ci/mmol, Amersham Corp.) for 2 h. The cells were washed twice in cold complete medium. At this point, the cells were lysed on the plate (20 mM Tris, pH 7.6, 120 mM NaCl, 4 mM $MgCl_2$, 1% NP-40, and 20 μg/ml PMSF). To clear the lysates from the cellular debris, the lysates were spun at 38,000 *g* for 30 min. The supernatants were incubated with 3 μl of normal rabbit serum on ice for 15 min. Protein A–Sepharose (100 μl of a 1:1 buffer/sepharose slurry) was then added, and the mixtures were incubated for an additional 60 min before being spun at 14,000 *g* for 5 min. Immunoprecipitations from the precleared lysates were carried out by incubating with 25 μl of the rabbit antisera fluid or 100 μl of mAb supernatant for 45 min, followed by 45 μl of protein A–Sepharose for 60 min. The beads were washed and the proteins were analyzed by 10–15% SDS-PAGE and autoradiographed as described previously (28, 29). When required, the beads were incubated for 30 h with Endo H buffer (70 mM NaCitrate, pH 5.5) with or without 2.5 mU of Endo H before analysis by SDS-PAGE.

Virus Propagation and Infection. Ad2 was propagated and titrated on 293 cells as described previously (30). To assess the effect of Ad2 on the phosphorylation of MHC molecules, 293 cells were infected with Ad2 at an MOI of 50 or mock infected using PBS. After 40 min of adsorption at 37°C, cells were incubated in DMEM containing calf serum and further incubated until metabolically labeled. HSV and CMV infection were carried out as described above for Ad2 except that infections were carried out with a MOI of 10. In the 0–2 h post-infection (hpi) time points, infection and phosphate starvation were done simultaneously and labeling media was added immediately after adsorption.

Antibodies. Rabbit antisera reacting with the E3/19k protein from Ad2 was a kind gift from Dr. William Wold (St. Louis University, St. Louis, MO). W6/32 (anti-HLA-A, -B, -C) (31) was prepared from hybridoma supernatant. Goat anti-mouse IgG FITC was obtained from Southern Biotechnology.

FACS®. To verify cell surface expression of the HLA antigen, FACS® analysis was carried out (Becton Dickinson & Corp., Mountain View, CA). Mock or virus-infected 293 cells or transfected 293.12 and 621.13 cells were harvested from petri dishes by washing the cells twice with PBS and then incubating with versene. A single cell suspension was prepared and the cells were then washed twice in PBS containing 0.5% (wt/vol) BSA, 20 mM NaN_3, and 20 mM Hepes. The cells were incubated with mAbs as previously described (28, 29), except that FITC-conjugated goat anti-mouse IgG was used as the second-step reagent. After labeling, the cells were fixed in 1.5% (vol/vol) *p*-formaldehyde. The fluorescent profiles were obtained by analyzing 5,000 cells in a semi-logarithmic plot using a FACScan® program. Conversion of log scale to linear scale mean fluorescent values was carried out using the equation: linear mean fluorescence = $10^{(\log \text{ mean fluorescence}/256 \text{ channels})}$.

Other Reagents. Protein A–Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Results

The FACS® analysis shown in Fig. 1 reveals that human 293 cells infected with Ad2 and then stained 20 h after infection with a mAb (W6/32), which binds to MHC class I heavy chains complexed to β_2-microglobulin (31), have a much reduced level of HLA at their cell surface in comparison to mock-infected 293 cells. The mean average linear fluorescence is 33 arbitrary fluorescence units (AFU) for Ad2-infected (MOI 50) 293 cells. The mean average fluorescence

[2] *Abbreviations used in this paper:* Ad, adenovirus; AFU, arbitrary fluorescence units; hpi, hours post-infection; RER, rough endoplasmic reticulum.

