Crystal structure of adenovirus E3-19K bound to HLA-A2 reveals mechanism for immunomodulation

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E3-19K binds to and retains MHC class I molecules in the endoplasmic reticulum, suppressing anti-adenovirus activities of T cells. We determined the structure of the adenovirus serotype 2 (Ad2, species C) E3-19K–HLA-A2 complex to 1.95-Å resolution. Ad2 E3-19K binds to the N terminus of the HLA-A2 groove, contacting the α1, α2 and α3 domains and β1m. Ad2 E3-19K has a unique structure comprising a large N-terminal domain, formed by two partially overlapping β-sheets arranged in a V shape, and a C-terminal α-helix and tail. The structure reveals determinants in E3-19K and HLA-A2 that are important for complex formation; conservation of some of these determinants in E3-19K proteins of different species and MHC I molecules of different loci suggests a universal binding mode for all E3-19K proteins. Our structure is important for understanding the immunomodulatory function of E3-19K.

Adenoviruses are widespread in the human population, with at least 51 known serotypes (Ad1–Ad51) classified into six species (A–F)1. Adenoviruses cause a number of infections that are linked to respiratory, gastrointestinal and ocular diseases2. Although primary adenovirus infection elicits antiviral cytotoxic T lymphocyte (CTL) immune responses, these responses are usually insufficient to clear the virus. Consequently, adenoviruses cause lifelong asymptomatic infections in healthy individuals and can be fatal in children and immunocompromised patients.

Large DNA viruses such as adenoviruses have evolved strategies for evading antiviral immune responses3,4. It was shown in the late 1980s that the E3-19K protein of adenovirus binds to and retains MHC class I molecules in the endoplasmic reticulum (ER) of infected cells, preventing their egress to the cell surface5–7. As a consequence, adenovirus-infected cells are less susceptible to lysis by adenovirus-specific CTLs8–10. At that time, this represented the first example of a viral protein capable of interfering with MHC I antigen presentation. It was also shown that when the lungs of cotton rats are infected with wild-type adenovirus, immunopathological responses are less severe than when the lungs are infected with a mutant adenovirus containing a deletion of the E3-19K gene11. It was suggested that the absence of E3-19K in the mutant virus activated CTLs as part of the host inflammatory response to the virus11. It is thought that E3-19K, through its association with MHC I, enables adenoviruses to establish persistence in host cells4,11,12.

E3-19K is a type I transmembrane glycoprotein that comprises an N-terminal ER-luminal domain, a transmembrane domain and a C-terminal cytosolic tail. The ER-luminal domain of E3-19K associates with the ER-luminal domain of MHC class I molecules8,13–16, whereas the dilysine motif in the cytosolic tail of E3-19K provides the signal for localization of the E3-19K–MHC I complex in the ER8,17,18. An analysis of immunoprecipitates from Ad2-, Ad5- and Ad19-infected mammalian cells suggested that E3-19K displays differential affinity with MHC I19–21. Using recombinant, soluble E3-19K proteins of species B, C, D and E and HLA-A, HLA-B and HLA-C molecules, we showed that E3-19K proteins associate with MHC I in a locus-specific manner22,23; E3-19K associates with HLA-A and HLA-B but displays essentially no affinity for HLA-C. We also showed that E3-19K proteins of species B, C and E associate with HLA-A and HLA-B in an HLA allele-dependent manner22,23.

The ER-luminal domain of E3-19K has been subdivided into three regions24,25; (i) residues 1 to ~78–81 are rather variable between E3-19K proteins of different species; (ii) residues ~79–82 to ~98 are rather conserved between E3-19K proteins of different species; and (iii) residues ~99 to 107 act as a linker between the ER-luminal domain and the transmembrane domain. Secondary-structure predictions of Ad2 E3-19K have provided some insights into potential architectural elements26–28. Structure-function relationship studies have identified specific residues in both the variable and conserved regions of E3-19K that are critical for its MHC I–binding function22,23,25,27,29. Evidence from several studies has suggested that E3-19K binds to the peptide-binding groove of MHC I13,14,22,23. We proposed recently a model of interaction in which E3-19K has contact sites on MHC I at the N-terminal end of the α1 helix and at the C-terminal end of the α2 helix23. To date, more than 20 years after the discovery of E3-19K as the first viral immunomodulatory protein, lack of knowledge of its three-dimensional structure when alone or in complex with MHC I severely limits understanding of its immunomodulatory function. Progress has been hampered largely by the fact that E3-19K is a protein recalcitrant to crystallization.

