Conserved Cysteine Residues within the E3/19K Protein of Adenovirus Type 2 Are Essential for Binding to Major Histocompatibility Complex Antigens

MARTINA SESTER AND HANS-GERHARD BURGERT*

Hans-Spemmann-Laboratorium, Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany

Received 1 March 1994/Accepted 1 June 1994

The E3/19K protein of human adenovirus type 2 is a resident transmembrane glycoprotein of the endoplasmic reticulum. Its capacity to associate with class I histocompatibility (MHC) antigens abrogates cell surface expression and the antigen presentation function of MHC antigens. At present, it is unclear exactly which structure of the E3/19K protein mediates binding to MHC molecules. Apart from a stretch of approximately 20 conserved amino acids in front of the transmembrane segment, E3/19K molecules from different adenovirus subgroups (B and C) share little homology. Remarkably, the majority of cysteines are conserved. In this report, we examined the importance of cysteine residues for the structure and function of E3/19K. We show that E3/19K contains intramolecular disulfide bonds. By using site-directed mutagenesis, individual cysteines were replaced by serines and mutant proteins were stably expressed in 293 cells. On the basis of the differential binding of monoclonal antibody Tw1.3 and cyanogen bromide cleavage experiments, a structural model of E3/19K is proposed, in which Cys-11 and Cys-28 as well as Cys-22 and Cys-83 are linked by disulfide bonds. Both disulfide bonds (all four cysteines) are absolutely critical for the interaction with human MHC antigens. This was demonstrated by three criteria: loss of E3/19K coprecipitation, lack of transport inhibition, and normal cell surface expression of MHC molecules. Mutation of the three other cysteines had no effect. This indicates that a conformational determinant based on two disulfide bonds is crucial for the function of the E3/19K molecule, namely, to bind and to inhibit transport of MHC antigens.

Human adenoviruses (Ad), comprising 47 different serotypes, typically cause acute disease of the eyes, the respiratory tract, and the gastrointestinal tract (31). However, some members of human Ad, in particular, members of subgroup C, e.g., Ad type 2 (Ad2), can establish persistent infections in a proportion of people which may last months or even years (19, 53). The genes implicated in this phenomenon are encoded in early region 3 (E3) of the Ad genome. Although this region is dispensable for virus replication in tissue culture cells, it is conserved among Ad subtypes (43), suggesting an important role during pathogenesis *in vivo*. In support of this idea, several proteins encoded in this transcription unit modulate immune response mechanisms leading to lysis of infected cells. The E3-14.7 and/or E3-10.4 and E3-14.5 proteins counteract the lytic function of the immune mediator tumor necrosis factor alpha (reviewed in reference 57), while the E3/19K protein protects infected cells from the cytolytic attack by cytotoxic T cells (1, 9, 12, 47, 54).

In recent years the mechanism by which E3/19K inhibits the action of cytotoxic T cells has been elucidated in some detail (2, 7). Cytotoxic T cells recognize their antigen as peptides (6, 14, 55) presented on the cell surface by major histocompatibility class I (MHC) antigens. Experimental evidence suggests that peptides produced in the cytosol are transported to the lumen of the endoplasmic reticulum (ER) where they assemble with the polymorphic heavy chain of MHC molecules (M_r ~45,000) and an invariant light chain (M_r 12,000), β_2 microglobulin (β_2m) (24, 33, 41, 58). Additional molecules, such as chaperones, may assist the assembly process (13, 15, 30, 38).

The trimolecular complex is then efficiently transported out of the ER to the cell surface for recognition by cytotoxic T cells.

The ER resident protein E3/19K interferes with this process by binding to MHC molecules. The Ad2 E3/19K protein is a transmembrane glycoprotein with a molecular mass of approximately 25 kDa. It consists of 142 amino acids. The luminal portion comprising ~105 amino acids is glycosylated at positions 12 and 61 and is separated by a transmembrane segment (~22 amino acids) from a 15-amino-acid long cytoplasmic tail, containing the ER retention signal (21, 42). We and others have demonstrated that binding of E3/19K to human MHC class I antigens (HLA) interferes with transport of MHC molecules to the cell surface (2, 7). Consequently, allogeneic and antigen-specific T-cell recognition *in vitro* is drastically suppressed (1, 9, 12, 47, 54). Consistent with those data, the immunopathology observed upon wild-type Ad infection of cotton rat lungs *in vivo* is less severe than that induced by a mutant virus lacking E3/19K (25). In Ad-infected animals, tumor necrosis factor alpha is upregulated. Interestingly, Ad can compensate for the tumor necrosis factor alpha-mediated increase of MHC expression by concomitantly upregulating E3/19K expression (36). Inhibition of MHC function by E3/19K seems to be universal, as binding has been detected with human (2, 7, 51), monkey (17), rat (34), and mouse MHC antigens (8, 9, 12, 47, 51, 54). Also, no significant functional difference was observed by using various cell types, such as embryonic kidney cells (7, 9, 36, 43), transformed and untransformed fibroblasts (2, 7, 12, 47), or, more recently, lymphoid cells, which may serve as a reservoir during persistent virus infections (35).

To fulfill the antigen presentation function, MHC molecules have evolved a structure enabling them to tightly bind a great variety of peptides. Crystallographic analyses of MHC class I molecules (5, 20, 23, 39) revealed two membrane-proximal

* Corresponding author. Mailing address: Hans-Spemmann-Laboratorium, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany. Phone: 49-761-5108487. Fax: 49-761-5108468.

FIG. 1. Alignment of E3/19K amino acid sequences from Ad2 (31) and Ad5 (10) (group C) and from Ad3 (50), Ad7 (56), Ad11 (40), and Ad35 (18) (group B). All amino acid sequences are predicted from the DNA sequence. Dots denote identity with the Ad2 sequence; dashes indicate missing residues. The cysteines mutated in this study are marked by arrowheads (positions 11, 22, 28, 83, 101, 109, and 122); the conserved region and the glycosylation sites are indicated by a line above the letters and by asterisks, respectively.

immunoglobulin-like domains ($\alpha 3$ and $\beta_2 m$) and distal to those the $\alpha 1/\alpha 2$ superdomain forming the peptide-binding groove. Each of these domains contains ~90 amino acids. The transmembrane segment is ~30 residues in length and is followed by a hydrophilic cytoplasmic tail of 30 to 40 amino acids (4).

Our understanding about the nature of the interaction and the domains of MHC molecules that are crucial for complex formation with E3/19K has advanced considerably. The association is noncovalent and does not involve the carbohydrates of the molecules (8). By using exon shuffling between a nonbinding and a binding MHC allele, we previously showed that the luminal peptide-binding domains of murine MHC molecules ($\alpha 1/\alpha 2$) are important for binding to the E3/19K protein (8, 16, 32). Consequently, it is predicted that the luminal domain of E3/19K mediates the contact to the MHC molecule. Most previous studies attempted to identify the structural features of E3/19K molecules required for interaction with MHC molecules by deleting portions of the molecule (11, 17, 21, 22, 28, 44). Although it became clear that the cytoplasmic tail of E3/19K is not essential for the ability of E3/19K to associate (11, 17, 21, 42, 44), a further dissection of the important structures became complicated because of discrepancies between the different studies and between in vitro and in vivo binding assays. For instance, the transmembrane segment might be a prerequisite for the interaction in vivo; however, it does not appear to be absolutely necessary for complex formation in vitro (22, 44).