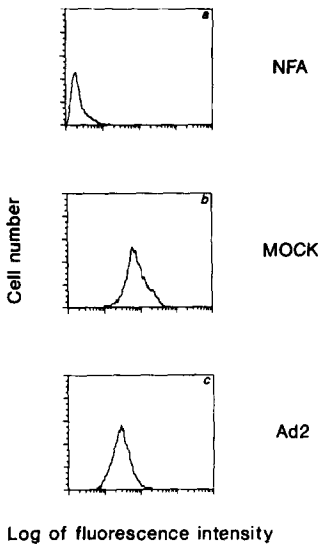

Figure 1. Inhibition of MHC class I surface expression by Ad2. 293 cells were mock treated (*b*) or infected with Ad2 at a MOI of 50 (*c*). At 20 hpi, cells were trypsinized and incubated with W6/32 (anti HLA-A, -B, -C) and labeled with goat anti-mouse IgG-FITC. The samples were then analyzed by flow cytometry. The negative control (*a*; no first antibody [NFA]) was mock-treated cells incubated with the secondary antibody only. The log values converted to linear values are: (*a*) NFA = 2 AFU; (*b*) mock = 93 AFU; and (*c*) Ad2-infected cells = 33 AFU. The inhibition of MHC surface expression was 66% in this particular experiment and 61% + 4 on average (six independent experiments).

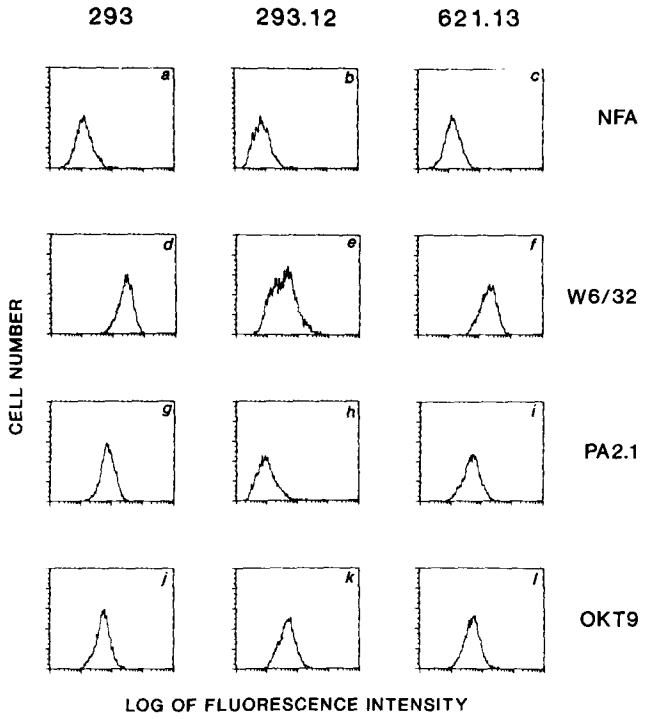

Figure 2. Inhibition of MHC class I surface expression by transfected cells expressing E3/19k. 293, 293.12 cells expressing the E3/19k protein, and 621.13 cells expressing the truncated E3/19k protein called 621 were trypsinized, washed, and analyzed for the surface-expression of MHC using W6/32 (anti-HLA-A, -B, -C; *d–f*) or PA2.1 (anti-HLA-A2; *g–i*) as the primary antibody and goat anti-mouse IgG-FITC as the secondary antibody. Cells were analyzed by flow cytometry. Controls included NFA (incubated with the secondary antibody only; *a–c*) and OKT9, which binds to the human transferrin receptor (*j–l*). Converted linear values are: (*a*) NFA = 15 AFU; (*d*) W6/32 = 286 AFU; (*g*) PA2.1 = 87 AFU; and (*j*) OKT9 = 60 AFU for 293 cells; (*b*) NFA = 9 AFU; (*e*) W6/32 = 50 AFU; (*h*) PA2.1 = 13 AFU; and (*k*) OKT9 = 55 AFU for 293.12 cells; and (*c*) NFA = 16 AFU; (*f*) W6/32 = 228 AFU; (*i*) PA2.1 = 57 AFU; and (*l*) OKT9 = 55 for 621.13 cells. In this experiment, inhibition of HLA-A, -B, -C surface expression in 293.12 cells was 85% as assessed with W6/32. HLA-A2 inhibition was 94% as analyzed with PA2.1. In two separate experiments, the inhibition was on average 84% ± 2 and 94% ± 1, respectively. Similarly, inhibition of MHC surface expression in 621.13 cells was 22% (26% ± 4) with the W6/32 antibody and 43% (45% ± 4) with the PA2.1 antibody.