We set out to determine the X-ray three-dimensional structure of soluble recombinant Ad2 E3-19K bound to HLA-A2–Tax peptide. We describe a new rescue refolding strategy that allowed formation of Ad2 E3-19K–HLA-A2, from which we generated crystals...
RESULTS
Structure of the Ad2 E3-19K–HLA-A2 complex
The ER-lumenal domain of Ad2 E3-19K has two N-linked glycans (Asn12 and Asn61)\(^3\). In previous studies, we showed that treatment of Ad2 E3-19K with the glycan-cleaving enzymes PNGase F and Endo H\(^1\) leads to precipitation of Ad2 E3-19K. This suggested an important role for the glycans in the stabilization of Ad2 E3-19K. When Ad2 E3-19K is bound to HLA-A2–Tax, it can more easily withstand hydrolysis of its glycans; crystallization trials of Ad2 E3-19K–HLA-A2 treated with PNGase F and Endo H failed to produce crystals (data not shown).

The expression of Ad2 E3-19K in *Escherichia coli* led to the production of inclusion bodies from which we were unable to refold E3-19K (discussed in ref. 31). In marked contrast, the addition of submolar amounts of folded HLA-A2–Tax in the oxidative refolding buffer containing urea-solubilized inclusion bodies of Ad2 E3-19K generated the Ad2 E3-19K–HLA-A2 complex in solution. Most likely, upon refolding, Ad2 E3-19K can bind immediately to HLA-A2 and form a stable Ad2 E3-19K–HLA-A2 complex before it can aggregate.

Using this strategy, we determined the structure of the complex between the ER-luminal domains of Ad2 E3-19K and HLA-A2–Tax to 1.95-Å resolution by molecular replacement (Online Methods and Table 1). Only minor differences were observed between the two molecules of E3-19K in the asymmetric unit (r.m.s. deviation of 0.52 Å for 97 equivalent Cα atoms), so only chain G will be discussed further. Clear electron density was observed for nearly all residues of Ad2 E3-19K–HLA-A2; residues 2–100 of Ad2 E3-19K, residues 1–275 of HLA-A2 heavy chain, residues 0–99 of β₂-microglobulin (β₂m) and the HTLV-1 Tax peptide (LLFGYPVYV). The overall structure of the Ad2 E3-19K–HLA-A2 complex is shown in Figure 1a.

The structure of Ad2 E3-19K contains a large N-terminal domain that forms two antiparallel β-sheets defined by β-strands A, B and E and β-strands C, C’, F and G (Fig. 1b). The C terminus of this domain is connected to an α-helix and a tail (Fig. 1b). The two β-sheets of the N-terminal domain overlap with each other only partially, with an angle of approximately 30° between each other, such that overall this domain resembles a V-shaped structure. The apex of the V shape is formed by contact between β-strands C’ and E, and the opening is defined by β-strands A and G. This unusual relative arrangement of β-sheets confines the hydrophobic core to the deepest part of the V shape. The hydrophobic core comprises residues of β-strands B (Cys22, Thr24,

