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The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition

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The E3/19K protein of human adenovirus type 2 binds to HLA class I antigens and blocks their terminal glycosylation and cell surface expression. The nature of this interaction is non-covalent and involves neither disulfide bridges between the two molecules nor their carbohydrates. The murine H-2 K^d antigen associates with the E3/19K protein in a similar fashion to human HLA antigens whereas the allelic product H-2 K^k does not. Hybrid genes between the K^d and K^k alleles were constructed and their products were expressed in embryonic kidney cells together with the E3/19K protein. This allowed us to identify the α1 and α2 domains as the essential structures of the histocompatibility antigens for binding the viral protein. Interestingly, these domains are also crucial for T cell recognition. The implications for the evolution of adenoviruses and their ability to cause persistent infections are discussed.

Key words: cellular immune interference/E3-19K of Ad-2/histocompatibility–viral protein interaction/intracellular transport/oligomeric assembly

Introduction

The human adenoviruses comprise a group of viruses with more than 40 different subtypes. Normally, these viruses cause a mild respiratory infection but for some serotypes, of which adenoviruses 2 and 5 (Ad-2 and Ad-5) are the best characterized, a latent and/or persistent infection may develop (Straus, 1984). The genome of Ad-2 contains a double-stranded DNA molecule of ~36 000 bp. Most of the genes for the 40–50 encoded proteins have been mapped (Flint, 1980). For several of these proteins the function(s) have been established by using adenovirus mutants (for reviews see Shenk and Williams, 1984; Ginsberg, 1984).

The infection cycle can be divided into an early and a late phase, which are defined by the onset of viral transcription and subsequent DNA replication (Persson and Philipson, 1982). One of the early regions of Ad-2, the E3, is located between map units 75.9 and 86.0 (Berk and Sharp, 1978; Chow *et al.*, 1979). The transcription and splicing of this region is complex and several mRNAs are produced (Bhat and Wold, 1986). One of the most abundant proteins encoded by these mRNAs is the E3/19K (Ross and Levine, 1979; Persson *et al.*, 1980b). This protein contains 159 amino acids and has the characteristics of a membrane protein (Persson *et al.*, 1980a; Ahmed *et al.*, 1982). It has a signal sequence of 17 amino acids which is cleaved after translocation through the rough endoplasmic reticulum (rER) (Persson *et al.*, 1980a; Wold *et al.*, 1985). Two carbohydrate groups are attached to asparagines at positions 11 and 63 (Persson *et al.*, 1980b; Kornfeld and Wold, 1981; Wold *et al.*, 1985). A putative membrane-spanning segment is present 15–40 amino acids from the carboxy terminus. It has been suggested that the E3/19K protein is expressed on the cell surface (Chin and Maizel, 1976; Pääbo *et al.*, 1983). However, we have recently demonstrated that most of the E3/19K protein remains intracellular (Burgert and Kvist, 1985).

The function of the E3/19K protein is not known. It has been shown that the proteins encoded by the E3 region are dispensable for virus growth in cultured cells (Shenk and Williams, 1984). However, several laboratories have demonstrated that the E3/19K protein associates with class I antigens of the major histocompatibility complex (Kvist *et al.*, 1978; Signäs *et al.*, 1982; Kämpe *et al.*, 1983; Pääbo *et al.*, 1983). These antigens (termed HLA and H-2 in man and mouse, respectively) are cell surface glycoproteins comprising a heavy chain (mol. wt 45 kd) non-covalently linked to β_2-microglobulin (mol. wt 12 kd) (Ploegh *et al.*, 1981). The heavy chain can be divided into three extracellular domains (α1, α2 and α3) each of which contains ~90 amino acids, a transmembrane domain ~30 residues long, followed by a hydrophilic cytoplasmic tail of 30–40 amino acids (Lew *et al.*, 1986). One function of the HLA and H-2 class I antigens is to present foreign (viral) antigens to cytotoxic T lymphocyte precursors. These lymphocytes can develop into cytolytic T lymphocytes (CTL) with the ability to eliminate the infected cells. It has been shown that the recognition by CTL of the infected cells is mediated via the class I antigens. This phenomenon is referred to as H-2 or HLA restriction (Klein, 1979; Zinkernagel and Doherty, 1979). The major target structures of H-2 antigens are composed of the α1 and α2 domains (Reiss *et al.*, 1983; Allen *et al.*, 1984; Arnold *et al.*, 1984a, 1985; Schøller *et al.*, 1986).

We have demonstrated that the association between the E3/19K protein and the HLA antigens interferes with terminal glycosylation and transport of HLA molecules. Furthermore, the cell surface expression of HLA class I antigens is drastically reduced in the presence of the E3/19K protein (Burgert and Kvist, 1985). We have suggested that this may have an effect on T cell recognition. In a recent study we showed that T cell recognition of target cells expressing the E3/19K protein is markedly reduced (Burgert *et al.*, 1987).