for mock-treated cell is 93 AFU. The no first antibody control is the same for both cell treatments (2 AFU). Thus, the inhibition of MHC surface expression after Ad2 infection in the experiment shown was 66%. In six independent experiments, the inhibition was on average 61% ± 4. Similarly, 293 cells stably transfected with an EcoRI D fragment containing the E3/19k gene (20) also have reduced expression of MHC molecules at the cell surface (Fig. 2). The average mean linear fluorescence of MHC class I molecules on 293 cells is 271 AFU, as determined by the W6/32 antibody (subtracting the background fluorescence of 15 AFU). The average mean fluorescence of the HLA-A2 molecule expressed on 293 cells and analyzed with the PA2.1 antibody is 72 AFU. After subtraction of the background fluorescence of 9 AFU, the mean fluorescence of MHC class I molecules on 293.12 cells is 41 AFU with the W6/32 antibody and 4 AFU with the PA2.1 antibody. Thus, the overall MHC class I inhibition was 85% with the W6/32 antibody and 94% with the antibody PA2.1. In two separate experiments, the inhibition of the W6/32 binding to 293.12 as compared to 293 cells was 84% ± 2. The inhibition of binding with the PA2.1 antibody was 94% ± 1. These data show that Ad2 reduces the expression of HLA molecules at the cell surface of 293 cells. Furthermore, the EcoRI D fragment appears to contain the gene or genes that are sufficient to mediate this process. In addition, Fig. 2 shows that the level of inhibition of HLA expression on 293.12 cells in comparison with 293 cells as detected with an antibody that recognizes the HLA-A2 molecule (PA2.1) is similar to that seen with the W6/32 antibody that recognizes a common determinant on all HLA molecules so far tested. Thus, the inhibition of HLA expression in 293.12 cells is not likely to be a result of the masking of the W6/32 epitope by the binding of the E3/19k protein. Finally, we analyzed the cell surface expression of HLA molecules in 293 cells that have been transfected with a gene encoding a mutant of the E3/19k protein (32), which lacks a RER retention signal (amino acids 134–142). This protein, designated 621, retains its ability to bind HLA molecules, but the complex is transported through the cell (32). In 293 cells stably transfected with the EcoRI D fragment containing the 621 gene, the cell surface expression of HLA molecules is lower than in untransfected 293 cells (W6/32 = 22% inhibition; PA2.1 = 43%) with a mean in two experiments of 26% ± 4 with the W6/32 antibody and 45% ± 4 with the PA2.1 antibody. Thus, the cell surface expression of HLA molecules is intermediate in 621 expressing 293 cells compared to untransfected and wild-type E3/19k-transfected 293 cells. It is likely that HLA molecules form a complex with the 621 molecule at the cell surface (32). Extrapolating from this and previous results (20–22), it is reasonable to conclude that the E3/19k protein is mediating the cell surface inhibition of HLA molecules in 293.12 and infected 293 cells and

that this inhibition is mediated by the presence of a functional RER retention sequence in the E3/19k protein.

To analyze the state of phosphorylation of HLA molecules in 293 cells infected with Ad2, cells were infected with Ad2 and then metabolically labeled with ^{32}P-orthophosphate from 20–22 hpi. The cells were lysed in NP-40 containing lysis buffer, and the cellular debris was removed by ultracentrifugation at 38,000 *g* for 30 min. Antibodies against HLA molecules were used to specifically immunoprecipitate these proteins from equal numbers of TCA-precipitable counts, as assessed by scintillation counting. The immunoprecipitates were analyzed on a 10–15% gradient SDS-PAGE followed by autoradiography. In mock-infected 293 cells labeled with ^{32}P-orthophosphate (Fig. 3 *A*), MHC proteins are clearly phosphorylated. In cells infected with Ad2, which encodes a functional E3/19k protein, the phosphorylated form of MHC proteins is substantially inhibited. This inhibition of phosphorylation mirrors the maximum inhibition of HLA surface expression, which takes place 16–24 hpi (Fig. 1). Densitometry traces indicate that the phosphorylation of MHC class I molecules is reduced by >60% in Ad2-infected cells compared to mock-treated 293 cells. In five independent experiments, the decrease of phosphorylation was 66% ± 4.