Surprisingly, apart from a small stretch of conserved amino acids in front of the transmembrane segment, which are supposed to be buried within the E3/19K molecule (17), there is little homology between MHC-binding proteins of Ad subgroups B and C (Fig. 1). This indicates that instead of a linear sequence, a certain conformation of the E3/19K proteins might be necessary for the interaction with MHC molecules. Remarkably, the majority of cysteines are conserved, suggesting the presence of disulfide bonds with an important role in the structure and function of the MHC-binding proteins.

In this report, we confirm this hypothesis. First, we show that intramolecular disulfide bonds exist. Furthermore, we present

evidence regarding which cysteines are linked to each other by using a combination of biochemical methods and site-directed mutagenesis. Most importantly, we demonstrate that the four N-terminal cysteines are absolutely required for the association of E3/19K with MHC molecules. All these data are incorporated in a model for the E3/19K structure.

MATERIALS AND METHODS

Cell lines and culture conditions. The 293 cell line expressing Ad5 E1A and E1B genes was established by transformation of human embryonic kidney cells with Ad5 DNA (26). This cell line was chosen since E1A gene products support efficient expression of E3 genes (37, 49). 293E3-45 and 293E22.7 cells (36) were derived from 293 cells by transfection with the Ad2 *EcoRV* C and *EcoRI* D fragment (27), respectively. The mutant cell lines were established by transfection of 293 cells with Ad2 *EcoRI* D fragments containing single amino acid substitutions. With each construct, the neomycin-phosphotransferase gene (52) was cotransfected as a selectable marker. The 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics. For routine culture, the medium of the transfectants was supplemented with 200 μ g of G418 (GIBCO, Eggenstein, Germany) per ml.

MAbs and antisera. The following monoclonal antibodies (MAbs) were used in this study: W6/32, anti-HLA-A, -B, and -C (3); BB7.2, anti-HLA-A2 and -Aw69 (45); and Tw1.3, anti-E3/19K (11). The polyclonal antiserum C-tail was raised against the synthetic peptide (CKYKSRRSFIDEKKMP) corresponding to the cytoplasmic tail of Ad2 E3/19K. The N-terminal Cys has been added to allow Cys-mediated coupling to keyhole limpet hemocyanin. Coupling was performed with preactivated keyhole limpet hemocyanin, according to the manufacturer's instructions (Pierce, Rockford, Ill.). A mixture of keyhole limpet hemocyanin-coupled peptide and free peptide was emulsified with complete Freund's adjuvant and injected subcutaneously into a chinchilla rabbit. Subsequently, the rabbit was immunized in 4-week intervals with the same mixture with incomplete Freund's adjuvant. The unpurified serum was used after the second boost.

Construction of mutant genes. The Ad2 *EcoRI* D fragment (2,674 bp; genomic map units 76.2 to 83.6) containing the E3/19K-coding region was ligated into pBluescript II KS- (Stratagene, Heidelberg, Germany), and single amino acid mutations were inserted by using the PCR-mediated oligonucleotide-directed mutagenesis protocol of Higuchi et al. (29). Alterations comprise cysteine-to-serine mutations at amino acid positions 11, 22, 28, 83, 101, 109, and 122 of the E3/19K protein. In construct Ala-22 (referred to as 22A), the cysteine at position 22 was replaced by an alanine. Briefly, mutagenesis was carried out in two steps with two mutant oligonucleotides and two oligonucleotides located 5' and 3' of the site to be mutagenized. In the first step, two separate PCRs were set up with the respective flanking and mutant oligonucleotides in sense and antisense orientations. In a second PCR, the gel-purified amplification products of both reactions were combined with the flanking (or a nested) set of oligonucleotides. Oligonucleotides used for site-directed mutagenesis are shown in Table 1 (mutated nucleotides are underlined). For mutants Ser-11, Ser-22, Ser-28, and Ala-22, E3-6.7-5' and E3/19K-Ser-109A were used as flanking primers; the remaining mutants were established with flanking oligonucleotides E3/19K-Ser-28 and E3/19K-3'. The mutagenized double-stranded fragment was cleaved with *PacI* and *SylI* for mutants Ser-11, Ser-22, Ala-22, and Ser-28 and with *SylI* and *BclI* for mutants Ser-83,

TABLE 1. Oligonucleotides used for site directed mutagenesis

Oligonucleotide	Nucleotide sequence ^a
E3/19K-Ser-11	CCAGCTTCCAAATGTTACATTTA
E3/19K-Ser-11A	GTAACATTGGAAGCTGGTTCCT
E3/19K-Ser-22	CTAATGAATCCCACTACTCTTATAA
E3/19K-Ser-22A	GAGTAGTGGATTTCATTAGCTTC
E3/19K-Ala-22	CTAATGAAGCCACTACTCTTATAA
E3/19K-Ala-22A	GAGTAGTGGCTTCATTAGCTTC
E3/19K-Ala-28	CTTATAAAATCCACCACAGAACAT
E3/19K-Ala-28A	CTGTGGTGGATTTTATAAGAG
E3/19K-Ser-83	TATGAAATGTCCGATATTACCATG
E3/19K-Ser-83A	AATATCGGACATTTTATAAAATGG
E3/19K-Ser-101	CACAAAAGGTTTAGAGAACAC
E3/19K-Ser-101A	CTCTAAATCTTTTGTGGGGGCC
E3/19K-Ser-109	GCACCTTTAGTTCCACCGCTCTG
E3/19K-Ser-109A	GGTGAACATAAGGTGCCAGTG
E3/19K-Ser-122	GCTTTGGTAAAGTCCCTTACTTAT
E3/19K-Ser-122A	AAGTACTTACCAAGCAAGCG
E3-6.7-5'	ATTCGATTCTCGAGGCATTTGCGGGGTGCTGGG
E3-19K-3'	TAGTGAATCTAGACATAGAGTAAATGTCCAGGGG

^a Underlining denotes altered nucleotides.

Ser-101, Ser-109, and Ser-122. *PacI*, *StyI*, and *BclI* restriction sites are located at positions 1262, 1697, and 2073, respectively, within the *EcoRI* D fragment. Mutagenized fragments of 437 bp (*PacI*-*StyI*) and 376 bp (*StyI*-*BclI*), respectively, were ligated into the wild-type plasmid cut with the same enzymes. The double mutant 22A/83 was constructed by ligating the *StyI*-*BclI* fragment of mutant Ala-22 into the *StyI*-*BclI* cleaved plasmid of Ser-83. Subsequently, the PCR-amplified portions of the *EcoRI* D fragment were analyzed by double-strand DNA sequencing with Sequenase according to the manufacturer's instructions (U.S. Biochemicals, Bad Homburg, Germany).