The aim of the present study was to examine the nature of the histocompatibility–E3/19K protein complex in greater detail and identify the structures involved in the interaction. We report that neither disulfide bridges between the proteins nor carbohydrates are of importance for the interaction. Furthermore, we have identified a murine H-2 molecule which does not interact with the viral protein. This finding allowed us to determine the domains of the histocompatibility antigens involved in the association with the E3/19K protein. The aspects of adenovirus evolution as they relate to immune surveillance are discussed.

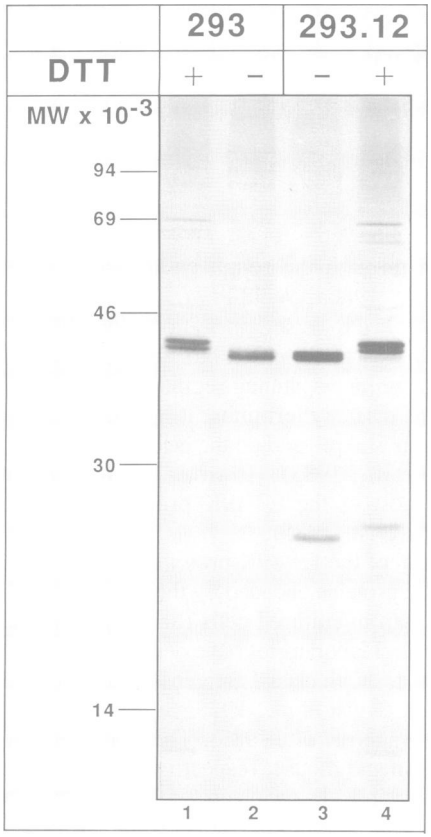

Fig. 1. SDS−PAGE analysis of the disulfide-bridge formation between E3/19K protein and HLA antigens. The cell lines 293 **(lanes 1** and **2)** and 293.12 **(lanes 3** and **4)** were labelled for 30 min with 200 μCi/ml of [^{35}S]methionine, lysed and HLA antigens were immunoprecipitated with the monoclonal antibody W6/32. To stabilize the existing disulfide bonds, lysis and subsequent immunoprecipitations were carried out in the presence of 7 mM iodoacetamide **(lanes 2** and **3)**. In contrast, immunoprecipitates analysed under reducing conditions **(lanes 1** and **4)** were treated with DTT before being loaded onto the gel. Mol. wt marker proteins were: phosphorylase b (94 kd), bovine serum albumin (69 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd) and lysozyme (14 kd).

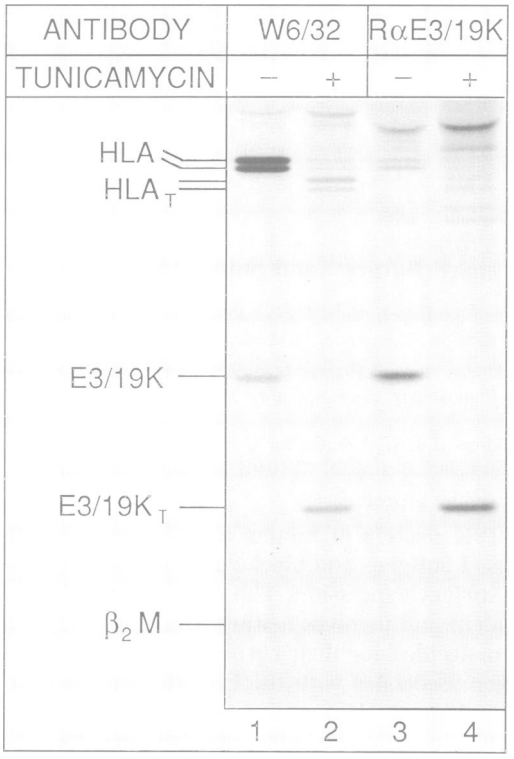

Fig. 2. Co-immunoprecipitation of E3/19K with HLA antigens in the presence of tunicamycin. 293.12 cells were pre-incubated for 1 h in complete medium with and without 7 μg/ml of tunicamycin **(lanes 2, 4** and **1, 3** respectively). The medium was changed to DMEM without methionine but containing the same concentration of tunicamycin and the incubation was continued for 45 min. The cells were then labelled for 45 min with 150 μCi/ml of [^{35}S]methionine in the presence of tunicamycin. The antibodies used for immunoprecipitation were W6/32 **(lanes 1** and **2)** and a rabbit antiserum against the cytoplasmic tail of the E3/19K protein from Ad-5 **(lanes 3** and **4**; Wold *et al.*, 1985). The positions for the proteins, with (HLA and E3/19K) and without (HLA_T and $E3/19K_T$) carbohydrates as well as for β_2-microglobulin (β_2m) are indicated.

Results

The interaction between E3/19K and HLA molecules is non-covalent and does not involve intermolecular disulfide bridges

We have examined whether or not a covalent association involving disulfide bridges exists between the HLA and the E3/19K proteins. The human embryonic kidney cell line 293 and its derivative 293.12 were used. The 293.12 cells have been transfected previously with the *Eco*RI D fragment of Ad-2 (Hérissé *et al.*, 1980), and express the E3/19K protein constitutively (Burgert and Kvist, 1985). These two cell lines were metabolically labelled with [^{35}S]methionine, lysed and HLA antigens were immunoprecipitated with the monoclonal antibody W6/32. The immunoprecipitates were analysed by SDS−PAGE.

