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Determinants of the endoplasmic reticulum (ER) lumenal-domain of the Adenovirus serotype 2 E3-19K protein for association with and ER-retention of major histocompatibility complex class I molecules

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Abstract

The E3-19K immunomodulatory protein from adenoviruses (Ads) inhibits antigen presentation by major histocompatibility complex (MHC) class I molecules. As a result, the ability of Ad-specific cytotoxic T lymphocytes (CTLs) to lyse infected cells is suppressed. The ER-lumenal domain of E3-19K is subdivided into a variable (residues 1 to ~78/81) and conserved (residues ~79/82 to 98) region followed by a linker (residues 99–107). Using molecular and cellular approaches, we characterized in detail the properties of the ER-lumenal domain of E3-19K that enable it to target MHC class I molecules. Proteolysis of recombinant serotype 2 E3-19K (residues 1–100) (with six His residues) generated a large N-terminal (residues 1–88) and a small C-terminal fragment (residues 94–100) in solution. Neither of these fragments associates with HLA-A*1101 as shown by a native gel band-shift assay. In contrast, the N-terminal 1–93 residues of Ad2 E3-19K exhibited the same binding affinity to HLA-A*1101 as E3-19K. Using a site-directed mutational analysis and flow cytometry, we show that Tyr⁹³, but not Tyr⁸⁸, critically modulates the cell-surface expression of MHC class I molecules. Taken together, these results indicate that the sequence comprising residues 89 to 93 (M⁸⁹SKQY⁹³), and in particular Tyr⁹³, in the conserved region of E3-19K is critical for its immunomodulatory function. Residues 89 to 93 likely form a linker or loop in E3-19K. Overall, our data provide novel insights into the structure of E3-19K and identify key determinants for association with and ER-retention of its cellular target protein. This knowledge is important for our understanding of the molecular basis of Ad pathogenesis.

Keywords

E3-19K; Adenovirus; persistent viruses; immunomodulatory proteins; immune evasion mechanisms; MHC class I molecules; and antigen presentation

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1. Introduction

MHC class I molecules present viral peptides to CTLs as part of a process that activates the immune system to lyse infected cells. In turn, viruses have evolved mechanisms to suppress the surface presentation of viral peptides on infected cells. It is now well established that the viral proteins involved in these mechanisms suppress cellular immune responses by interfering with key steps of the class I antigen presentation pathway (Hansen and Bouvier, 2009).

Ads cause acute and persistent infections of the respiratory, gastrointestinal, and urinary tracts and eyes (Horwitz, 1990). There are at least 51 human Ad serotypes (Ad1 to Ad51) classified into six subgroups (A to F) (Green et al., 1979; Wadell, 1984). The genome of Ad of all subgroups, with the exception of subgroups A and F, codes for an E3-19K immunomodulatory protein in the *early 3* (*E3*) transcription unit (Pääbo et al., 1986a). E3-19K abolishes the presentation of viral peptides by newly synthesized MHC class I molecules (Burgert and Kvist, 1985, 1987; Cox et al., 1990; Kvist et al., 1978) and, consequently, suppresses the activity of Ad-specific CTLs (Andersson et al., 1987; Burgert et al., 1987; Flomenberg et al., 1996; Rawle et al., 1989). *In vivo* data strongly support a role for E3-19K in Ad infections (Ginsberg et al., 1989); lungs of cotton rats infected with a mutant Ad containing a deletion of the E3-19K gene caused a more severe immunopathology than lungs infected with wild-type Ad. It was suggested that the absence of E3-19K in the mutant virus activated Ad-specific CTLs as part of the inflammatory response to viral infection (Ginsberg et al., 1989).