Transfection of cells. For stable expression of mutant genes, constructs were transfected into 293 cells together with the pSV2neo^r plasmid conferring G418 resistance (52). Plasmids containing mutant constructs were linearized with the restriction enzyme *SalI*, the plasmid containing the resistance gene was cut with *PvuI*. The 293 cells were transfected by electroporation (35). Cells (10^7) were washed twice with DMEM without FCS and resuspended in 1 ml of DMEM. After sterilization of the DNA, 15 μ l of the mutant construct (1 μ g/ μ l) and 5 μ l of the G418 resistance gene (0.3 μ g/ μ l) were added to 800 μ l of the cell suspension and incubated at room temperature for 10 min. Transfection was carried out at 960 μ F and 320 V in a 0.4-cm cuvette with a genepulser (BIO-RAD, Munich, Germany). Immediately after the pulse, the cells were resuspended in 2 ml of complete DMEM containing FCS, incubated for 5 min, and plated in seven petri dishes with a diameter of 10 cm. At 48 h after transfection, selection was initiated with 600 μ g of G418 per ml. G418-resistant clones were screened initially by fluorescence-activated cell sorter (FACS) analysis in the presence of saponin (11), as outlined below.

Cell labeling, immunoprecipitation, and SDS-PAGE. Labeling of the cells with [³⁵S]methionine, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out essentially as described previously (7, 8).

Endo H treatment. After immunoprecipitation of HLA-A2 with MAb BB7.2, protein A-Sepharose-bound material was treated with 0.08 U of endoglycosidase H (endo H; Boehringer, Mannheim, Germany) for 20 h at 37°C. Incubation was done under semisterile conditions in 70 mM sodium citrate (pH 5.5)-8 μ g of phenylmethylsulfonyl fluoride (Sigma, Deisenhofen, Germany). Before release of the bound material

with SDS sample buffer, the beads were washed once with 10 mM Tris-HCl (pH 7.4).

Cyanogen bromide digestion. Radioactive lysates of 293E3-45 cells were incubated with Tw1.3 and protein A-Sepharose, and E3/19K was released from protein A-Sepharose by incubation with 88% formic acid for 15 min at room temperature (46). E3/19K was digested in 150 μ l of 1 mM cyanogen bromide (Sigma) in 77% formic acid. Incubation was carried out at room temperature for 20 h, and the reaction was stopped by adding 10 volumes of H₂O. After lyophilization, the digested fragments were heated in SDS sample buffer with or without 10 mM dithiothreitol (DTT) prior to adding 50 mM iodoacetamide to each sample. Subsequently, fragments were analyzed by SDS-17.5% PAGE.

Western immunoblot analysis. Tw1.3-immunoprecipitated material was run on an SDS-12% polyacrylamide minigel and blotted for 1 h at 190 V in Tris-glycine blotting buffer. Nonspecific binding sites on the membrane were blocked for 1 h in Tris-buffered saline (140 mM NaCl, 10 mM Tris-HCl [pH 7.4]) containing 5% FCS. The membrane was then incubated for an additional hour with 1 μ g of Tw1.3 per ml or a 1:500 dilution of Cytoplasmic tail serum in the same buffer. Specific bands were detected with alkaline phosphatase-conjugated antibodies against mouse or rabbit immunoglobulin G (Dako and Dianova, respectively, Hamburg, Germany). BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium (Promega, Heidelberg, Germany) were used as specific substrates for alkaline phosphatase.

Flow cytometry analysis. Cells (4×10^5 to 2×10^6) were resuspended in phosphate-buffered saline containing 2.5% FCS and 0.07% sodium azide. One-half of the cells were incubated with MAb W6/32 for 45 min at 4°C; the other half were treated the same way but did not receive primary antibody. Before applying a fluorescein isothiocyanate-conjugated antibody (50 μ l of a 1:60 dilution; Sigma), excess primary antibody was removed by washing three times with ice-cold incubation buffer. After 45 min of incubation with secondary antibody, cells were washed three more times. For internal staining of E3/19K, incubation was carried out with Tw1.3 in the presence of 0.1% saponin (Sigma). This concentration was kept throughout the following washing and incubation steps. Fluorescence profiles were obtained by analyzing 5,000 viable cells in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

RESULTS

The E3/19K protein contains intramolecular disulfide bonds. To investigate the role of the cysteines in the structure and function of the E3/19K protein, we first examined whether the cysteines are involved in intramolecular disulfide bonds. The presence of disulfide bonds can be monitored by following the migration of the reduced and the nonreduced protein in SDS-PAGE. The cell line 293E3-45, which expresses E3/19K, was labeled for 45 min with [³⁵S]methionine, and the E3/19K protein was immunoprecipitated with MAb Tw1.3. Separation of the precipitated material by SDS-PAGE revealed a nonreduced form of E3/19K which migrated faster than the reduced form, suggesting the presence of intramolecular disulfide bonds (Fig. 2, lanes 3 and 4). The difference in apparent molecular masses corresponds to about 700 to 1,000 Da. Compared with the altered migration seen upon reduction of HLA heavy chain and β_2m (Fig. 2, lanes 1 and 2), known to contain two and one cysteine bridges, respectively, a crude estimate can be made about the size of the loops between the connecting cysteines. As the apparent molecular masses of the

FIG. 2. Intramolecular disulfide bond formation of E3/19K and HLA antigens as analyzed by SDS-PAGE. 293E3-45 cells were labeled for 1 h with 100 μ Ci of [35 S]methionine per ml and lysed with detergent. HLA and E3/19K proteins were immunoprecipitated with MAbs W6/32 (lanes 1 and 2) and Tw1.3 (lanes 3 and 4). To prevent oxidation of free cysteines, lysis and subsequent immunoprecipitation under nonreducing conditions were carried out in the presence of 7 mM iodoacetamide (lanes 1 and 3). Under reducing conditions (lanes 2 and 4) iodoacetamide was omitted from the lysis buffer. Treatment with (+) and without (–) DTT and subsequent blocking with iodoacetamide occurred just before loading of the gel. Asterisks mark the lower nonreduced species of each protein. Molecular masses (in kilodaltons) are indicated on the right.

nonreduced forms differ by 800 to 1,100 Da (HLA) and 400 Da (β_2m), respectively, we envisage the existence in E3/19K of either one disulfide loop larger than that in β_2m or, alternatively, two with similar sizes.

A disulfide bond linking Cys-11, Cys-22, and Cys-28 with Cys-101, Cys-109, and Cys-122 apparently does not exist. Further information as to which cysteines are linked to each other was obtained by cyanogen bromide digestion of E3/19K. Cyanogen bromide cleaves proteins at the carboxyl groups of methionines. Conveniently, four of the five methionines in E3/19K are concentrated between amino acids 74 and 89 (Fig. 1), while the fifth methionine is the penultimate amino acid. Thus, complete cleavage by cyanogen bromide will produce a total of five fragments (Fig. 3). Two of these (fragments 1 and 5) with molecular masses greater than 6 kDa, containing three cysteines each, might be visualized by SDS-PAGE. By labeling cells with cysteine and performing SDS-PAGE under reducing and nonreducing conditions, we should be able to determine whether a disulfide bond exists between fragments 1 and 5 (Fig. 3D). In the presence of a disulfide bond, one protein band with a molecular mass of 22 to 23 kDa should be detectable under nonreducing conditions and should be cleavable by the addition of a reducing agent. In the absence of a covalent linkage, faster-migrating bands (~ 17 and ~ 6 kDa), which should not or only slightly be affected by reduction, should be visualized.