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Table 1 Data collection and refinement statistics

| Data collection | Ad2 E3-19K–HLA-A2 complex |
|-----------------|---------------------------|
| Space group     | I212121                   |
| Cell dimensions | a, b, c (Å) 113.19, 133.67, 196.72 |
| Resolution (Å) | 50.00–1.95 (2.02–1.95)b |
| Rmerge          | 0.148 (0.540)             |
| I / σ(I)        | 15.97 (3.20)              |
| Completeness (%)| 99.7 (97.4)               |
| Redundancy      | 11.0 (7.1)                |
| Refinement      |                           |
| Resolution (Å) | 37.06–1.95                |
| No. reflections | 102,933                   |
| Rwork / Rfree  | 0.210 / 0.241             |
| No. atoms       |                           |
| Protein         | 7,968                     |
| Water           | 472                       |
| β factors       |                           |
| Protein         | 35.6                      |
| Water           | 34.4                      |
| R.m.s. deviations |                           |
| Bond lengths (Å) | 0.006                    |
| Bond angles (°) | 1.3                      |

a Data were collected from a single crystal. bValues in parentheses are for highest-resolution shell.

diffacting to 1.95-Å resolution. Our structure offers precise explanations for the mechanism by which E3-19K modulates antiviral cellular immunity.
Ile26 and Cys28) and E (Val48 and Ala50) of the ABE β-sheet and β-strands C (Leu35, Ile37 and His39), C’ (Gly44) and F (Val62 and Val64) of the CC’FG β-sheet (Fig. 1c); only one residue of the edge β-strand A (Val13), and none of the edge β-strand G, is part of the hydrophobic core. Other residues including Pro9 and Trp52 of the large N-terminal domain, Phe79 and Met87 of the α- helix and Trp96 of the tail also participate in forming the hydrophobic core (Fig. 1c).

In addition to those within the hydrophobic core, a number of interactions keep the α-helix packed against the N-terminal domain. These include the disulfide bond between Cys83 and Cys22 and also several mostly water-mediated (water G271) hydrogen bonds between Ser90 (α-helix) and Ser17 (β-strand A) and Asn20 (AB loop, the loop connecting β-strands A and B). The well-ordered electron density of the N-terminal residues Lys2–Pro9 reveals that the backbone oxygen atoms of these residues participate in forming the hydrophobic core (distance ≤4.0 Å) and salt bridges are indicated by dashed green lines; hydrophobic contacts (distance <4.0 Å) are represented by dashed magenta lines. Water molecules are shown as red spheres. The backbone oxygen atoms of these residues.

Features of Ad2 E3-19K, consistent with results from mutational analysis of cysteine residues in Ad2 E3-19K32.

The coordinates of Ad2 E3-19K were submitted to the DALI server33 to establish how E3-19K might be related to other proteins of known structure in the Protein Data Bank (PDB). The search revealed a relatively low degree of structural similarity with gp130. The closest structural relative is D2 of D2D3 gp130 (PDB 1PVH, r.m.s. deviation of 3.4 Å), with a Z score of 6.6. The next two best matches are D1 of D1D2 gp130 (PDB 1BQU, r.m.s. deviation of 3.5 Å) and D2 of D1D2D3 gp130 (PDB 111R, r.m.s. deviation of 3.7 Å), with Z scores of 6.5 and 6.4, respectively. These domains adopt fibronectin type III folds. The superposition of these gp130-based structures onto the Ad2 E3-19K structure revealed that similarities are limited to one or two β-strands of the CC’FG β-sheet; no protein could be identified that aligned well with E3-19K in its entirety. No closely related structures were also found with the SSM server34. We conclude that the E3-19K protein has a unique tertiary structure.

The E3-19K–HLA-A2 binding interface
Ad2 E3-19K binds to the N terminus of the α1-helix on HLA-A2, also contacting the C terminus of the α2 helix and prominent loops of the α3 domain and βm (Fig. 2). The E3-19K–HLA-A2 binding interface shields a molecular surface area of 1,929 Å² from solvent. The shape-complementarity coefficient, S_c, of the complex was determined to be 0.68 (S_c = 1 for perfect geometrical fits35).

Each interaction site (referred to as sites 1, 2, 3 and 4) is discussed separately (Fig. 2). Site 1 is located at the N terminus of the α1 helix on HLA-A2 and contributes 36% to the total buried surface area.