E3-19K is a type I transmembrane glycoprotein that comprises a N-terminus ER-luminal domain and a short C-terminus cytosolic tail. The ER-luminal domain of E3-19K binds directly to the ER-luminal domain of MHC class I molecules (Beier et al., 1994; Burgert and Kvist, 1987; Feuerbach et al., 1994; Flomenberg et al., 1994; Gabathuler et al., 1990; Herminston et al., 1993) and the dilysine motif in the cytosolic tail of E3-19K provides the signal for localization in the ER (Burgert and Kvist, 1987; Cox et al., 1991; Pääbo et al., 1987). Based on sequence comparisons of E3-19K proteins of serotypes from different subgroups, the ER-luminal domain has been subdivided into three distinct regions with loosely defined boundaries (Flomenberg et al., 1992; Herminston et al., 1993): (1) residues 1 to ~78/81 are rather variable between E3-19K proteins of different subgroups; (2) residues ~79/82 to 98 are rather conserved between E3-19K proteins of different subgroups; and (3) residues 99 to 107 link the ER-luminal domain to the transmembrane domain. To date, our knowledge of how the variable and conserved regions of E3-19K interact with MHC class I molecules is unclear. Several residues have, however, been identified to be critical in modulating E3-19K function: in the variable region, Trp⁵² in Ad2 E3-19K (Sester et al., 2010) and in the conserved region, Glu¹⁰⁴, Asp¹⁰⁷, Met¹¹⁰, and Lys¹¹⁴ in Ad35 E3-19K (Flomenberg et al., 1992) (equivalent to positions 81, 84, 87, and 91 in Ad2 E3-19K) and Met⁸⁷ and Trp⁹⁶ in Ad2 E3-19K (Sester et al., 2010). Evidence has also been provided that E3-19K targets the $\alpha 1$ - and $\alpha 2$ -domains (peptide-binding groove) of MHC class I molecules (Beier et al., 1994; Feuerbach et al., 1994; Flomenberg et al., 1994). Consistent with this, we showed previously that MHC residue 56 (located at the N-terminal end of the $\alpha 1$ -helix) is part of the E3-19K/MHC I binding interface (Liu et al., 2007).

To date, although the molecular basis of Ad pathogenesis is not well understood, it is thought that the E3-19K/MHC I association plays an important role in enabling Ads to cause acute and persistent infections in the human host (Ginsberg et al., 1989; Wold and Gooding, 1989; Burgert, 1996). A thorough understanding of the structure/function relationship of E3-19K is therefore important for advances in the pathogenesis of Ads. Here, we used molecular and cellular approaches to identify determinants of the ER-luminal domain of

Ad2 E3-19K that are critical for its immunomodulatory function. We show that Tyr⁸⁸ and Tyr⁹³ are highly susceptible to cleavage by proteases. Neither of the proteolytic fragments (residues 1–88 and residues 94–100) generated in solution associates with HLA-A*1101. However, the N-terminal 93 residues of E3-19K are sufficient to retain high-affinity binding to HLA-A*1101. This identifies residues 89 to 93 in the conserved region as critical for the function of E3-19K. Based on our data, we suggest that residues 89 to 93 are likely to form a linker or loop in E3-19K.

2. Materials and methods

2.1. Cloning and expression

The cDNAs encoding the ER-luminal domain (residues 1 to 100) (with six His residues) of Ad2 E3-19K[Y88G] and Ad2 E3-19K[Y93G] were generated by the QuikChange site-directed mutational approach using the Ad2 E3-19K (residues 1 to 100)/pFastBac plasmid as template (Liu et al., 2005). The cDNA encoding the ER-luminal domain (residues 1 to 93) of Ad2 E3-19K was similarly generated. Recombinant E3-19K proteins were expressed using the Bac-to-Bac baculovirus expression system (Invitrogen) and purified as described previously (Liu et al., 2005).

Expression plasmids for the ER-luminal domains of HLA-A*0301, -A*1101, -A*3301, and -Aw*6801 heavy chains were described previously (Bouvier and Wiley, 1998; Liu et al., 2007). Recombinant class I heavy chains and β_2 -microglobulin (β_2m) were expressed as inclusion bodies in BL21(DE3)pLysS cells and purified from the cell lysates. Inclusion bodies were washed and solubilized in urea as described previously (Garboczi et al., 1992).

The P631 plasmid (gift from Dr. W. Wold, Saint Louis University School of Medicine, Saint Louis, MO; Toth et al., 2002) containing the 6.7K and Ad2 E3-19K genes was used for expression of full-length Ad2 E3-19K. The cDNAs of full-length Ad2 E3-19K[Y88G] and Ad2 E3-19K[Y93G] were generated by QuikChange (Stratagene) using the P631 plasmid as a template. To generate cell lines stably expressing E3-19K and the two mutants, plasmids harboring the correct DNA sequences were linearized with *pvu* I and transfected into human embryonic kidney (HEK) 293 cells (gift from Dr. B.S. Prabhakar, University of Illinois at Chicago, Chicago, IL) (10^6) using the Cell Line Nucleofector Kit V (Lonza). The E3-19K, E3-19K[Y88G], and E3-19K[Y93G] transfectants were incubated in DMEM medium (Invitrogen) supplemented with 10% FBS for 48 hours followed by plating into 10-cm petri dishes. The selection of transfectants was initiated with 300 μ g/ml Zeocin. After ~25 days, Zeocin-resistant clones were expanded and maintained in 150 μ g/ml Zeocin. The expression levels of E3-19K and mutant proteins in the transfectants were quantified by immunoblotting using the P33 antibody (directed against amino acids 45 to 49 of Ad2 E3-19K) from rabbit serum. MHC class I cell-surface expression was determined by flow cytometry (see below).