Therefore, E3/19K was precipitated from cysteine-labeled cell extracts with MAb Tw1.3 and protein-A Sepharose. Protein-A Sepharose-bound material was released by formic acid, and one-half was incubated for 20 h with cyanogen bromide (Fig. 3E, lanes 3 and 4) while the other half was mock treated (Fig. 3E, lanes 1 and 2). Both samples were analyzed under reducing and nonreducing conditions. Under both conditions, E3/19K is cleaved into smaller fragments (Fig. 3E). The largest, most prominent one migrates at ~ 16.5 kDa and corresponds almost certainly to fragment 1. The protein backbone of fragment 1 has a calculated molecular mass of 8.6 kDa. Adding the apparent molecular masses of the two carbohy-

FIG. 3. Potential disulfide bonds within and between CNBr fragments. (A to D) Schematic representations of possible disulfide bonds upon CNBr cleavage of E3/19K. Complete digestion gives rise to five fragments, two of which might be visualized in SDS-PAGE after metabolic labeling with [35 S]cysteine (fragments 1 and 5). The calculated molecular masses of fragments 1 to 5 are 8,572 Da (including carbohydrates, 16 to 17 kDa), 1,124, 582, 312, and 6,044 Da, respectively. Only the fragments containing cysteines are shown in the figure. The positions of the cysteines are depicted as numbers. Dashed lines indicate possible disulfide linkages. The closed areas represent the transmembrane regions. (E) Nondigested (lanes 1 and 2) and CNBr-digested (lanes 3 and 4) E3/19K protein analyzed under reducing (+ DTT) and nonreducing (– DTT) conditions. Cells were labeled for 1 h with 100 μ Ci of [35 S]cysteine per ml. Cell extracts were prepared, and E3/19K was immunoprecipitated with MAb Tw1.3. Fragment 1 indicates the N-terminal fragment containing amino acids 1 to 74 and two carbohydrates. The smaller fragments a, b, and c are explained as follows. Fragment a comigrates with a fragment generated after HLA immunoprecipitates were treated with CNBr (data not shown). Therefore, because of the small amount of HLA coprecipitated, it is likely to represent an HLA-derived fragment. Fragments b and c have M_r s of 6,400 to 6,700 and $\sim 5,600$ respectively. The former may represent a partially cleaved product (fragment 5 still connected to fragment 4 and/or fragment 3), while band c might correspond to fragment 5. The small molecular weight difference compared with the expected one might be due to an unusual migration of fragment 5 (mainly the transmembrane part of E3/19K) or to the limited precision of the measurement. We excluded the possibility that the CNBr treatment resulted in reduction of the proteins by treating a protein with known disulfide bonds (the murine MHC K d molecule) in the same way (data not shown). The molecular masses (in kilodaltons) of the protein standard (lane 5) are indicated on the right, while the positions of the reduced (E3/19K) and nonreduced (E3/19K*) forms of E3/19K and those of the different fragments are indicated on the left.

drates, which account for ~ 7.5 kDa (see Fig. 7, and data not shown), the predicted molecular mass is ~ 16.1 kDa, which is very similar to that of the most slowly migrating band (lanes 3 and 4; fragment 1). Fragment 5 probably corresponds to bands b and/or c (M_r , 6,400 to 6,700 and $\sim 5,600$, respectively), while band a, with an M_r of 9,400, seems to be HLA derived (data not shown; see the legend to Fig. 3 for further explanations). Although it is difficult to assign each individual band in the low-molecular-weight range, the data exclude disulfide bonds outlined in Fig. 3D. First, the relative molecular mass of the most slowly migrating fragment is 16.5 kDa and not 22 to 23 kDa. Secondly, this band is not sensitive to reduction, and therefore no evidence was found for a disulfide-linked complex between fragments 1 and 5 (Fig. 3D). Thus, we conclude that none of the cysteines at position 11, 22, or 28 is involved in a disulfide bond to 101 or 109. On the basis of this experiment, possibilities A to C (Fig. 3) cannot be distinguished.

FIG. 4. Capacity of MAb Tw1.3 and C-tail serum to bind to reduced and nonreduced E3/19K. Lysates derived from 5×10^6 293E3-45 cells were incubated with Tw1.3. Immunoprecipitated material was separated by SDS-PAGE and transferred to nitrocellulose. As a control for unspecific binding of Tw1.3, equal numbers of 293 cells were treated as described above. The band marked with an asterisk (E3/19K*) represents an E3/19K dimer (molecular mass, ~45 kDa) while the unspecific band migrating more slowly than the dimer (molecular mass, ~55 kDa) apparently represents the heavy chains of MAb Tw1.3 used for immunoprecipitation. Cell lines and antibodies are indicated at the top of the figure. Reduction by DTT is indicated by a plus.

Generation, expression, and detection of mutant E3/19K molecules with cysteines replaced by serines and alanine. To study the role of the cysteines for binding to HLA antigens, we individually replaced each cysteine (Cys) with serine (Ser). We chose to replace cysteine residues with serine in order to maintain the polarity and size of the amino acid side chain as closely as possible. As the replacement of Cys-22 by Ser creates a new site for N-linked glycosylation (NXC to NXS), Cys-22, in addition, was mutated to alanine. All the available data suggest that interaction of MHC antigens with E3/19K occurs with the luminal portion (8, 11, 17, 28, 44), thus, mutation of the conserved Cys-122 located close to the C-terminal end of the transmembrane region was considered as a control.

Mutations were introduced by PCR directly into the *EcoRI* D fragment of Ad2. In doing so, the Ad-specific control elements, such as the promoter, donor and acceptor splice sites, translation regulatory elements, and poly(A) signals, are retained. By choosing appropriate restriction enzymes, it was necessary to sequence only a small portion of *EcoRI* D (see Materials and Methods for details). Mutant constructs were transfected into human embryonic kidney cells (293) together with the neomycin resistance gene. Prior to screening for E3/19K expression, we tested whether MAb Tw1.3 is suitable for detection of mutated E3/19K proteins. Lysates of 293 and 293E3-45 cells were incubated with Tw1.3, and the immunoprecipitated material either was (Fig. 4, lanes 7 and 8) or was not (Fig. 4, lanes 5 and 6) treated with DTT, separated on SDS-PAGE, and transferred to nitrocellulose. Incubating the blot with Tw1.3 reveals a specific E3/19K band only in the nonreduced sample (Fig. 4, lane 5). Thus, although the Tw1.3 epitope is detectable in the SDS-denatured protein, it does not withstand reduction of intramolecular disulfide bonds. Therefore, it is predicted that Tw1.3 binding is affected by mutations that destroy disulfide bonds.

In order to be able to detect all mutants, we developed an antiserum against the cytoplasmic tail of E3/19K (C-tail), which is not affected by any of the mutations. With this antiserum, E3/19K was also recognized under reducing conditions (Fig. 4, lanes 2 and 4). Thus, expression of all mutated E3/19K proteins could be monitored. Interestingly, under

nonreducing conditions, another E3-specific band with an apparent molecular mass approximately twice that of E3/19K (~45 kDa) was detected by both antibodies. This E3/19K species (E3/19K*, lanes 4 and 5) appears to represent a disulfide-linked E3/19K dimer since it is lost upon reduction. This putative E3/19K dimer was also observed when immunoprecipitations were carried out under nonreducing conditions. The amount of dimer in the lysates compared with that of the monomeric form varied between 30 and 50% (data not shown).