Immunoprecipitated material from 293.12 cells was isolated under reducing conditions and revealed proteins with apparent mol. wts of 42 kd (HLA antigens), 25 kd (E3/19K) and 12 kd [β_2-microglobulin (Figure 1, lane 4)]. The E3/19K protein is absent in immunoprecipitates from the parental 293 cells used as a control (Figure 1, lane 1). If disulfide bridges were involved in the HLA−E3/19K interaction, one would expect high mol. wt bands in the material analysed under non-reducing conditions. In addition, a concomitant reduction in the intensity of the protein bands seen in lane 4 should occur. No high mol. wt products can be observed under these conditions (Figure 1, lanes 2 and 3). Instead, the apparent mol. wt of the isolated proteins decreased by ~1100 daltons and 400 daltons for HLA antigens and β_2-microglobulin respectively, as expected for proteins with intramolecular disulfide bridges. The apparent mol. wt of the E3/19K protein decreased by 900 daltons, indicating structural changes by disulfide bridge(s) formed within the molecule.

Thus, although both the E3/19K protein and HLA antigens contain intramolecular disulfide bridges, intermolecular disulfide bonds did not seem to contribute to the formation of the complex.

The E3/19K and HLA molecules interact via their protein portions

To elucidate which structural moieties of the glycoproteins are responsible for their association, immunoprecipitations were performed after treatment of 293.12 cells with tunicamycin, an inhibitor of N-glycosylation. When antibodies against HLA antigens were used, HLA, E3/19K and β_2-microglobulin were precipitated (Figure 2, lanes 1 and 2). In the tunicamycin-treated sample the apparent mol. wt of the two glycoproteins decreased whereas that for β_2-microglobulin remained constant (cf. lanes 1 and 2). This indicates the loss of N-linked sugars in the HLA and E3/19K glycoproteins.

To confirm that the co-immunoprecipitated band is derived from the E3/19K protein, we used an antiserum raised against the cytoplasmic tail of E3/19K (Wold *et al.*, 1985). A protein