2.2 Flow cytometry

E3-19K, E3-19K[Y88G], and E3-19K[Y93G] transfectants were characterized by flow cytometry using a LSR instrument (Becton Dickinson). Cells were washed twice with PBS containing 1% BSA and 0.1% NaN₃. After blocking with rabbit IgG (Sigma-Aldrich), cells were stained with the W6/32 mAb (recognizes HLA-A, -B, and -C molecules) for 45 minutes, washed three times, and stained with the FITC-conjugated IgG for 45 minutes. Cells were then analyzed for MHC class I cell-surface expression by gating to exclude dead cells. Data analysis was performed using the Summit V4.3 software (Beckman Coulter). The levels of MHC I cell-surface expression on transfectants are expressed relative to levels on untransfected HEK 293 cells (100%).

2.3. In vitro assembly of MHC class I molecules

Recombinant MHC class I molecules were assembled from the inclusion bodies of class I heavy chains and β_2m (or biotinylated β_2m) in the presence of synthetic peptides in an oxidative refolding buffer (Garboczi et al., 1992). The MHC I-restricted peptides that were added to the refolding buffer have been described previously (Liu et al., 2007).

Reconstituted MHC class I molecules were purified on a Superdex 200 HR 10/30 column in 20 mM Tris and 150 mM NaCl (pH 7.5).

2.4. Limited proteolysis

Ad2 E3-19K (residues 1 to 100) (with six His residues; referred to hereafter as E3-19K) (Liu et al., 2005) was incubated with chymotrypsin, elastase, pronase, trypsin, V8, and thermolysin (Roche) at enzyme:substrate ratio (w/w) of 1:100 at 20°C in 20 mM Hepes, 150 mM NaCl (pH 7.5) for 4 hours. The reactions were then quenched with the SDS gel loading buffer and samples boiled at 100°C for 5 minutes followed by analysis on a Tris-tricine SDS-PAGE gel (16.5%) (0.1 M Tris, 0.1 M tricine (pH 8.3)) (Tris-tricine SDS-PAGE gels are useful for resolving low molecular weight proteins and peptides). Proteins were visualized with Coomassie Blue R-250. For the kinetic experiments, aliquots were taken at different times from the mixture and quenched with aprotinin (to 0.3 μ M). Samples corresponding to 0.3 hour and 24 hours hydrolysis were characterized by N-terminal amino acid sequencing (7 cycles) and MALDI mass spectrometry after electroblotting the SDS-PAGE gel onto a polyvinylidene difluoride membrane (Millipore).

2.5. Native gel band-shift assay

E3-19K proteins (14 μ g) were incubated with MHC class I molecules (20 μ g) (2:1 molar ratio) on ice in 20 mM Tris and 150 mM NaCl (pH 7.5) for 30 minutes. The incubation mixtures were analyzed on native PAGE gel (10%) at 4°C in 25 mM Tris and 200 mM glycine (pH 8.4). Proteins were visualized with Coomassie blue R-250.

2.6. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed at 20°C using a Biacore T100 instrument as described previously (Liu et al., 2007). Refolded β_2m was biotinylated as described previously (Liu et al., 2007). Biotinylated HLA-A*1101 molecules were immobilized on a streptavidin-coated sensor chip (Series S Sensor chip SA, certified) at a density of ~750–800 resonance unit using a stock solution (~30 nM) in HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.05% Surfactant P20 (pH 7.4)) at a flow rate of 60 μ l/minute. Uncoupled streptavidin sites were blocked with biotin (30 μ l/minute for 1 minute). Solutions of E3-19K proteins (2.5 to 2500 nM) were injected over the sample and control flow cells (60 μ l/minute for 4 minutes) and binding responses (with control responses automatically subtracted) were recorded. Binding surfaces were regenerated with HBS-EP. Kinetics dissociation constants were determined by globally fitting the association and dissociation phases of the sensorgrams with the Biacore T100 evaluation software (version 2.0.1; Biacore) assuming a 1:1 Langmuir binding model. Equilibrium dissociation constants were determined by plotting binding responses at steady state versus E3-19K concentrations based on a 1:1 Langmuir binding model.