Differential binding of MAb Tw1.3 to mutant E3/19K molecules. A total of 20 to 25 clones derived from transfection of each E3/19K mutant gene into 293 cells were screened for expression of E3/19K by immunoprecipitation and FACS analysis using the detergent saponin. One clone of each transfection was chosen for further analysis. First, expression of the Tw1.3 epitope was monitored. Tw1.3 was unable to recognize mutants Ser-11 and Ser-28, whereas all the other mutant proteins were detected (Fig. 5A and B, compare lanes C-tail and Tw1.3). This indicates that the common phenotype of these two mutants might be due to a cysteine bridge between Cys-11 and Cys-28 in the wild-type molecule. Moreover, by measuring the amount of E3/19K precipitated by Tw1.3 versus that by C-tail with a PhosphorImager, a characteristic 50 to 60% reduction for mutants Ser-22, Ala-22, and Ser-83 was revealed, which was not observed for the other mutants (Fig. 5C). This identical alteration in affinity together with the cyanogen bromide data (no disulfide bond between the N-terminal cysteines and Cys-101 and Cys-109) suggests that Cys-22 is linked to Cys-83.

To test this hypothesis, the double mutant 22A/83 was generated. If Cys-83 is a partner for Cys-22, the additional elimination of Cys-83 should not have any further effect than that observed for mutants with the single mutation (Ala-22 or Ser-83); that is, the double mutant should exhibit a reduction of Tw1.3 binding (approximately 50%) similar to that of Ala-22 or Ser-83. In contrast, if Cys-83 (or Cys-22) is linked to Cys-11 or Cys-28, a complete loss of Tw1.3 binding is expected. Figure 5C (22A/83) shows that the double mutant still binds Tw1.3 with an affinity similar to that of the single mutants, thus confirming the proposed disulfide bond between Cys-22 and Cys-83 (see also Fig. 8).

The capacity of E3/19K to bind to MHC molecules depends on correct disulfide bond formation. We then investigated the binding properties of the mutant E3/19K proteins to HLA antigens (Fig. 5A and B, lanes W6/32). HLA antigens were precipitated with an antibody against HLA-A, -B, and -C antigens. As can be seen in cells expressing wild-type E3/19K (Fig. 5A, lane 6), an additional band of 25 kDa which is not observed in untransfected 293 cells (lane 3) or in E3-negative cells (lane 18) is coprecipitated. Mutant E3/19K proteins exhibited a differential capacity to form complexes with HLA. Introducing serine at position 11, 22, 28, or 83 eliminates coprecipitation of E3/19K (Fig. 5A, lanes 9, 12, and 15, and B, lane 13). Replacing Cys-22 with alanine has the same effect (compare Fig. 5A, lane 12, and B, lane 16). Thus, loss of E3/19K coprecipitation for mutant Ser-22 is not due to the newly introduced carbohydrate chain (note the different molecular weight of this mutant [Fig. 5A, lanes 10 and 11]). In contrast, mutant E3/19K proteins with Ser at position 101, 109, or 122 showed a capacity to bind HLA similar to that of the wild-type E3/19K. This indicates that the four N-terminal cysteines are critical for MHC binding.

Lack of coprecipitation does not always correlate with lack of transport inhibition (6a, 48). To test whether the E3/19K mutants interfere with transport of HLA out of the ER, we performed pulse-chase experiments monitoring the processing

of the HLA-A2 allele, which has a high affinity to E3/19K. Analyzing only one allele should allow us to detect the small molecular weight changes accompanying the acquisition of complex-type sugars in the medium/trans-Golgi stack. The 293 cells and transfected 293 cells expressing wild-type and mutant E3/19K proteins were pulse-labeled for 20 min and either chilled on ice (Fig. 6, lanes 1 and 10) or further incubated for 3 h with an excess of unlabeled methionine (Fig. 6, lanes 2 to 9 and 11 to 18). Subsequently, HLA-A2 was immunoprecipitated, and the samples were divided. One-half of each was treated with endo H (lanes 10 to 18) while the other half was mock treated (lanes 1 to 9). Endo H cleaves N-linked carbohydrate chains in the high mannose form but does not affect terminally glycosylated forms attached in the medium/trans-Golgi cisternae. Although the migration of the terminally glycosylated HLA-A2 molecule is only slightly slower than that of the core-glycosylated form, the results are already evident in the untreated samples. However, by endo H treatment, endo H-resistant (HLA-A2^R) and endo H-sensitive (HLA-A2^S) forms can easily be distinguished. HLA-A2 molecules in cells transfected with mutants Ser-11, Ser-22, Ser-28, and Ser-83 migrate with the same electrophoretic mobility as do HLA-A2 molecules in untransfected 293 cells (compare lanes 4 to 7 and 13 to 16 with lanes 2 and 11). In contrast, in cells expressing mutants Ser-109 and Ser-122, HLA-A2 molecules remain endo H sensitive and thus do not become terminally glycosylated. Likewise, HLA-A2 molecules in mutant Ser-101 cells are sensitive to endo H digestion while mutants 22A and 22A/83 have the same phenotype as Ser-22 (data not shown). Thus, the four N-terminal cysteine mutants are unable to interfere with transport of HLA-A2.

In addition, we examined the cell surface expression of all

FIG. 5. Folding and capacity of mutant E3/19K molecules to form complexes with HLA. The 293, 293E3-45, and mutant cell lines were labeled for 1 h with 100 μ Ci of [³⁵S]methionine per ml. After the cells were lysed, the samples were divided into three aliquots for immunoprecipitation with three different antibodies, C-tail, W6/32, and Tw1.3, as indicated above the lanes. The positions of HLA (heavy chain), E3/19K, and β_2m are indicated on the right; those of molecular mass markers (in kilodaltons) are indicated on the left. E3/19K* indicates the position of the E3/19K mutant containing an additional carbohydrate at position 20, as ascertained by endoglycosidase F treatment, which removes all N-linked glycans and thereby eliminates the molecular weight difference (data not shown). Cell lines with the respective Cys-to-Ser mutation are denoted by the amino acid number above the lanes: (A) Ser-11, Ser-22, and Ser-28; (B) Ser-83, Ser-101, Ser-109, Ser-122, and Ala-22. Lanes labeled E3 neg represent a 293 cell line expressing the *neo*^r gene only. (C) Affinity of E3/19K mutants to Tw1.3 and C-tail. The amount of E3/19K precipitated by Tw1.3 and C-tail was determined by measuring the radioactivity of the E3/19K band with a PhosphorImager (Fujix Bas1000, Fuji, Japan). The relative binding affinity of Tw1.3 to the different mutants is expressed as the ratio of radioactivity detected by Tw1.3 versus that by C-tail. The ratio obtained for wild-type E3/19K (WT) was set at 100%. The bars represent the means for three independent experiments except for mutants Ser-22, 22A, 22A/83, and Ser-101 for which only two experiments were analyzed.

HLA alleles. Four different clones derived from each transfection were stained with the HLA-A-, -B-, and -C-specific antibody W6/32 and analyzed by flow cytometry. The mean value of fluorescence of 293 cells and eight neomycin-resistant transfectants which do not express E3/19K proteins was set at 100%. The results shown in Fig. 7 confirms the coprecipitation and pulse-chase data. All the mutant cell lines with which coprecipitation was undetectable and HLA-A2 transport was observed express normal levels of HLA on their cell surface, whereas E3/19K mutants Ser-101, Ser-109, and Ser-122 down-regulate HLA to an extent similar to that of wild-type E3/19K (Fig. 7). This demonstrates that serines at positions 101, 109, and 122 do not interfere with binding, and therefore HLA molecules are retained. In contrast, the N-terminal mutations at positions 11, 22, 28, and 83 disrupt complex formation of E3/19K with HLA, resulting in efficient transport of MHC molecules to the cell surface. Therefore, the cysteines at those positions are absolutely critical for the function of E3/19K.