In an attempt to generalize these findings to other viral systems, the phosphorylation of MHC molecules expressed in HSV- and CMV-infected cells was examined. Vero cells, which are permissive for HSV-1 infections, were infected with HSV-1 and then metabolically labeled with ^{32}P-orthophosphate 22–24 hpi. The cell lysates were prepared as before and immunoprecipitations were carried out from equal numbers of TCA precipitable counts with the W6/32 antibody. Similarly, CMV was used to infect MRC5 cells, and the phosphorylation of MHC molecules was compared between virus-infected and noninfected cells. Fig. 3, *B* and *C* shows that there is little difference between the virus-infected and the mock-treated cells up to 22 hpi nor at times before this time (data not shown). Thus, we conclude that two members of the herpes virus group, CMV and HSV-1, do not inhibit the phosphorylation levels of MHC molecules up to 22 hpi.

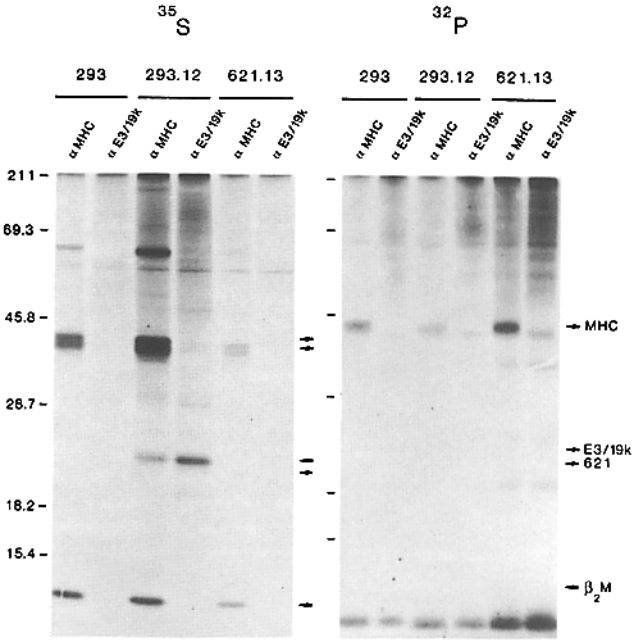

Figure 4. Reduction of the level of MHC phosphorylation in transfected cells expressing E3/19k. 293, 293.12 cells expressing the E3/19k protein, and 621.13 cells expressing the 621 protein were pulse labeled for 30 min with ^{35}S-methionine or 2 h with ^{32}P-orthophosphate and lysed. MHC (αMHC) or E3/19k (αE3/19k) molecules were immunoprecipitated from equal trichloroacetic counts and analyzed by SDS-PAGE and autoradiography. A longer exposure of the ^{35}S autoradiogram reveals the presence of both MHC and β_2M in the immunoprecipitates using the αE3/19k antibody (data not shown). Separate experiments were analyzed by densitometry and indicated a reduction of phosphorylation of the MHC molecules of 81% ± 4 in 293.12 cells (six experiments) and a level of phosphorylation of 134% ± 37 in 621.13 cells (four experiments) comparable to 293 cells).

We next wanted to determine if the Ad2 E3/19k protein was able to inhibit the phosphorylation of the MHC molecules or if there are other Ad-encoded proteins involved in this process. Human 293 cells, which had been transfected with the EcoRI D fragment of Ad2 containing the E3/19k