Figure 2 Interaction surface between Ad2 E3-19K and HLA-A2. Interaction sites 1, 2, 3 and 4 of Ad2 E3-19K–HLA-A2 are shown in separate panels, using the same color code as in Figure 1a. Nitrogen, oxygen and sulfur atoms are colored blue, red and light orange, respectively. Hydrogen bonds (distance ≤3.5 Å) and salt bridges are indicated by dashed green lines; hydrophobic contacts (distance <4.0 Å) are represented by dashed magenta lines. Water molecules are shown as red spheres. The backbone oxygen atoms of these residues.
The site features a salt bridge between Lys27 (β-strand B) of E3-19K and Glu53 of HLA-A2 as well as hydrogen bond interactions involving Lys16 and Tyr49 (β-strands A and E, respectively) of E3-19K and Arg48 and Gln54 of HLA-A2, respectively. These interactions are supplemented by a number of solvent-mediated hydrogen bonds and hydrophobic contacts. Site 2 is located at the C terminus of the α2 helix on HLA-A2 and occupies 16% of the total buried surface area. The key interaction at site 2 is a salt bridge between Lys42 (β-strand C′) of E3-19K and Glu177 of HLA-A2. Site 3 involves prominent loops of E3-19K and the α3 domain of HLA-A2 and contributes 17% of the total buried surface area. The interface at that site is defined by a number of mostly water-mediated hydrogen bonds and hydrophobic contacts. Site 4 involves the α-helix of E3-19K and β2m and represents 31% of the total buried surface area. Interactions at site 4 include mostly hydrogen bonds involving Gln92 and Tyr93). Notably, sequence alignment reveals strict conservation of the WPP motif (residues 96–98) (Supplementary Fig. 1). Trp96 is part of the hydrophobic core (Fig. 1c compared to Fig. 4) and participates in a network of solvent-mediated hydrogen bonds, as mentioned above. This effect, together with the exceptional conformational rigidity of proline residues, suggests that the WPP motif has an important structural role.

Conserved MHC I residues at the binding interface
A sequence alignment of consensus HLA-A, HLA-B and HLA-C heavy chains at each of the three interaction sites on the HLA-A2 heavy chain is shown in Figure 5. The analysis shows that Ad2 E3-19K binds to conserved residues of the HLA-A2 heavy chain at sites 1 and 2 but to polymorphic residues at site 3. Site 4, which involves β2m, is obviously strictly conserved in HLA molecules. Overall, our structure shows that Ad2 E3-19K interacts largely with conserved residues of HLA-A2.
DISCUSSION

It was previously speculated that the E3-19K protein resembles an immunoglobulin-like domain; however, our structure shows that this is not the case. Structural-alignment searches against the DALI and SSM servers did not identify any structures that aligned well with Ad2 E3-19K in its entirety, which indicated a unique tertiary structure.

E3-19K proteins share relatively low levels of sequence homology in their N-terminal 100 residues (Supplementary Fig. 1). In spite of this, several residues in this region that are part of the hydrophobic core are strictly or highly conserved (Supplementary Fig. 1). This includes residue Trp96, which together with Pro97 and Pro98 likely provides conformational rigidity to the tail region of Ad2 E3-19K. Such rigidity of the tail should restrict conformational freedom of the membrane-anchored ER-luminal domain. The strictly conserved WPP motif may thus be important for correctly aligning E3-19K onto MHC I. It is of note that a mutation of Trp96 in Ad2 E3-19K decreased interaction with MHC I. Thus, it is clear that the conservation of key structural determinants in E3-19K proteins reflects a strong evolutionary pressure to maintain a function-specific tertiary structure, which is consistent with our knowledge that all E3-19K proteins display an MHC I-binding function.