DISCUSSION

The aim of this study was to further characterize the structural requirements of E3/19K for binding to MHC molecules. Consistent with our previous data that carbohydrates in E3/19K and MHC molecules are not essential for the interaction (8), variable N-glycosylation sites are found in E3/19K proteins from different adenovirus subgroups. Amino acid

FIG. 6. Transport of HLA-A2 antigens in cells expressing mutant and wild-type E3/19K molecules. The 293, 293E3-45 and mutant cell lines were pulse-labeled for 20 min with 200 μ Ci of [35 S]methionine per ml, chased for 3 h (lanes labeled 3) in an excess of cold methionine, and lysed. In addition, 293 cells were lysed immediately (lanes labeled 0) after the pulse. HLA-A2 antigens were immunoprecipitated with MAb BB7.2. The precipitated material was split; one half was treated with endo H (lanes 10 to 18) while the other one was mock treated (lanes 1 to 9). The positions of HLA-A2 (heavy chain), E3/19K, and β_2 m are indicated on the right; those of molecular mass markers (in kilodaltons) are indicated on the left. Asterisks denote endo H-cleaved products; superscript indicates endo H-resistant molecules.

sequence comparisons of E3/19K from Ad groups B and C (Fig. 1) reveal only a low overall identity of around 30% (18, 28, 50). However, as noted previously (17), a stretch of amino acids between positions 77 and 98 is rather conserved (~75%). Strikingly also, several of the cysteines are conserved: Cys-11, Cys-22, and Cys-83 in the luminal domain and Cys-122 in the

transmembrane domain close to the cytoplasm are found in all E3/19K proteins examined. With the exception of Ad35 E3/19K, Cys-28 is conserved as well, whereas Cys-101 and Cys-109, localized in front and at the beginning of the transmembrane segment, respectively, are found only in Ad of subgroup C.

To examine the role of these cysteines in the structure and function of the Ad2 E3/19K protein, we individually replaced each cysteine with serine. In addition, Cys-22 was mutated to alanine since the serine mutation created an additional N glycosylation site. Our previous data (8) demonstrated that complex formation does not involve intermolecular disulfide bonds. However, some of the cysteines form intramolecular disulfide bonds, as indicated by the shift in molecular weight upon reduction (Fig. 2 and 3). Further information as to which cysteines might be linked to each other was obtained by the combination of cyanogen bromide cleavage (Fig. 3) and site-directed mutagenesis (Fig. 5). The former method clearly demonstrated that there is no disulfide bond between any of the N-terminal cysteines (at positions 11, 22, and 28) and Cys-101 or Cys-109, leaving only Cys-83 as a potential partner. This notion was further corroborated and extended by the common phenotype of the four N-terminal E3/19K mutants Ser-11, Ser-22, Ser-28, and Ser-83 with regard to complex formation with MHC. Most remarkably, two pairs of mutants exhibit a differential ability to bind MAb Tw1.3. Substitution of Cys-11 and Cys-28 is associated with the complete loss of the Tw1.3 epitope, while substitution of Cys-22 and Cys-83, individually or in conjunction, is accommodated (Fig. 5), causing only an ~60% reduction in antibody reactivity. We suggest

FIG. 7. Cell surface expression of HLA in mutant and wild-type cell lines. Cells were stained for flow cytometry with MAb W6/32. At least four clones of each transfection were analyzed in at least two independent experiments. The mean value of fluorescence was calculated for each cell line and related to the mean values for 293 cells and eight E3/19K⁻ transfectant clones (293/E5). The latter was set at 100%. Error bars represent standard deviations ($\sigma_n - 1$).

FIG. 8. Schematic model of the Ad2 E3/19K protein incorporating the newly defined disulfide bond linkages. The E3/19K structure is drawn as a line. Carbohydrates are depicted as hatched circles. The positions of the cysteines are numbered. The @ symbols linked by dashes represent the lipid bilayer. The regions of the molecule implicated in MHC binding and ER retention are indicated by straight lines. The broken line represents the conserved stretch of amino acids; the double arrow denotes the Tw1.3 epitope. According to our results, Cys-11 and Cys-28 as well as Cys-22 and Cys-83 are linked via disulfide bonds, both of which are important for binding to MHC molecules. In addition, the disulfide bond between Cys-11 and Cys-28 is essential for the Tw1.3 epitope. Cys-101, Cys-109, and Cys-122 are not involved in intramolecular cysteine bridges nor do they influence binding of Tw1.3 or complex formation with MHC molecules.

that the identical phenotype of each pair of mutants reflects a disulfide bond between the two respective cysteines (Cys-11 and Cys-28; Cys-22 and Cys-83). On the basis of these results, we propose a model for the E3/19K structure (Fig. 8).

We suggest that Tw1.3 recognizes a noncontiguous epitope which requires a disulfide bond between Cys-11 and Cys-28. As Tw1.3 recognizes both Ad2 and Ad5 E3/19K proteins, the limited sequence variation between the two proteins at positions 1, 2, 16, 17, 41, 58, and 68 to 70 (conservative changes were not considered) may suggest that these amino acids are not part of the epitope. We believe that amino acids 16 to 22, one part of the small loop in our model, might not be essential for Tw1.3 binding because (i) amino acids 16 and 17 are variable, (ii) Cys-22 is of minor importance, and (iii) the additional carbohydrate at position 20, introduced by the replacement of Cys-22 by serine, did not influence recognition by Tw1.3 (Fig. 5). Consistent with this idea, an E3/19K mutant containing a deletion spanning amino acids 16 to 21 has still some residual reactivity with Tw1.3 (28). Taken together, we suggest that the N-terminal amino acids 3 to 15 and a significant portion of the loop structure proposed to exist between Cys-22 and Cys-83 (amino acids 23 to 67) might be important for the Tw1.3 epitope.

In general, our conclusions are compatible with and extend those reached by Hermiston et al. (28), deleting portions of the E3/19K luminal domain. They showed that deletions of 4 to 12 amino acids from the N terminus to amino acid 83 causes, with one exception, a loss of antibody reactivity while the deletions of those between 84 and 107 had no effect. In their study, cysteines were not eliminated. However, no evidence was provided for the presence of normal disulfide bonding in their deletion mutants. Thus, it is possible that some of these deletions, in particular those within the proposed loop spanning Cys-22 and Cys-83, prevented correct disulfide bonding and therefore correct folding and function of E3/19K.

In this study, evidence is presented that most or perhaps all

of the cysteine mutants do not have a grossly altered structure. First, all the mutants were fully glycosylated in contrast to previous truncation mutants which were only partly glycosylated (22). Secondly, seven of the nine cysteine mutants retained the epitope of an MAb directed to the luminal portion of E3/19K, indicating limited and distinct structural alterations. Thirdly, replacement of one particular cysteine did not lead to the elimination of all intramolecular cysteine bonds in E3/19K, as evidenced by the typical shift to a higher apparent molecular weight of mutant proteins upon reduction (data not shown).