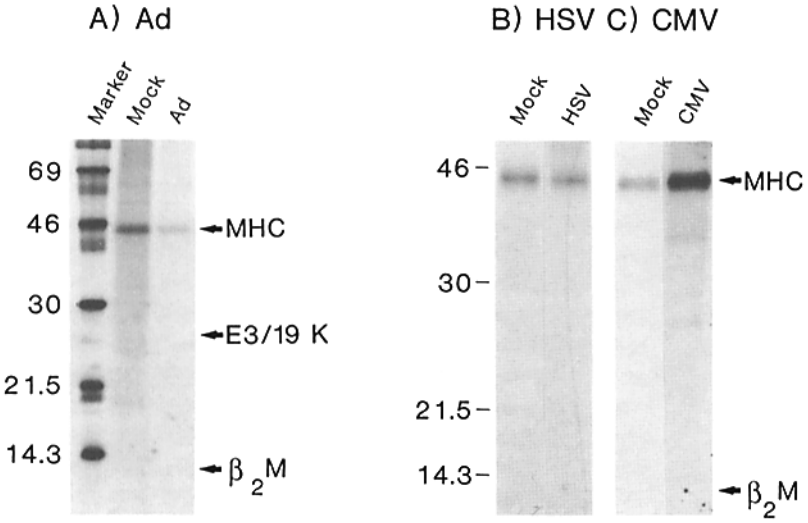

Figure 3. Effect of different viruses on the phosphorylation of MHC class I. 293, Vero, or MRC5 cells were infected with Ad2 (MOI = 50), HSV-1 (MOI = 10), or CMV (MOI = 10), respectively, or mock treated. The cells were labeled for 2 h with ^{32}P-orthophosphate at 20 h (Ad2) or 22 h (HSV-1 and CMV) post-infection. The cells were lysed and MHC molecules were immunoprecipitated using the W6/32 antibody and analyzed by SDS-PAGE and autoradiography. Equal TCA-precipitable counts were used for the immunoprecipitations. The position of the ^{32}P-labeled heavy chain of MHC (MHC) as well as the virtual migration positions of E3/19k (E3/19k) in the adenovirus immunoprecipitates and the light chain (β_2M) are shown on the right side. The molecular masses of the protein standards (in kD) are indicated on the left side. Densitometry analysis indicated that the reduction of phosphorylation of MHC by Ad2 is 66% ± 4 on average (five independent experiments).

gene, called 293.12 cells (20), with the EcoRI D fragment containing the 621 gene (32), or untransfected 293 cells, were metabolically labeled with either ^{35}S-methionine or with ^{32}P-orthophosphate and immunoprecipitations were carried out as described above. In ^{35}S-methionine-labeled 293.12 cells, the antibody against MHC proteins clearly immunoprecipitates the MHC molecule with the E3/19k bound to it, whereas in the untransfected 293 cells HLA, but not the E3/19k protein, was detected (Fig. 4). In addition, the 621 protein can be detected by coimmunoprecipitation with HLA molecules in 621-transfected cells. The 621 runs at a slightly lower molecular weight than the E3/19k protein in coimmunoprecipitation with HLA molecules (Fig. 4). Unfortunately, the rabbit antisera we are using was raised against a synthetic peptide (33) that corresponds to the RER retention signal absent in the 621 protein (32). Thus, we can not analyze this protein directly.

In the 293 cells metabolically labeled with ^{32}P-orthophosphate, HLA class I molecules are phosphorylated. However, in the E3/19k transfectant the phosphorylation of the MHC is dramatically inhibited. The weak signal that does appear represents 24% of the HLA phosphorylation level in 293 cells (mean of 19% ± 4 in five independent experiments). The immunoprecipitations were carried out from equal numbers of TCA-precipitable counts. Normalization of the ^{32}P labeling with the ^{35}S-methionine labeling reveals an even greater level of inhibition of HLA phosphorylation. The small amount of phosphorylated HLA in 293.12 cells may be molecules that avoid binding to the E3/19k protein or they may represent a form of the HLA molecules that are phosphorylated at an alternative site other then serine 335, which is not affected by the E3/19k protein. The identity of this molecular form of phosphorylated HLA molecules is under investigation. We conclude that the EcoRI D fragment of Ad2 contains the information necessary to transfer the inhibition of phosphorylation of HLA molecules. In addition, we conclude that the E3/19k protein is not itself phosphorylated (Fig. 4).

We next wanted to determine whether the retention of HLA molecules in the RER was responsible for the inhibition of HLA phosphorylation or whether it was a result of an additional activity associated with the E3/19k protein. We therefore analyzed the phosphorylation of HLA molecules in the presence of the 621 protein. We find that the phosphorylation of HLA molecules is not effected in these cells (Fig. 4). The apparent increase in the level of phosphorylation of the MHC molecules shown in this particular experiment (239% compared to 293 cells) is not reproducible (134% ± 37 in four experiments). We infer from this data that the inhibition of phosphorylation is a consequence of inhibiting HLA transport through the cell. Alternatively, the COOH-terminal RER retention signal absent in the 621 protein contains an activity that directly mediates the hypophosphorylation of HLA molecules.