3. Results

3.1. Proteolysis of Ad2 E3-19K generates two major fragments in solution

To obtain information on E3-19K that may help decipher the roles of the variable and conserved regions in supporting its immunomodulatory function, we first used a limited proteolysis approach. The use of enzymes as structural probes is a useful way to identify

solvent-exposed linker or loop regions in proteins (Fontana et al., 2004). A series of enzymes comprising chymotrypsin, elastase, pronase, trypsin, V8, and thermolysin was used to probe the structure of Ad2 E3-19K. Proteolysis was carried out at 20°C and aliquots were taken from the incubation mixtures after 4 hours followed by analysis of samples on a Tris-tricine SDS-PAGE gel (Fig. 1A). Note that the glycosylated nature of E3-19K (lane C) cause the protein to migrate as three closely spaced bands on the SDS-PAGE gel as demonstrated previously from a digestion of the protein with PNGase F (Liu et al., 2005). Results show that all enzymes hydrolyzed E3-19K to different extent, except for elastase (lane 2) to which E3-19K was resistant. The hydrolysis of E3-19K by chymotrypsin was further investigated to gain insights into the kinetics of the reaction (Fig. 1B). Results show that cleavage occurred rapidly upon incubation with the enzyme and the two major fragments generated in solution were stable over time.

The identity of the chymotryptic fragments generated after 24 hours (bands 1–4 in Fig. 1B) was determined from *N*-terminal amino acid sequencing and MALDI mass spectrometry analysis. Results showed that the large fragment (bands 1–3) corresponds to the N-terminus 1–88 residues of E3-19K (Fig. 1C) and the small fragment (band 4) corresponds to the C-terminus 94–100 residues (with six His residues) (Fig. 1C). Identical results were obtained from a characterization of the fragments generated after 0.3 hour (Fig. 1B). Thus, the large fragment comprises the entire variable region and several residues from the conserved region, while the small fragment consists of residues from the C-terminus of the conserved region.

Overall, the use of chymotrypsin as a structural probe pinpointed two critical proteolytic sites, Tyr⁸⁸ and Tyr⁹³, in the conserved region of E3-19K. The nature of these cleavage sites is consistent with the specificity of chymotrypsin to hydrolyze at the C-terminus of Tyr. These results suggest that the regions around Tyr⁸⁸ and Tyr⁹³ are readily accessible in E3-19K.

3.2. Chymotryptic fragments of Ad2 E3-19K do not associate with HLA-A*1101

We next examined whether the large and small chymotryptic fragments of E3-19K remain associated together in solution as this would provide valuable insights into their structural interplay in E3-19K. For this, the chymotryptic mixture (lane 1 in Fig. 2A) was concentrated in a centricon-3 (Amicon) and after numerous washes, the retentate and eluate fractions were analyzed individually on SDS-PAGE gel (lanes 2 and 3, respectively). Results show that the retentate fraction (lane 2) contained only the large fragment, while the eluate fraction (lane 3) contained only the small fragment. The two proteolytic fragments of E3-19K are therefore dissociated in solution.

In previous studies, using a native gel band-shift assay, we showed that the incubation of Ad2 E3-19K with HLA-A*1101 (Fig. 2B) yielded a new band on the gel that corresponds to the E3-19K/HLA-A*1101 complex (Liu et al., 2005). Here, we examined whether the small and large chymotryptic fragments also have the ability to associate with HLA-A*1101. Results show that incubation of the E3-19K digest with HLA-A*1101 failed to generate a new band on the native gel. Instead, bands corresponding to uncomplexed large N-terminal fragment of E3-19K and HLA-A*1101 were observed. The band corresponding to uncomplexed HLA-A*1101 was further analyzed by *N*-terminal amino acid sequencing and results convincingly confirmed the absence of the small C-terminal fragment. Thus, neither the 1–88 fragment nor the 94–100 fragment associates with HLA-A*1101. It is noteworthy that although the N-terminal 1–88 fragment is well behaved in solution (based on characterization by gel filtration chromatography and native gel) and comprises ~90% of the ER-luminal domain of E3-19K, it failed to associate with HLA-A*1101. This suggests that

residues from the conserved region that are currently missing in the 1–88 fragment, are absolutely critical for the MHC I binding function of E3-19K.