Strikingly, introducing serines at positions 11, 22, 28, and 83 abrogates binding of the Ad2 E3/19K protein to MHC antigens and thereby abrogates its transport inhibition function. This was demonstrated by a lack of E3/19K coprecipitation (Fig. 5), by expression of wild-type levels of MHC molecules on the cell surface in the presence of mutated E3/19K proteins (Fig. 7), and by a normal transport of class I molecules from the ER to the medium/trans-Golgi cisternae (Fig. 6). The two proposed disulfide bonds formed among these four cysteine residues (Fig. 8) seem to create a conformation required for binding to MHC molecules. Thus, the interaction of these two molecules is obviously based on a three-dimensional structure and not on a linear sequence.

Most previous studies (11, 17, 22, 28, 44) employed the strategy of introducing deletions in the E3/19K protein to map the binding site of the viral protein for MHC molecules. Interpretation of those data is not simple since the results are not always consistent. The studies agree in that the cytoplasmic tail of E3/19K is not required for the interaction. However, further deletions eliminating part (17, 22) or all (17, 22, 44) of the transmembrane segment disrupted complex formation in vivo (in cells) but not in vitro, after translation or on MHC-coated beads (22, 44). The differences may be due to the loss of membrane integration in vivo or the different geometry of binding in vitro versus that in vivo. In general, the aforementioned approach of introducing deletions is limited by the gross structural changes accompanying those deletions (17). This latter point also needs to be considered when interpreting the deletional analysis of the luminal domain by Hermiston et al., in which all deletions except one (deletion of amino acids 102 to 107) destroyed the ability of E3/19K to bind to HLA (28). To some extent, this latter construct was still able to bind to and partially inhibit the transport of HLA, although no effect on cell surface expression of HLA was visible in their infection system. As amino acids of very different natures can be accommodated in MHC-binding proteins of different Ad (Fig. 1), the study by Hermiston et al. (28) points perhaps to a length requirement (see above).

Flomenberg et al. (17) focused on the conserved region of the Ad35 E3/19K protein (analogous to amino acids 77 to 98 of Ad2). Four of the five point mutants exhibited a considerable reduction in their ability to bind to monkey MHC antigens, indicating that this portion may bind directly to a conserved part of MHC antigens, such as the $\alpha 3$ domain or $\beta 2m$. It is somewhat surprising that we were unable to assign a significant role to the $\alpha 3$ domain of MHC molecules but rather to the polymorphic $\alpha 1$ and $\alpha 2$ domains (8, 16, 32). As all our MHC constructs contained the conserved $\alpha 3$ domain, it is possible that contacts with this domain are necessary but not sufficient. Alternatively, the conserved amino acids of E3/19K might be an essential core structure required for correct folding of the more distal portions. Both possibilities are consistent with our model.

Assaying E3/19K immunoprecipitates under nonreducing conditions reveals specific bands at 24 and 45 kDa (Fig. 2 and

data not shown). Initially, we assumed that the upper band may represent only coprecipitating HLA. However, a band of similar molecular weight was detected in Western blots, with E3/19K-specific antibodies (Fig. 4). This establishes the nature of the material to be, at least in part, E3/19K derived. Most likely, it comprises a disulfide-linked E3/19K dimer, since reduction eliminates this band. The putative dimer is also detected when iodoacetamide or *N*-ethyl-maleimide are present in the lysis buffer, although to a lesser extent (data not shown). Therefore, we conclude that at least a portion is formed *in vivo*. The existence of such dimers was previously suggested by using vaccinia recombinants of E3/19K (11). At present, their functional role remains unclear. In any case, by quantitating the amount of dimer versus that of the monomer we could not clearly assign a critical role for dimerization to one particular cysteine (data not shown).

In summary, our mutational analysis of the cysteines within E3/19K allowed us to establish a structural model of E3/19K and suggests the existence of an important conformation for MHC binding, on the basis of correct disulfide bond formation. This would explain the observed sequence variation among E3/19K proteins as well as the importance of noncontiguous amino acids in the MHC $\alpha 2$ domain for binding E3/19K (16). Further studies are needed to clarify whether E3/19K proteins synthesized by Ad from different subgroups indeed bind with the same affinity to the same MHC antigens.

ACKNOWLEDGMENTS

We are grateful to J. Yewdell (Bethesda, Md.) for kindly providing antibody Tw1.3. We also thank H. U. Weltzien, F. Deryckere, D. Feuerbach, B. Menz, and P. Nielsen for critical reading of the manuscript and L. Lay for photographic work.

REFERENCES

- Andersson, M., A. McMichael, and P. A. Peterson. 1987. Reduced allorecognition of adenovirus-2 infected cells. *J. Immunol.* **138**: 3960–3966.
- Andersson, M., T. Pääbo, T. Nilsson, and P. A. Peterson. 1985. Impaired intracellular transport of class I MHC antigen as a possible means for adenoviruses to evade immune surveillance. *Cell* **43**:215–222.
- Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. William, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell* **14**:9–20.
- Bjorkman, P. J., and P. Parham. 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu. Rev. Biochem.* **59**:253–288.
- Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (London)* **329**:506–512.
- Braciale, T. J., and V. L. Braciale. 1991. Antigen presentation: structural themes and functional variations. *Immunol. Today* **12**:124–129.
- Burgert, H.-G., et al. Unpublished observations.
- Burgert, H.-G., and S. Kvist. 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell* **41**:987–997.
- Burgert, H.-G., and S. Kvist. 1987. The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition. *EMBO J.* **6**:2019–2026.
- Burgert, H.-G., J. L. Maryanski, and S. Kvist. 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc. Natl. Acad. Sci. USA* **84**:1356–1360.
- Cladaras, C., and W. S. M. Wold. 1985. DNA sequence of the E3 transcription unit of adenovirus 5. *Virology* **140**:28–43.
- Cox, J. H., J. R. Bennink, and J. W. Yewdell. 1991. Retention of adenovirus E19 glycoprotein in the endoplasmic reticulum is essential to its ability to block antigen presentation. *J. Exp. Med.* **174**:1629–1637.
- Cox, J. H., J. W. Yewdell, L. C. Eisenlohr, P. R. Johnson, and J. R. Bennink. 1990. Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. *Science* **247**: 715–718.
- Degen, E., M. F. Cohen-Doyle, and D. B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both beta 2-microglobulin and peptide. *J. Exp. Med.* **175**:1653–1661.
- Falk, K., and O. Rötzschke. 1993. Consensus motifs and peptide ligands of MHC class I molecules. *Semin. Immunol.* **5**:81–94.
- Feuerbach, D., and H.-G. Burgert. 1993. Novel proteins associated with MHC class I antigens in cells expressing the adenovirus protein E3/19K. *EMBO J.* **12**:3153–3161.
- Feuerbach, D., S. Etteldorf, C. Ebenau-Jehle, J.-P. Abastado, D. Madden, and H.-G. Burgert. Submitted for publication.
- Flomenberg, P., J. Szmulewicz, E. Gutierrez, and H. Lupatkin. 1992. Role of the adenovirus E3-19K conserved region in binding major histocompatibility complex class I molecules. *J. Virol.* **66**:4778–4783.
- Flomenberg, P. R., M. Chen, and M. S. Horwitz. 1988. Sequence and genetic organization of the adenovirus type 35 early region 3. *J. Virol.* **62**:4431–4437.
- Fox, J. P., C. E. Hall, and M. K. Cooney. 1977. The Seattle virus watch. VII. Observations of adenovirus infections. *Am. J. Epidemiol.* **105**:362–386.
- Fremont, D. H., M. Matsumura, E. A. Stura, P. A. Peterson, and I. A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science* **257**:919–927.
- Gabathuler, R., and S. Kvist. 1990. The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus type 2 consists of three separate amino acid segments at the carboxy terminus. *J. Cell Biol.* **111**:1803–1810.
- Gabathuler, R., F. Lévy, and S. Kvist. 1990. Requirements for the association of adenovirus type 2 E3/19K wild-type and mutant proteins with HLA antigens. *J. Virol.* **64**:3679–3685.
- Garrett, T. P., M. A. Saper, P. J. Bjorkman, J. L. Strominger, and D. C. Wiley. 1989. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature (London)* **342**:692–696.
- Germain, R. N., and D. H. Margulies. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* **11**:403–450.
- Ginsberg, H. S., U. Lundholm-Beauchamp, R. L. Horswood, B. Pernis, W. S. M. Wold, R. M. Chanock, and G. A. Prince. 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA* **86**:3823–3827.
- Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59–72.
- Hérissé, J., G. Courtois, and F. Galibert. 1980. Nucleotide sequence of the EcoRI D fragment of adenovirus 2 genome. *Nucleic Acids Res.* **8**:2173–2192.
- Hermiston, T. W., R. A. Tripp, T. Sparer, L. R. Gooding, and W. S. M. Wold. 1993. Deletion mutation analysis of the adenovirus type 2 E3-gp19K protein: identification of sequences within the endoplasmic reticulum luminal domain that are required for class I antigen binding and protection from adenovirus-specific cytotoxic T lymphocytes. *J. Virol.* **67**:5289–5298.
- Higuchi, R., B. Krummel, and R. K. Saiki. 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**:7351–7367.
- Höchstetbach, F., V. David, S. Watkins, and M. B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA* **89**:4734–4738.
- Horwitz, M. S. 1990. Adenoviruses, p. 1723–1740. In B. N. Fields, D. M. Knipe, et al. (ed.), *Virology*. Raven Press, Ltd., New York.
- Jefferies, W. A., and H.-G. Burgert. 1990. E3/19K from adenovirus 2 is an immunosubversive protein that binds to a structural motif