Finally, the glycosylation of the phosphorylated forms of HLA molecules in 293 and 293.12 cells was analyzed. HLA molecules from ^{32}P-labeled 293 and 293.12 cells were immunoprecipitated in duplicate. One of each of the samples was digested overnight in buffer containing Endo H. The duplicates of these samples were incubated in buffer alone. Endo H resistance is a marker of glycoproteins that have left the cis/medial Golgi compartment. At the end of the incubation SDS-PAGE loading buffer was added, and the samples were separated by SDS-PAGE. The gel was dried and autoradiography was performed. Fig. 5 shows that the vast majority of phosphorylated HLA molecules in 293 or 293.12 cells are not Endo H sensitive. This experiment suggests that HLA molecules become phosphorylated after they leave the cis/medial Golgi compartment. It also suggests that the majority of the phosphorylated form of the HLA molecules seen in the 293.12 cells are molecules that have escaped E3/19k-mediated retention in the RER.

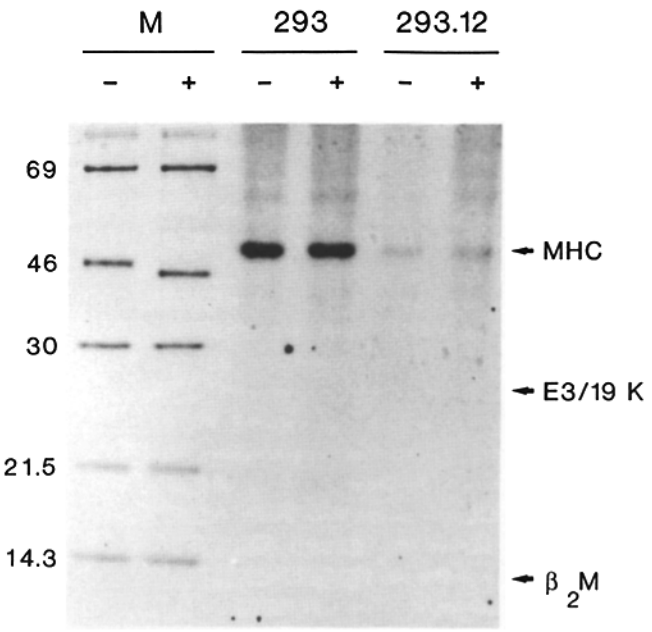

Figure 5. Glycosylation of the phosphorylated forms of MHC molecules in 293 and 293.12 cells. 293 and 293.12 cells expressing the E3/19k protein were pulsed labeled for 2 h with ^{32}P-orthophosphate, and MHC class I molecules were immunoprecipitated with the W6/32 antibody. The immunoprecipitates were mock treated (−) or digested with Endo H (+) overnight and analyzed by SDS-PAGE and autoradiography. Figure symbols are as previously noted. M, protein molecular mass standards. The OVA in the molecular mass standards is sensitive to Endo H digestion and was used as a control for the efficiency of digestion. In this experiment, the reduction of phosphorylation, as determined by densitometry tracing was 91%.

Discussion

Ads have developed several mechanisms for evading the host immune system (17, 20, 21, 34). In fact, the E3 region of Ad, which is dispensable for viral replication in tissue culture (34), may act as a specific expression cassette for down-regulating the host immune response. The E3/19k protein is likely to be the most abundant protein encoded by the E3 region (34). It binds to MHC class I molecules in the RER, thereby inhibiting their transport to the cell surface (20, 21). It appears that the E3/19k protein is arranged in functional

protein domains including an intraluminal domain that interacts with a region of the α_1 and α_2 domains of MHC class I molecules (28). This region of class I MHC molecules also controls their rate of transport to the cell surface. A second domain acts jointly as a stop transfer sequence and a transmembrane region. Finally, the cytoplasmic tail of the molecule appears to contain the information for confining the E3/19k molecule, and the MHC protein to which it is bound, to the RER (35).