The characterization of interaction in E3-19K–MHC I pairs is important because of the role that this association has in adenovirus pathogenesis. We suggest that Ad2 E3-19K residues Lys27 (site 1), which is strictly conserved (Supplementary Fig. 1), and Lys42 (site 2), which is conserved by charge, are key determinants of the MHC I-binding function of E3-19K proteins. We showed previously the importance of the electrostatic interaction mediated by Lys42 from a mutation of E177K in HLA-A*1101, which abolished interaction with E3-19K of species B, C, and D. Furthermore, we suggest that the highly conserved Ad2 E3-19K residues Glu92 and Tyr93 (site 4) are important determinants of interaction in E3-19K–MHC I pairs. Consistent with this, we showed that a Y93G mutation in Ad2 E3-19K abolished interaction with HLA-A molecules. We and others have also shown that mutation of noncontact Ad2 E3-19K residues such as Met87 and Lys91 (site 4) destabilized Ad2 E3-19K–MHC I interaction. Because the shape complementarity is high at site 4 (S4 = 0.81), any mutations that affect the geometrical fit between the α-helix of E3-19K and β3m is expected to disrupt the interconnected network of interactions at that site (Fig. 2). We have evidence in support of this view from studies in which we showed that for a given MHC class I molecule, Ad37 E3-19K of species D displays the weakest binding affinities relative to those of species B, C, and D. E3-19K proteins of species D are not only most divergent at site 4 (Supplementary Fig. 1) but also carry nonconserved substitutions at each of the three contact residues: M89V, Q92L, and Y93H. Taken together, this analysis suggests that the binding mode of Ad2 E3-19K onto HLA-A2 is likely to represent a universal mode of interaction for all E3-19K proteins. Finally, site 3 is a region of the Ad2 E3-19K–HLA-A2 interface where the consensus sequences of HLA heavy chains are most divergent (Fig. 5), and it also lacks the kind of key interaction involving conserved residues that are present at the other three sites. As such, interactions at site 3 are expected to be responsible for the variability in binding affinities that E3-19K proteins display for MHC I.

There are many particular features of the Ad2 E3-19K–HLA-A2 structure (Fig. 1a) that exemplify the fine-tuned nature of the E3-19K immunomodulatory function. First, E3-19K ensures ‘cross-reactivity’ with MHC I by binding largely to conserved MHC residues. Second, E3-19K has evolved to circumvent peptide specificity by binding away from the groove. Third, and importantly, E3-19K displays high-affinity interaction with MHC I. Fourth, Ad2 E3-19K has evolved an elongated structure that permits contacts over the entire length of HLA-A2, including the heavy chain and β2m. In this regard, we showed that the first N-terminal 93 residues of Ad2 E3-19K are sufficient to maintain high-affinity interaction with HLA-A*1101 (ref. 29), which is in perfect agreement with our structure. Finally, E3-19K binds to the N terminus, rather than the C terminus, of the groove. The C terminus of the groove is thought to represent the binding region for the class I assembly proteins onto immature MHC I. Thus, in targeting the N terminus of the groove, E3-19K likely ensures that it can maintain an association with both immature (peptide-free) and mature (peptide-filled) forms of MHC I. In support of this, we showed that Ad2 E3-19K binds to both peptide-free and peptide-filled HLA-A*1101 (ref. 31).

The conformationally sensitive monoclonal antibody (mAb) 64-3-7, which is specific for ‘open’ MHC I, was shown to bind to a region of MHC I comprising Arg48 and Pro50 (site 1 in Fig. 2). Because 64-3-7 can distinguish between open and ‘closed’ MHC I, it was suggested that the region of the groove to which 64-3-7 binds is folded differently in these two forms of MHC I. In the context of our structure, this is entirely consistent with our findings that Ad2 E3-19K displays differential binding affinities for peptide-free (open) and peptide-filled (closed) HLA-A*1101 (ref. 31). It is also consistent with a previous report showing that E3-19K cannot be co-immunoprecipitated with 64-3-7 (ref. 38). Taken together, this evidence suggests that the stretch of MHC residues between Arg48 to Glu58 is likely to undergo major peptide-induced conformational changes, reinforcing the notion that peptide-induced conformational changes occur at the N terminus of the groove.47,39

We showed previously that E3-19K proteins bind to HLA-A and HLA-B molecules but show no avidity for HLA-C molecules. Other immunomodulatory proteins of large DNA viruses display the same locus specificity. An analysis of interaction between Ad2 E3-19K and HLA-A2 at site 3 (Fig. 2), in the context of the crystal structure of free HLA-Cw*0403 (PDB 1QQD), suggests that residues Glu183 and Gly207 in HLA-Cw*0403 cannot mediate interactions shown at site 3. We therefore suggest that these two residues have a role in the inability of E3-19K to bind HLA-C. Notably, we have new data showing that Ad2 E3-19K and Ad4 E3-19K are unable to associate with HLA-E (data not shown). HLA-E carries Glu183 and Gly207, and in the context of the structure of free HLA-E*0103 (PDB 3BZE), both of these residues also cannot mediate interactions shown at site 3. This lends further support to the idea that MHC residues Glu183 and Gly207 modulate the inability of E3-19K to associate with HLA-C and HLA-E.