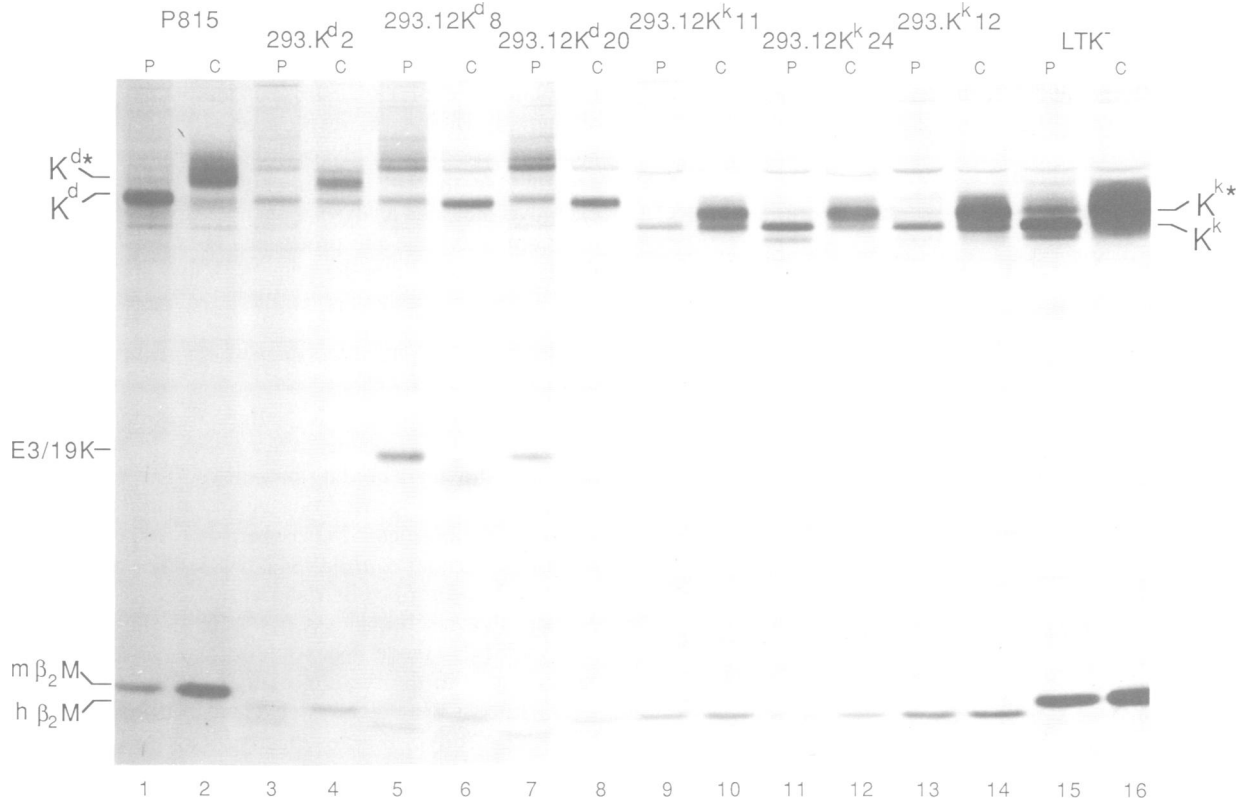

Fig. 3. Pulse-chase analysis of cells expressing H-2 K^d and K^k antigens. The cell lines indicated on top of the figure were pulse labelled for 20 min with 200 μCi/ml [^{35}S]methionine and either lysed immediately (P) or incubated for 2 h in medium containing an excess of cold methionine and then lysed (C). The K^d and K^k antigens were immunoprecipitated by the monoclonal antibodies 20-8-4S (**lanes 1–8**) and a mixture of 16-1-11N and H100-30 (**lanes 9–14**; Sachs *et al.*, 1981; Arnold *et al.*, 1985). Two different clones from each transfection were analysed: 293.12K^d8 (**lanes 5** and **6**), 293.12K^d20 (**lanes 7** and **8**), 293.12K^k11 (**lanes 9** and **10**) and 293.12K^k24 (**lanes 11** and **12**). Control cells were: P815 (**lanes 1** and **2**), 293 cells transfected with the K^d gene (293K^d2; **lanes 3** and **4**) or the K^k gene (293K^k12; **lanes 13** and **14**) and LTK$^-$ cells (**lanes 15** and **16**). The P815 cells were labelled for 15 min and immunoprecipitation of K^k antigens from LTK$^-$ cell extracts was carried out with the monoclonal antibody 16-1-11N. The symbols are as follows: K^d and K^k represent the high mannose forms of the H-2 antigens, whereas K^{d*} and K^{k*} denote the positions of the processed products containing complex type sugars. The positions for E3/19K and human (h-β_2M) and mouse (m-β_2M) β_2-microglobulin are indicated.

of the same apparent mol. wt as co-precipitated by the antibody W6/32 was detected with this antiserum (Figure 2, lanes 3 and 4; cf. lanes 2 and 4, 1 and 3). These results show that the proteins associate also in the absence of their carbohydrates, demonstrating that only the protein portions of the two molecules are important for their interaction. This finding allows mapping the domains of the class I histocompatibility antigens involved in the E3/19K association.

The E3/19K protein does not associate with all histocompatibility class I antigens

To obtain further knowledge about this protein–protein interaction between class I antigens and the E3/19K protein, we decided to determine which structures of the histocompatibility antigens are essential for the association. Preferably, this should be done in such a manner as to introduce a minimum of changes in the gross structure of the histocompatibility antigen. One possibility would be to use hybrid molecules between an interacting class I antigen and a non-interacting one. It has been shown that histocompatibility class I antigens from several species like rat (Kvist *et al.*, 1978), human (Burgert and Kvist, 1985) and mouse (Signäs *et al.*, 1982) all have the ability to associate with the viral protein. In a recent study, we demonstrated that the murine H-2 K^d antigen binds to the E3/19K molecule (Burgert *et al.*, 1987). However, preliminary experiments indicated that the H-2 K^k antigen did not. We decided to examine this fortuitous result further.

The H-2 K^d and K^k genes (Kvist *et al.*, 1983; Arnold *et al.*, 1984b) were separately introduced into the human 293.12 cells which expressed the E3/19K protein. We used the hygromycin-resistance gene as a selection marker (Hyg^R; Bernhard *et al.*, 1985). Several cloned cell lines expressing either H-2 K^d or K^k antigens were isolated and analysed as described below.

During their transport to the cell surface, HLA and H-2 antigens are normally processed by addition of terminal sugars such as galactose and sialic acid (Ploegh *et al.*, 1981). This can be observed in pulse-chase experiments as an increase in apparent mol. wt of the proteins during the chase. In the presence of the E3/19K protein this processing of the H-2 antigens does not occur. We can therefore identify interaction of the E3/19K protein by two different criteria: (i) the co-immunoprecipitation of the viral protein and (ii) the inhibition of processing of the histocompatibility antigen.

Cells were pulsed with [^{35}S]methionine for 20 min and chased in an excess of cold methionine for 2 h. Cell lysates were incubated with monoclonal antibodies against the murine H-2 antigens and immunoprecipitated material was analysed by SDS–PAGE. The two murine cell lines P815 (H-2^d) and LTK$^-$ (H-2^k) were used as controls for following normal processing of the K^d and K^k molecules. The 293 cells, the parental line of 293.12 cells, transfected with H-2 K^d and K^k respectively, were also included as controls in the experiment.