Protein phosphorylation is thought to regulate several cellular processes including signal transduction, mitosis, cell proliferation, cell motility, cell shape, and gene regulation (36). Its role in the trafficking of proteins through the exocytic pathway is only now being assessed. The study of MHC class I molecules provides an opportunity for determining the rules for phosphorylational control of protein transport. A great deal is known about how MHC class I assembly and conformation control their passage through the cell. For example, it is known that the variation between the MHC heavy chain alleles controls their rate of transport through the cell (28), binding to peptides (3), and the binding of the E3/19k protein (28). We also know that peptides bind during the assembly of class I heavy chains with β_2-microglobulin (37). Furthermore, site-specific phosphorylation including and in addition to the major site of phosphorylation at serine 335 may modulate distinct routing of MHC class I molecules through the cell (10, 11). Finally, alternative splicing of exon VII, which contains the major sites of phosphorylation in MHC class I molecules, may yet prove to have a profound effect on the antigen-presenting function of class I molecules (10, 11, 14). Unfortunately, we are still lacking a direct demonstration that HLA class I molecules become phosphorylated exclusively at the plasma membrane. The only real support for this is that PMA, a potent activator of plasma membrane–associated protein kinase C, causes hyperphosphorylation of HLA molecules (10) and that deletion of exon VII containing two phosphorylation sites alters the rate of endocytosis of HLA molecules (10, 11). In the present study, we sought to approach this issue.

During infection with Ad2, we find that the cell surface expression of MHC molecules decreases (Fig. 1). This effect is maximized 16–20 hpi. The level of phosphorylation of the MHC molecules was measured at the peak of the inhibition of MHC surface expression. To show that the inhibition of phosphorylation is not merely a result of the lowered expression of MHC molecules after infection with Ad2, we examined a cell line that constitutively expresses the E3/19k protein. We found that we could detect the binding of the E3/19k protein to MHC molecules. Furthermore, the phosphorylation of MHC molecules is dramatically reduced in the cells that express the E3/19k protein. We conclude from these studies that the E3/19k protein can inhibit the phosphorylation of MHC class I proteins. Recently, it was shown that the EcoRI D fragment of Ad2 encodes another protein, 6.7k (38). The role of this protein in the formation of the E3/19k-MHC class I complex is unknown but is under investigation.

The mechanism by which the E3/19k protein reduces the level of MHC class I heavy chain phosphorylation is still unclear. We decided to examine if the E3/19k protein inhibits the phosphorylation of MHC molecules by inhibiting their transport out of the RER, if it acts directly on the MHC proteins to inhibit phosphorylation, or if it actively dephosphorylates MHC molecules. For example, does phosphorylation of MHC molecules control their egress or transport to the cell surface. We therefore examined HLA phosphorylation in 293 cells expressing a form of the E3/19k protein called 621, which lacks a peptide from the COOH terminus, which confers RER retention to the HLA–E3/19k complex (35). The 621 protein retains its ability to bind to HLA class I proteins. We found that phosphorylation is not inhibited in cells expressing the 621 protein. Therefore, either the RER retention signal actively participates in dephosphorylating or inhibiting the phosphorylation of HLA molecules, or retention in the RER does not allow HLA molecules to gain access to enzymes that phosphorylate them. We favor the latter scenario, as it would suggest that HLA molecules must leave the RER/cis-Golgi compartments in order to become fully phosphorylated. In further support of this hypothesis is the demonstration that the vast majority of phosphorylated HLA molecules expressed in 293 and 293.12 cells are not Endo H sensitive (Fig. 5). This suggests that the HLA molecules must leave the cis/medial Golgi in order to become phosphorylated and that most of the residually phosphorylated HLA molecules expressed in 293.12 cells are molecules that have escaped E3/19k mediated retention in the RER. In this regard, this is the best data yet presented that HLA molecules are indeed phosphorylated in a cellular compartment beyond the medial Golgi compartment, and it supports the notion that HLA molecules may become phosphorylated at the cell surface.

In the future, our ability to modify MHC molecules and to express them in cell lines (28) and transgenic animals (29) should allow us to determine the exact role of MHC class I protein phosphorylation. In addition, the E3/19k protein may be useful as a tool to alter the phosphorylation of MHC class I molecules. Regardless of the outcome of these experiments, the results reported here add another level of complexity to the molecular pathogenesis of Ads.

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