- regulating the intracellular transport of major histocompatibility complex class I proteins. *J. Exp. Med.* **172**:1653–1664.
33. Kvist, S., and F. Levy. 1993. Early events in the assembly of MHC class I antigens. *Semin. Immunol.* **5**:105–116.
 34. Kvist, S., L. Östberg, H. Persson, L. Philipson, and P. A. Peterson. 1978. Molecular association between transplantation antigens and cell surface antigen in adenovirus-transformed cell line. *Proc. Natl. Acad. Sci. USA* **75**:5674–5678.
 35. Körner, H., and H.-G. Burgert. 1994. Down-regulation of HLA antigens by the adenovirus type 2 E3/19K protein in a T-lymphoma cell line. *J. Virol.* **68**:1442–1448.
 36. Körner, H., U. Fritzsche, and H.-G. Burgert. 1992. Tumor necrosis factor a stimulates expression of adenovirus early region 3 proteins: implications for viral persistence. *Proc. Natl. Acad. Sci. USA* **89**:11857–11861.
 37. Leff, T., R. Elkaim, C. R. Goding, P. Jalinot, P. Sassone-Corsi, M. Perricaudet, C. Kedinger, and P. Chambon. 1984. Individual products of the adenovirus 12S and 13S E1a mRNAs stimulate viral EIIa and EIII expression at the transcriptional level. *Proc. Natl. Acad. Sci. USA* **81**:4381–4385.
 38. Li, Z., and K. Srivastava. 1993. Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *EMBO J.* **12**:3143–3151.
 39. Madden, D. R., J. C. Gorga, J. L. Strominger, and D. C. Wiley. 1992. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* **70**:1035–1048.
 40. Mei, Y.-F., and G. Wadell. 1992. The nucleotide sequence of adenovirus type 11 early 3 region: comparison of genome type Ad11p and Ad11a. *Virology* **191**:125–133.
 41. Monaco, J. J. 1992. A molecular model of MHC class-I-restricted antigen processing. *Immunol. Today* **13**:173–179.
 42. Pääbo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson. 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50**:311–317.
 43. Pääbo, S., T. Nilsson, and P. A. Peterson. 1986. Adenoviruses of subgenera B, C, D, and E modulate cell-surface expression of major histocompatibility complex class I antigens. *Proc. Natl. Acad. Sci. USA* **83**:9665–9669.
 44. Pääbo, S., F. Weber, T. Nilsson, W. Schaffner, and P. A. Peterson. 1986. Structural and functional dissection of an MHC class I antigen-binding adenovirus glycoprotein. *EMBO J.* **5**:1921–1927.
 45. Parham, P., and F. M. Brodsky. 1981. Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum. Immunol.* **3**:277–299.
 46. Peter, M. E., C. Hall, A. Rühlmann, J. Sancho, and C. Terhorst. 1992. The T-cell receptor ζ chain contains a GTP/GDP binding site. *EMBO J.* **11**:933–941.
 47. Rawle, F. C., E. Tollefson, W. S. M. Wold, and L. R. Gooding. 1989. Mouse anti-adenovirus T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. *J. Immunol.* **143**:2031–2037.
 48. Severinsson, L., I. Martens, and P. A. Peterson. 1986. Differential association between two human MHC class I antigens and an adenoviral glycoprotein. *J. Immunol.* **137**:1003–1009.
 49. Shenk, T., and J. Williams. 1984. Genetic analysis of adenoviruses. *Curr. Top. Microbiol. Immunol.* **111**:1–39.
 50. Signäs, C., G. Akusjärvi, and U. Pettersson. 1986. Region E3 of human adenoviruses: differences between the oncogenic adenovirus-3 and the non-oncogenic adenovirus-2. *Gene* **50**:173–184.
 51. Signäs, C., M. G. Katze, H. Persson, and L. Philipson. 1982. An adenovirus glycoprotein binds heavy chains of class I transplantation antigens from man and mouse. *Nature (London)* **299**:175–178.
 52. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**:327–341.
 53. Straus, S. E. 1984. Adenovirus infections in humans, p. 451–496. *In* H. S. Ginsberg (ed.), *The adenoviruses*. Plenum Press, New York.
 54. Tanaka, Y., and S. S. Tevethia. 1988. Differential effect of adenovirus 2 E3/19K glycoprotein on the expression of H-2D^b-restricted SV40-specific CTL-mediated lysis. *Virology* **165**:357–361.
 55. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* **7**:601–624.
 56. Wold, W. S. M., C. Cladaras, S. L. Deutscher, and Q. S. Kapoor. 1985. The 19-kDa glycoprotein coded by region E3 of adenovirus. Purification, characterization, and structural analysis. *J. Biol. Chem.* **260**:2424–2431.
 57. Wold, W. S. M., and L. R. Gooding. 1991. Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions. *Virology* **184**:1–8.
 58. Yewdell, J. W., and J. R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* **52**:1–123.