The cell lines 293.12K^d8 and 293.12K^d20 express the K^d

antigen and the E3/19K protein is co-precipitated by antibodies against the K^d antigen (Figure 3, lanes 5–8). No processing of the class I antigens can be observed (cf. lanes 5 and 6, 7 and 8 with 1 and 2, 3 and 4 respectively). In contrast, the K^k molecule does not associate with the E3/19K protein in the two cell lines 293.12K^k11 and 293.12K^k24 (lanes 9–12). Furthermore, the K^k antigens are processed in a normal fashion, as in the 293K^k12 and LTK$^-$ cells (cf. lanes 9 and 10, 11 and 12 with 13 and 14, 15 and 16, respectively). The fact that the H-2 K^k antigens are terminally glycosylated in the presence of the E3/19K protein, but the H-2 K^d antigens are not, was confirmed by endoglycosidase H analysis (data not shown). Both the co-immunoprecipitation and the processing data are in agreement and, thus, we conclude that the K^d antigen interacts strongly with the E3/19K protein, whereas its allelic counterpart K^k does not.

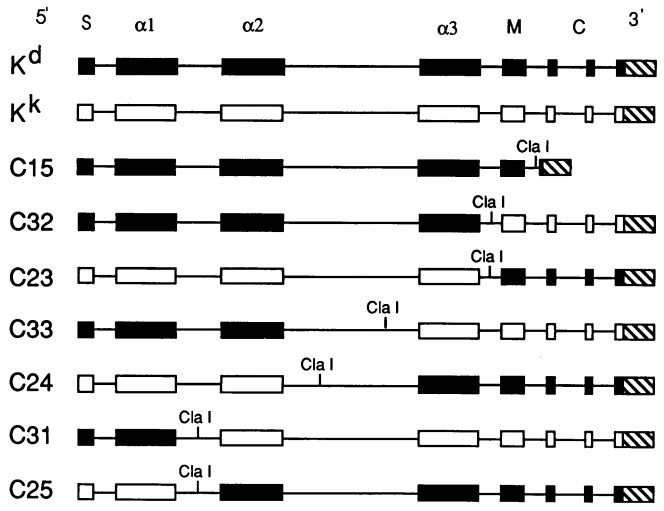

Fig. 4. Schematic representation of parental and hybrid H-2 genes. Exons are shown as either filled boxes (H-2 K^d origin) or open boxes (H-2 K^k origin). Introns are denoted by the horizontal line connecting the exons. The hatched boxes correspond to the 3′ non-coding region. Domains of the corresponding protein are assigned: S = signal sequence; α1, α2 and α3, the three extracellular domains; M, the transmembrane exon; C, the exons encoding the cytoplasmic portion. The *Cla*I site indicates the position of recombination.

Domains of histocompatibility class I antigens essential for association with the E3/19K protein

The finding that the H-2 K^k antigen does not interact with the E3/19K protein allowed us to test a series of H-2 K^d–K^k hybrid molecules for their binding properties. The construction of the hybrid genes was carried out using a modification of the subcloning deletion method (Frischauf *et al.*, 1980) and has been published (Arnold *et al.*, 1984a, 1985). The generation of construct C15 is described in Materials and methods. The constructs used are shown schematically in Figure 4. This series of recombinant molecules made it possible to analyse the contribution of each domain to the binding of the E3/19K protein.

293.12 cells were co-transfected independently with all the H-2 K^d–K^k DNA hybrid genes together with the hygromycin resistance gene. Hygromycin-resistant cell colonies were labelled with [^{35}S]methionine and hybrid proteins were immunoprecipitated with monoclonal antibodies against the H-2 antigens and analysed by SDS–PAGE.