3.3. A key role for residues 89 to 93 in the conserved region of Ad2 E3-19K

It is possible that residues 89 to 93 (M⁸⁹SKQY⁹³), which are neither part of the large nor the small chymotryptic fragment (Fig. 1C), are required for the proteolytic fragments to remain associated together in solution and/or associate with HLA-A*1101. To address this issue, and as a way to gain further structural insights into E3-19K, the proteolytic sites Tyr⁸⁸ and Tyr⁹³ were individually mutated. Because chymotrypsin almost never cleaves at the C-terminus of Asp, Glu, Gly, and Pro residues, we generated the E3-19K[Y88G] and E3-19K[Y93G] mutants. The treatment of E3-19K[Y88G] and E3-19K[Y93G] with chymotrypsin generated two fragments in solution (Fig. 3) that migrated similarly on the gel as those of E3-19K (lane 1 in Fig. 2A). The characterization of these fragments by N-terminal amino acid sequencing and MALDI mass spectrometry showed that the large fragment (bands 5–7) of E3-19K[Y88G] corresponds to the N-terminal 1–87 residues (Fig. 1C) and the smaller fragment (band 8) corresponds to the C-terminal 94–100 residues (with six His residues) (Fig. 1C). Thus, surprisingly, chymotrypsin hydrolyzed the C-terminus of Met⁸⁷ in E3-19K[Y88G], generating the 1–87 fragment instead of the expected 1–93 fragment (dashed line in Fig. 1C). In contrast, the large fragment (bands 9–11) of E3-19K[Y93G] was identified as the expected N-terminal 1–88 residues (Fig. 1C) and the small fragment (band 12) as the C-terminal 89–100 residues (with six His residues) (Fig. 1C). Importantly, although the proteolytic fragments of E3-19K[Y93G] form a contiguous sequence (Fig. 1C), they were dissociated in solution and failed to gel-shift HLA-A*1101 (data not shown).

Overall, these results strongly suggest that the stretch of residues M⁸⁹SKQY⁹³ in the conserved region is critical for the MHC I binding function of E3-19K; the folding of these residues into their native conformation is key for this effect to be manifested.

3.4. The N-terminal 93 residues of Ad2 E3-19K are sufficient for high-affinity binding to HLA-A*1101

Because the chymotryptic treatment of E3-19K[Y88G] failed to generate the N-terminal 1–93 fragment in solution (dashed line in Fig. 1C), we engineered a baculovirus system for expression of E3-19K(1–93). E3-19K(1–93) was expressed in reasonably high yields in High Five cells and was well-behaved in solution. Notably, E3-19K(1–93) gel shifted HLA-A*1101 efficiently as evidenced by the presence of a new band on native gel (Fig. 4A) and the disappearance of the band corresponding to uncomplexed HLA-A*1101. This is in contrast to the 1–88 fragment which failed to associate with HLA-A*1101 (Fig. 2B).

These results were extended by a quantitative analysis of interaction between E3-19K(1–93) and HLA-A*1101 by SPR. Optimal fittings of the sensorgrams yielded a kinetics dissociation constant, $K_{d,k}$, of 15.9 ± 0.2 nM (Fig. 4B). Binding responses measured at equilibrium were plotted against concentrations of E3-19K(1–93) and the optimal fit of the curve yielded an equilibrium dissociation constant of 16.2 ± 0.3 nM. Thus, both the kinetics and equilibrium dissociation constants are in excellent agreement. In previous studies, we showed by SPR that the avidity of Ad2 E3-19K for HLA-A*1101 is 14.5 ± 2.1 nM (kinetics) (Liu et al., 2007), a value very similar to that determined here for the N-terminal 93 residues. The high binding affinity of E3-19K(1–93) for HLA-A*1101 provides further evidence that residues 89 to 93 are critical for this association.

3.5. Residue Tyr⁹³ in the conserved region Ad2 E3-19K is a key determinant of its immunomodulatory function

We next characterized the binding properties of E3-19K[Y88G] and E3-19K[Y93G] towards HLA-A*1101 using a native gel band-shift assay (Fig. 5A). The incubation mixture of E3-19K[Y88G] with HLA-A*1101 yielded a new band on the gel that migrates at a different position from uncomplexed proteins. Identical results were obtained with other HLA alleles (HLA-A*0301, -A*3301, and -Aw*6801) of the HLA-A3 superfamily. In marked contrast, E3-19K[Y93G] failed to gel-shift HLA-A3-like molecules as indicated by the lack of new bands at the expected position for complexes and the presence of strong bands corresponding to uncomplexed MHC class I molecules. Thus, this study identifies Tyr⁹³ as a key residue in enabling E3-19K to associate with MHC class I molecules.