Because of the effects that E3-19K has on suppressing the expression of the MHC I homologs MICA and MICB on adenovirus-infected cells, it is presumed that E3-19K binds to MICA and MICB and that E3-19K residues Thr14 and Met82 play a part in this interaction.27 We have mapped the position of the side chains of Thr14 and Met82 in the structure of Ad2 E3-19K (Fig. 4). The implication of this analysis is that E3-19K likely uses a different surface to sequester MICA and MICB from its MHC I binding surface (Fig. 4). Furthermore, that mutation of Thr14 and Met82 in Ad2 E3-19K had essentially no effect on the MHC I binding function of E3-19K is entirely consistent with our structure (Figs. 1 and 4).

In conclusion, the structure of Ad2 E3-19K–HLA-A2 represents an example of coevolution between a viral immunomodulatory protein and its host MHC I ligand. Our studies provide structural explanations for how E3-19K achieves promiscuous and high-affinity binding to HLA-A and HLA-B and give insights into its inability to engage with HLA-C and HLA-E. Notably, a comparison of our structure with that of
of US2 immunomodulatory protein from human cytomegalovirus bound to HLA-A2 (ref. 44) shows that both proteins have common contact residues on HLA-A2 at site 3 (Fig. 2). This has implications for understanding the molecular basis of co-infection of the same host cell by two different persistent viruses.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The atomic coordinates and structure factors of the Ad2 E3–19K–HLA-A2 complex have been deposited in the PDB with ID code 4E5X.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.L., crystallographic and biochemical studies and manuscript preparation; Y.M., biochemical studies; M.B., project supervisor and manuscript preparation and principal manuscript author.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cloning and protein expression. The cDNA encoding the ER-luminal domain (residues 1–100) of Ad2 E3-19K (referred to as E3-19K) was generated by polymerase chain reaction. This was carried out using the plasmid pBS-Ad2E3-19K (gift from H.-G. Burgert, University of Wisconsin, Coventry, UK) containing the cDNA of full-length Ad2 E3-19K as a template and the appropriate forward and reverse primers. The amplified cDNA was ligated into the PLM1 vector (gift from D.C. Wiley, deceased) by using the BamHI and EcoRI restriction sites. Ad2 E3-19K, HLA-A2 heavy chain (residues 1–275) and β2m (residues 0–99) were expressed in the Escherichia coli strain BL21(DE3)pLysS (Stratagene) as inclusion bodies. Inclusion bodies were isolated, purified from cell pellets and solubilized as described previously46.

Assembly of HLA-A2–Tax and Ad2 E3-19K–HLA-A2. HLA-A2–Tax was reconstituted in vitro from the urea-solubilized inclusion bodies of HLA-A2 heavy chain (1 µM) and β2m (2 µM) in the presence of the Tax peptide (LLFGYPVYV) (10 µM) in an oxidative refolding buffer46. Stock solutions of purified HLA-A2–Tax (10–30 mg ml–1) in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl were kept at –80 °C. The Ad2 E3-19K–HLA-A2 complex was assembled by using a modified version of this approach. The urea-solubilized inclusion bodies of Ad2 E3-19K were added at 4 °C, under rapid stirring, to a final concentration of 1 µM to an oxidative refolding buffer consisting of 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 400 mM l-arginine, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 1 µM phenylmethylsulfonyl fluoride and submolar amounts (0.2 µM) of refolded, purified HLA-A2–Tax. The presence of refolded HLA-A2–Tax in the buffer of Ad2 E3-19K was absolutely essential for formation of the Ad2 E3-19K–HLA-A2 complex. The refolding mixture was incubated at 4 °C. After 24 h, the mixture was concentrated in an Amicon stirred cell and then purified on a Superdex 200 HR 10-30 column in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. The purified Ad2 E3-19K–HLA-A2 complex generated from this rescue refolding strategy was characterized by SDS-PAGE and native PAGE. Stock solutions of purified Ad2 E3-19K–HLA-A2 (20–40 mg ml–1) were kept at –80 °C. The rescue refolding strategy described here is applicable to other complexes involving recalcitrant proteins such as Ad2 E3-19K.