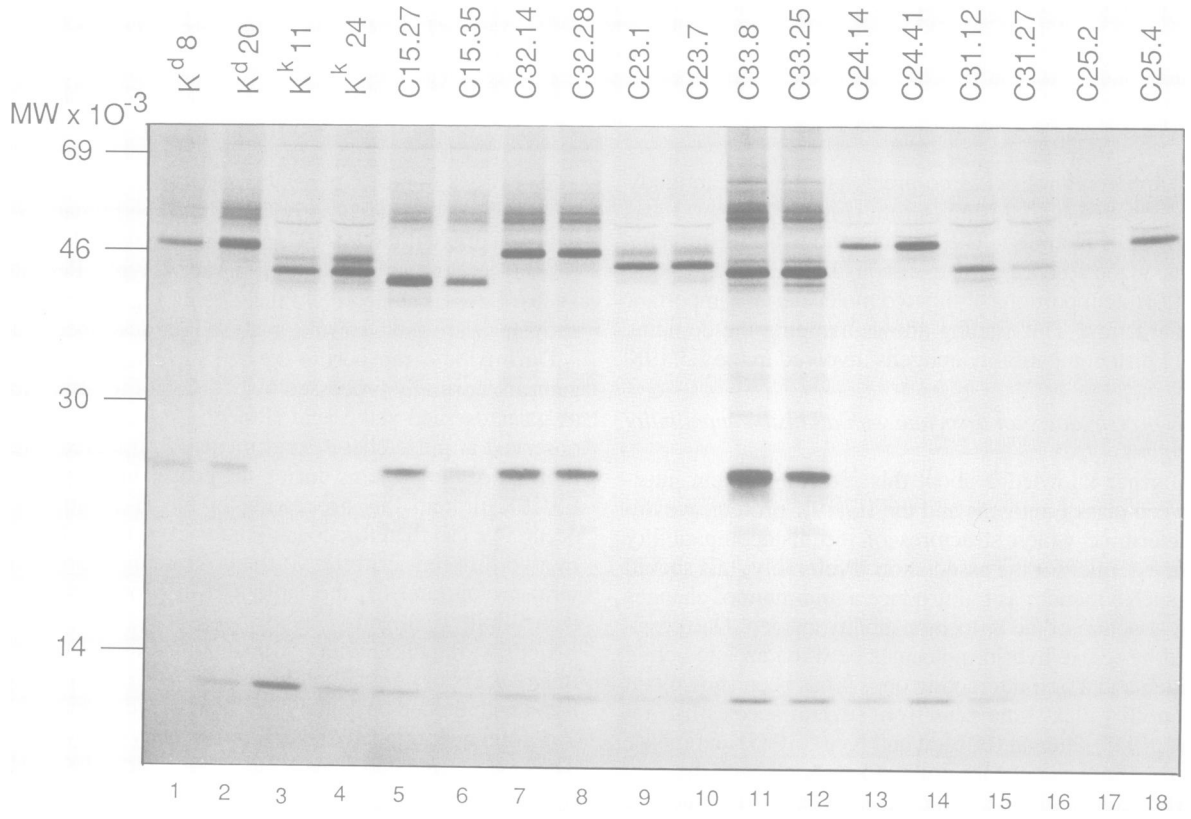

Fig. 5. SDS–PAGE analysis of hybrid H-2 antigens associated with the E3/19K protein. Cells were labelled for 1 h with 150 μCi/ml of [^{35}S]methionine. Cell extracts were prepared and class I antigens were immunoprecipitated with the following monoclonal antibodies: 20-8-4S **(lanes 1, 2, 5–8, 11, 12, 15** and **16)**; H100-30 and 16-1-11N **(lanes 3, 4, 9** and **10)**; K9-18 **(lanes 13, 14, 17** and **18)**. The cell clones used are indicated at the top. The mol. wt markers are the same as in Figure 1.

The C15 molecule lacks the cytoplasmic portion of the H-2 K^d antigen (exons 6–8 have been deleted, Figure 4) so that only the basic cluster of amino acids immediately following the transmembrane domain is present. Co-precipitation of the E3/19K protein occurs in both C15-expressing cell lines shown (Figure 5, lanes 5 and 6). Thus, the H-2 antigens and the viral protein do not interact via their cytoplasmic portions. This is confirmed by the examination of the C32 and C23 hybrid molecules. The former molecule is H-2 K^d in the extracellular part whereas the transmembrane and the cytoplasmic parts are of K^k origin. This antigen strongly binds to the E3/19K molecule (lanes 7 and 8). In contrast, the C23 antigen which has the reciprocal configuration containing K^d sequences in the membrane and cytoplasmic portions, does not bind to E3/19K (Figure 5, lanes 9 and 10). Thus, neither the cytoplasmic nor the transmembrane parts of the H-2 antigens appear to contribute to the binding of the viral protein.

Analysis of the extracellular portion of the histocompatibility antigens reveals that the E3/19K protein is co-precipitated with the C33 antigen whereas it is not with the C24 antigen (Figure 5, lanes 11 and 12, 13 and 14, respectively). This result shows that the α1 and α2 domains are essential for association whereas the α3 domain does not appear to be involved. We further analysed whether or not the α1 and α2 domains can interact independently with the E3/19K protein. Neither the C31 nor the C25 molecules bind to the E3/19K molecule (Figure 5, lanes 15–18). 293.12 cells expressing K^d or K^k antigens were used as positive and negative controls respectively (Figure 5, lanes 1–4).

From these experiments we conclude that the α1 and α2 domains of the H-2 K^d antigen are crucial for the association of the H-2 molecule with the adenovirus-encoded protein E3/19K.