A quantitative analysis of interaction between E3-19K[Y88G] and E3-19K[Y93G] and HLA-A*1101 was carried out by SPR (Fig. 5B). Optimal fittings of the sensorgrams yielded kinetics and equilibrium dissociation constants, K_d , of 36.4 ± 0.1 nM and 39.8 ± 0.2 nM, respectively, for E3-19K[Y88G]. However, no reliable K_d value could be determined for interaction between E3-19K[Y93G] and HLA-A*1101 due to the weak (and thus more unspecific) binding responses. Therefore, results from SPR are consistent with those from native gel and further demonstrate the important contribution of Tyr⁹³ to the MHC I binding function of E3-19K.

To confirm that our *in vitro* binding data translate into cells, HEK 293 cells stably expressing E3-19K, E3-19K[Y88G], and E3-19K[Y93G] were analyzed by flow cytometry (Fig. 5C). Analysis of the data show that, relative to untransfected HEK 293 cells (100%), the levels of MHC I cell-surface expression on E3-19K, E3-19K[Y88G] and E3-19K[Y93G] transfectants were ~32%, 53%, and 116%, respectively. Note that similar levels of expression for E3-19K, E3-19K[Y88G], and E3-19K[Y93G] in the transfectants were determined from immunoblots (Fig. 5C). The results thus demonstrate that MHC class I molecules are more readily expressed on cells stably expressing E3-19K[Y93G] relative to cells expressing E3-19K[Y88G]. This is consistent with the enhanced ability of E3-19K[Y88G] to form complexes with HLA-A3-like molecules as determined by native gel and SPR.

4. Discussion

In this report, we presented novel biochemical and structural results on E3-19K in an effort to better understand the properties that enable it to target MHC class I molecules for retention in the ER.

Limited proteolysis of E3-19K showed that the protein is readily cleaved at Tyr⁸⁸ and Tyr⁹³, generating two fragments in solution that are resistant to further hydrolysis. The large fragment was identified as residues 1 to 88, comprising the entire variable region and a few residues from the conserved region. The small fragment corresponded to residues 94 to 100 (with six His residues) from the conserved region. The susceptibility of Tyr⁸⁸ and Tyr⁹³ to cleavage by enzymes strongly suggests that the stretch of residues from 89 to 93 (M⁸⁹SKQY⁹³) possesses a flexible backbone as typically seen in linker or loop regions in proteins. Moreover, since neither the 1–88 fragment nor the 94–100 fragment of E3-19K associated with HLA-A*1101 (similar observations were made with the contiguous 1–88 and 89–100 chymotryptic fragments of E3-19K[Y93G]), we conclude that the putative linker or loop formed by residues 89 to 93 is a critical determinant for the MHC I binding function of E3-19K.

The ability of residues 1 to 93, but not residues 1 to 88, to form a high-affinity complex with HLA-A*1101 further highlighted the critical role of residues 89 to 93 in E3-19K. That this truncated form of E3-19K is biologically active implies that residues 89 to 93 adopt a native conformation in E3-19K(1–93). It is possible that Tyr⁹³, which we have shown to be critical for the immunomodulatory function of E3-19K, not only in binding assays but also in transfectants stably expressing E3-19K[Y93G], is responsible for maintaining residues 89 to 92 in a functional conformation. This could be achieved for example through intramolecular hydrogen bonds and/or hydrophobic interactions between Tyr⁹³ and residues from the variable and/or conserved region. We therefore suggest that Tyr⁹³, which is conserved in E3-19K proteins of all Ad serotypes (except for those of subgroup D where a His residue is found) acts as a structural determinant by maintaining residues 89 to 92 in a native conformation, whether as a linker or loop. Interestingly, it was reported previously that immunoprecipitates of MHC class I molecules from Ad-infected 293 cells co-precipitated Ad2 E3-19K but not Ad19a E3-19K of subgroup D (Deryckere and Burgert, 1996). This result demonstrates that Ad19a E3-19K has a weaker affinity relative to Ad2 E3-19K for MHC class I molecules, a property that could be contributed to (at least in part) by the Tyr-to-His substitution at residue 93. It is of note that an in-frame virus deletion mutant of Ad2 E3-19K lacking residues 92 to 100 was unable to bind to MHC class I molecules in spite of being correctly folded (Hermiston et al., 1993). This is consistent with our view that residues 89, 90, and 91 (which were part of this deletion mutant) are unable to support the MHC I binding function of E3-19K unless Tyr⁹³ is present, allowing these residues to adopt their functional conformation in E3-19K.