Crystallization and data collection. Crystallization conditions of the Ad2 E3-19K–HLA-A2 complex were searched with the aid of a Tecan Freedom EVO 200 robot using sitting drops. Complex solution (10 µl, 10 mg ml–1) was dispensed into each well of a Corning 96-well plate, mixed with an identical volume of Crystal Screen Index (Hampton Research) as solution no. 22, 0.8 M sucrose, 0.1 M sodium citric acid, pH 7.0. Optimization of the crystallization condition was carried out using the Tecan Freedom EVO 200 HR 10-30 column in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. The purified Ad2 E3-19K–HLA-A2 complex generated from this rescue refolding strategy was characterized by SDS-PAGE and native PAGE. Stock solutions of purified HLA-A2–Tax. The presence of refolded HLA-A2–Tax in the buffer of Ad2 E3-19K was absolutely essential for formation of the Ad2 E3-19K–HLA-A2 complex. The refolding mixture was incubated at 4 °C. After 24 h, the mixture was concentrated in an Amicon stirred cell and then purified on a Superdex 200 HR 10-30 column in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. The purified Ad2 E3-19K–HLA-A2 complex generated from this rescue refolding strategy was characterized by SDS-PAGE and native PAGE. Stock solutions of purified Ad2 E3-19K–HLA-A2 (20–40 mg ml–1) were kept at –80 °C. The rescue refolding strategy described here is applicable to other complexes involving recalcitrant proteins such as Ad2 E3-19K.

Structure determination and refinement. The structure of Ad2 E3-19K–HLA-A2 complex was determined by molecular replacement in AMoRe48 by using free HLA-A2–Tax (PDB 1HHK) as a search model. Two solutions were identified for both the rotation and translation functions, corresponding to two molecules of Ad2 E3-19K–HLA-A2 in the asymmetric unit. After molecular replacement, the electron density maps were calculated, initial refinements were examined manually and residues with a poor fit to the electron density map were omitted from the model. The electron density improved with use of the DM program from the CCP4 package49. Subsequent model rebuilding was carried out in Coot50. The extended model was then refined using simulated annealing, energy minimization and restrained individual B factor in CNS51. In the first refinement cycles, noncrystallographic symmetry (NCS) restraints were applied to the main chain atoms (loops excluded), using data to 3.0 Å, resulting in Rfree and Rwork values of 0.43 and 0.39, respectively. In the final refinement cycles, NCS restraints were released, and a composite omit map was calculated to eliminate model bias. Water molecules were added by using the CNS program51 and were checked with the Fc – Fs electron density map (>3σ) at 3.5 Å and less from hydrogen bond donors or acceptors. A total of 472 water molecules were added to the model. Throughout refinement, agreement between the model and the observed data was monitored by calculating Rwork on the basis of 10% of the reflections. The final Rwork and Rfree values (with a bulk solvent correction) are 21.0% and 24.1%, respectively, for all reflections between 37.06 and 1.95 Å (Table 1). In the final model, the structural geometry was checked by using PROCHECK52. All backbone φ–ω torsion angles of the model were within allowed regions of the Ramachandran plot. Because of poor quality or missing electron density, the final model lacks residue 1 of chain G and residues 1, 99 and 100 of chain H. Buried surface areas were calculated with the program ArealMol in the CCP4 package, using a probe radius of 1.4 Å. All structural figures were prepared using PyMOL (http://www.pymol.org)53.

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