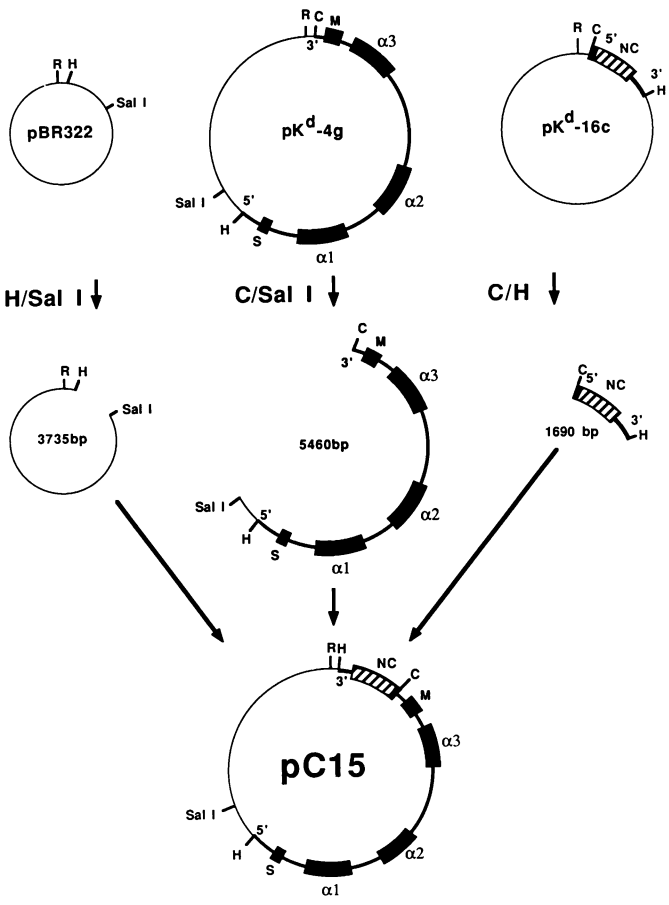

Fig. 6. Construction of the C15 gene. Two subclones of the H-2 K^d gene, pK^d-4g and pK^d-16c, were digested with *ClaI*/*SalI* and *ClaI*/*Hin*dIII restriction enzymes respectively. Fragments containing K^d gene sequences (thick lines and boxes) were isolated from low melting point agarose and subsequently ligated to the pBR322 vector which has undergone *Hin*dIII/*SalI* digestion. Plasmid DNA containing the C15 gene was isolated and the DNA sequence was determined over the recombination position. For details see Materials and methods. Restriction enzymes were: C, *ClaI*; H, *Hin*dIII; R, *Eco*RI; S, *SalI*. Exons are denoted and designated as in Figure 4.

Discussion

We have shown earlier that E3/19K, an early glycoprotein of adenovirus type 2, interacts with human histocompatibility class I antigens in a specific manner. In the human embryonic kidney cell line 293, as well as in the non-transformed cells WI38, MRC-5 and 3229, this interaction prevents the class I antigens from being terminally glycosylated and inhibits their transport and cell surface expression (Burgert and Kvist, 1985). These results have been confirmed for HeLa cells (Andersson *et al.*, 1985). In this paper we have studied the nature of this specific binding and determined the structural domains of the histocompatibility antigens which are essential for the association.

Immunoprecipitations performed under non-reducing conditions do not reveal any high mol. wt bands on SDS–PAGE (Figure 1). However, the E3/19K protein migrates slightly faster in the absence of the reducing agent, indicating the presence of intramolecular disulfide bridges. We conclude that the association between the HLA antigens and the E3/19K protein consists of non-covalent bonds and does not involve intermolecular disulfide bridges.

The E3/19K protein is a glycoprotein with two asparagine-linked carbohydrate groups (Kornfeld and Wold, 1981). Tunicamycin, an inhibitor of N-glycosylation, was used to examine whether or not the carbohydrate groups of the molecules were responsible for the interaction (Figure 2). We could show that co-immunoprecipitation of the E3/19K protein occurs also in the presence of tunicamycin. Thus, the carbohydrate groups are not required for the interaction.

We have analysed several murine histocompatibility class I antigens for their ability to interact with the E3/19K protein. The genes encoding the H-2 K^d and K^k antigens were transfected into 293.12 cells which constitutively express the E3/19K protein. Immunoprecipitation and SDS–PAGE analysis clearly demonstrated that in these cells the K^d but not the K^k antigens associated with the E3/19K protein (Figure 3). Hybrid genes between the K^k and K^d alleles were constructed, expressed in the 293.12 cells and then the hybrid proteins were examined for their ability to associate with the adenovirus E3/19K protein (Figures 4 and 5). We make the following conclusions from these experiments: (i) the cytoplasmic and transmembrane portions of the histocompatibility antigens are not essential for interaction with E3/19K; (ii) the α3 domain is not directly involved and can be exchanged between the two H-2 antigens without affecting the E3/19K binding; (iii) the α1 and α2 demains contain the structure necessary for association with the E3/19K protein; and (iv) this essential structure is abrogated by exchanging α1 and α2 domains between the two alleles and, therefore, these domains cannot independently interact with the E3/19K protein.

It has been suggested that the transmembrane or the cytoplasmic domains of the histocompatibility class I antigens are involved in the interaction with the E3/19K protein (Signäs *et al.*, 1982). Recently, it has been demonstrated that, *in vitro*, a truncated E3/19K molecule which lacks the membrane and cytoplasmic

parts, retains its ability to bind the extracellular papain-fragment of HLA molecules (Pääbo *et al.*, 1986). We have shown here that it is the α1 and α2 domains of histocompatibility antigens which are crucial for binding the viral protein. It is possible to obtain a better understanding of which amino acids are involved in the interaction by comparing the protein sequences of the α1 and α2 domains of the H-2 K^d, HLA-A2 and B7 with that of the H-2 K^k. The former antigens all bind to the E3/19K protein whereas the latter antigen does not (Severinsson *et al.*, 1986; H.-G.Burgert and S.Kvist, this report and unpublished results). We find that 5−8 residues for each individual domain can be considered as either unique for the K^k molecule or represent a non-conservative change. Site-directed mutagenesis might help to identify the individual residues crucial for binding to the E3/19K protein.

Do all human class I antigens bind to the E3/19K protein?