Several mutational studies have been carried out to identify residues that are critical to the function of E3-19K. It was demonstrated, for example, that Met⁸⁷ is important for the MHC I binding properties of Ad2 E3-19K (Sester et al., 2010). The equivalent Met¹¹⁰ in Ad35 E3-19K was also shown to severely reduce binding to MHC class I molecules when mutated to a Lys residue (Flomenberg et al., 1992). Considering these results, and because we showed that Met⁸⁷ is susceptible to hydrolysis by chymotrypsin in E3-19K[Y88G], it is therefore likely that the putative linker or loop region that we propose here includes residue Met⁸⁷ as well. It was also reported that a Lys¹¹⁴-to-Thr¹¹⁴ mutation in Ad35 E3-19K moderately inhibited MHC I binding while a Gln¹¹⁵-to-Glu¹¹⁵ had no effect on binding (equivalent to residues Lys⁹¹ and Gln⁹², respectively, in Ad2 E3-19K) (Flomenberg et al., 1992). It is not immediately clear why residues 114 and 115 are only weakly critical to Ad35 E3-19K function given that the equivalent residues 91 and 92 in Ad2 E3-19K are within the region 89 to 93 identified here. It is possible that the conservative nature of these mutations allowed Ad35 E3-19K mutants to maintain similar interactions (e.g., hydrogen bonds or hydrophobic contacts) with MHC residues. It is also possible that, in order to establish high-affinity complexes with a given MHC class I molecule, E3-19K proteins of different Ad serotypes mediate interactions with different MHC residues within the binding site, thus making it somewhat inaccurate to compare results from site-directed mutational analysis between serotypes.

Finally, it has been shown previously that residues 1 to 96 of Ad2 E3-19K can bind to MHC class I molecules (Gabathuler et al., 1990; Pääbo et al., 1986b). Here, we have extended these observations by demonstrating in a qualitative and quantitative manner that residues 1 to 93 are sufficient for high-affinity binding to HLA-A*1101. To the best of our knowledge, this is the shortest form of E3-19K capable of MHC I binding that has been reported so far. This raises interesting questions on the role of residues 94 to 100 in E3-19K. Our data suggest that, at least in the soluble form of E3-19K, these residues are dispensable for MHC I binding. However, it is possible that in the context of a lipid membrane, these residues serve to orientate the ER-luminal domain of E3-19K for optimal interaction with the ER-luminal domain of MHC class I molecules. Consistent with this view, Trp⁹⁶ was shown to

be important for MHC I binding in transfectants that stably express Ad2 E3-19K (Sester et al., 2010).

The E3-19K/MHC I association is thought to be a critical component of acute and persistent Ad infections. Thus, a delineation of the properties of Ad2 E3-19K that confer MHC I association and ER-retention as contributed here, is an important step to advance our understanding of the molecular basis of Ad pathogenesis. Similar studies aimed at characterizing the binding properties E3-19K proteins of different Ad serotypes and subgroups would help further extend our findings. It may also provide insights into variations in the ability of E3-19K proteins of different Ad serotypes to target MHC class I molecules and cause diseases in the human host.

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Figure 1.

Susceptibility of Ad2 E3-19K to proteases. (A) SDS-PAGE (16.5%) analysis of E3-19K incubated with chymotrypsin (lane 1), elastase (lane 2), pronase, (lane 3), trypsin (lane 4), V8 (lane 5), and thermolysin (lane 6). The digests were carried at 20°C in 20 mM Hepes, 150 mM NaCl (pH 7.5) at enzyme:substrate ratio of 1:100 (w/w) for 4 hours. The reactions were quenched with SDS gel loading buffer and heating samples at 100°C. (B) SDS-PAGE (16.5%) analysis of E3-19K incubated with chymotrypsin as described in (A). Aliquots were taken from the mixture at different times as indicated on top of the gel. In (A) and (B), lane C (control) represents E3-19K without added enzymes and loaded onto the gel at the same concentration as in other lanes. The gels were run in 0.1 M Tris, 0.1 M tricine (pH 8.3) and stained with Coomassie Blue R-250. (C) Stable chymotryptic fragments (bands 1 to 4 in Fig. 1B) are mapped within the domain organization of E3-19K (residues 1–100) (Flomenberg et al., 1992; Herminston et al., 1993); residues 1 to ~78/81 are rather variable between E3-19K of different subgroups and residues ~79/82 to 98 are rather conserved between E3-19K of different subgroups. Bands 5 to 12 correspond to the stable chymotryptic fragments of both E3-19K[Y88G] and E3-19K[Y93G] shown in Fig. 3. The expected large chymotryptic fragment from E3-19K[Y88G] is denoted by a dashed line. The disulfide bonds Cys²²/Cys⁸³ (open triangle) and Cys¹¹/Cys²⁸ (closed triangle), N-linked glycans Asn¹² and Asn⁶¹, and the (His)₆-tag sequence are labeled.

Figure 2.

Chymotryptic fragments of Ad2 E3-19K do not associate with HLA-A*1101. (A) SDS-PAGE (16.5%) analysis of fragments generated from the incubation of E3-19K with chymotrypsin. The quenched proteolytic mixture (lane 1) was concentrated in a centricon-3 and washed extensively with 20 mM Tris, 150 mM NaCl (pH 7.5) after which the retentate (lane 2) and eluate (lane 3) were loaded onto the gel. Lane C (control) is E3-19K without added chymotrypsin. The gel was run in 0.1 M Tris, 0.1 M tricine (pH 8.3). (B) E3-19K (14 μ g) and the E3-19K chymotryptic digest (14 μ g) were both incubated with HLA-A*1101 (20 μ g) (2:1 molar ratio) on ice in 20 mM Tris, 150 mM NaCl (pH 7.5) for 30 minutes followed by the addition of native gel loading buffer. The incubation mixtures were immediately loaded onto the native gel (10%) and run at 4°C in 25 mM Tris, 200 mM glycine (pH 8.4). Note that the pI of E3-19K is 8.85 and the pIs of the small and large fragments are 10.0 and 7.85, respectively; when pI values are above the pH of the running buffer (pH 8.4), proteins do not penetrate into the native gel (see Liu et al., 2005, for a native gel showing the well-behaved nature of Ad2 E3-19K).

Figure 3.

Site-directed mutational analysis of Tyr⁸⁸ and Tyr⁹³. SDS-PAGE (16.5%) analysis of fragments from the incubation of E3-19K[Y88G] and E3-19K[Y93G] with chymotrypsin. The digests were carried out as described in Fig. 1A. The gel was run in 0.1 M Tris, 0.1 M tricine (pH 8.3).

Figure 4.

Ad2 E3-19K(1–93) associates with HLA-A*1101. (A) Samples of E3-19K (14 μ g) and E3-19K(1–93) (11 μ g) were incubated with HLA-A*1101 (20 μ g) (2:1 molar ratio) as described in Fig. 2B. The incubation mixtures were loaded onto the native gel (10%) that was run as described in Fig. 2B. See legend of Fig. 2B for why E3-19K does not penetrate into the native gel; because the pI of E3-19K(1–93) is 8.36, it can penetrate slightly into the native gel. (B) Solutions of E3-19K(1–93) (2.50 to 150 nM) were injected through the control (immobilized biotin) and sample (immobilized HLA-A*1101) flow cells. Association and dissociation time courses were recorded with responses from the control cell being automatically subtracted. Solid lines through the sensorgrams were generated from globally fitting the data to a 1:1 Langmuir binding model, yielding a kinetics dissociation constant of 15.9 ± 0.2 nM.

Figure 5.

MHC residue Tyr⁹³ influences the immunomodulatory function of E3-19K. (A) Samples of E3-19K[Y88G] (14 μg) and E3-19K[Y93G] (14 μg) were incubated with HLA-A3-like molecules (20 μg) (2:1 molar ratio) as described in Fig. 2B. The controls and mixtures were loaded into the native gel (10%) that was run as described in Fig. 2B. The pIs of both mutants are 8.87, thus neither of these proteins penetrate into the native gel. (B) Solutions of E3-19K[Y88G] (2.50 to 250 nM) and E3-19K[Y93G] (400 to 2500 nM) were injected through the control (immobilized biotin) and sample (immobilized HLA-A*1101) flow cells. Association and dissociation time courses were recorded with responses from the control cell being automatically subtracted. Solid lines through the sensorgrams were generated from globally fitting the data to a 1:1 Langmuir binding model, yielding a kinetics dissociation constant of 36.4 ± 0.1 nM for E3-19K[Y88G]. No reliable K_d value could be determined for E3-19K[Y93G] due to weak (and thus more unspecific) binding responses. (C) The cell-surface expression of MHC class I molecules on human embryonic kidney (HEK) 293 cells (solid histogram) and on transfectants stably expressing E3-19K, E3-19K[Y88G], and E3-19K[Y93G] (solid histograms) was determined by flow cytometry after staining with the W6/32 MAbs. The incubation of HEK 293 cells and transfectants with FITC conjugated IgG alone (empty histogram) served as controls. The insert shows comparable levels of protein expression in all transfectants as determined by immunoblotting using the P33 antibody.