It is interesting that the murine H-2 K^k antigen does not show any obvious affinity for the E3/19K protein. We have used a panel of monoclonal antibodies for the K^k molecule to confirm this finding. These antibodies recognize different domains of the K^k antigen (Arnold *et al.*, 1985). We were unable to co-precipitate the E3/19K protein (data not shown). In contrast, all monoclonal antibodies against the K^d molecule so far examined, co-precipitate equimolar amounts of the E3/19K protein. These antibodies recognize either the α1 domain (antibodies 20-8-4S and 34-1-2S; Sachs *et al.*, 1981) or the α3 domain (antibody K9-18; Arnold *et al.*, 1985). The K9-18 antibody precipitates the C24 and C25 antigens (Figure 4) without co-precipitating the E3/19K proteins (Figure 5). However, it does co-precipitate the E3/19K from cells expressing either K^d, C15 or C32 antigens (Figure 5). Therefore, the co-precipitation is not dependent on the antibody used. The pulse-chase experiment shows that the K^k antigen is processed in a normal fashion in the presence of the E3/19K protein, whereas the K^d antigen is not (Figure 3). Thus, we are confident that the H-2 K^k antigen lacks the ability to associate with the E3/19K protein and is the first example of a class I antigen that does not interact.

It would be of great interest to know whether or not similar differential binding properties exist among the human class I antigens, the histocompatibility antigens of the natural host. This could have implications for the ability of adenoviruses to establish persistent infections. Only 10−20% of infected people have symptoms of a persistent infection related to adenoviruses (Straus *et al.*, 1984). This phenomenon might be linked to certain HLA class I alleles. To determine this, we are presently investigating a large number of human HLA allelic products for their ability to bind E3/19K.

What causes inhibition of intracellular transport?

The interaction of the E3/19K protein with the first (α1) and second (α2) domains of the C33 antigen results in a similar inhibition of transport and cell surface expression as for the K^d antigen (data not shown). In contrast, 293 and 293.12 cells which have been transfected with the H-2 K^k gene have similar amounts of K^k antigen on their cell surface (data not shown). Therefore, we feel confident that it is the association of the E3/19K protein, or a process linked to it, which causes the inhibition in intracellular transport of the HLA and H-2 K^d antigens.

We can envisage several possible mechanisms responsible for blocking the transport of the HLA-E3/19K complex. (i) The E3/19K protein is synthesized on membrane-bound ribosomes (rER) and its final destination seems to be intracellular (Burgert and Kvist, 1985, and unpublished results). The interaction with the histocompatibility antigens occurs immediately after synthesis and, therefore, HLA molecules bound to the viral protein may no longer be transported independently but rather have to follow the destination of the E3/19K protein. (ii) It is possible that the E3/19K protein interferes with the assembly process of HLA antigens with β_2-microglobulin. Oligomerization has been shown to be essential for the G protein of vesicular stomatitis virus and haemagglutinin of influenza A virus (Gething *et al.*, 1986; Kreis and Lodish, 1986). If multimeric forms of HLA and β_2-microglobulin molecules are required for efficient transport from the rER, the E3/19K molecule might block this process. Alternatively, the binding of the E3/19K protein might interfere with a *trans*-acting factor during normal assembly of H-2 antigens (DeMars *et al.*, 1985; Salter and Cresswell, 1986). (iii) It is likely that a particular conformation of H-2 and HLA antigens is necessary for transport to occur. Inability of a mutant of the H-2 L^d antigen to form an intramolecular disulfide bridge in the α3 domain inhibits its cell surface expression (Miyazaki *et al.*, 1986). Also, considerably different rates of transport (5- to 10-fold) have been demonstrated for the very homologous H-2 K^k and D^k antigens (Williams *et al.*, 1985). Conformational differences could then contribute to the efficiency of transport. At present, we have no experimental data favouring any one of these possibilities. We are currently using site-directed mutagenesis to change several amino acids in the cytoplasmic part of the E3/19K protein. These experiments may help to clarify whether or not the cytoplasmic portion of the viral protein is important for the destination of HLA-E3/19K complex.

Implications for evolution of adenoviruses and their interference with the cellular immune response

It has been convincingly shown that the restriction elements for CTL are present within the α1 and α2 domains of class I histocompatibility antigens (Reiss *et al.*, 1983; Allen *et al.*, 1984; Arnold *et al.*, 1984a, 1985; Nathenson *et al.*, 1986). In this report, we have shown that the same domains are essential for binding the E3/19K protein. We present for the first time evidence of direct binding of a viral protein to the α1 and α2 domains of class I antigens. It is tempting to speculate that these domains are also involved in binding other viral proteins or peptides during virus infection (Townsend *et al.*, 1986). The unusually strong binding of the E3/19K protein to class I antigens and its ability to stay in rER, might have offered the adenoviruses (and perhaps other viruses) a unique opportunity to interfere and escape the cellular immune response. In a recent study we have shown that CTL clones restricted to the H-2 K^d antigen do not recognize and lyse target cells expressing the E3/19K protein (Burgert *et al.*, 1987). As the transport and cell surface expression of class I antigens in E3/19K positive cells is inhibited, one could envisage that other viral products can be exposed at the cell surface without being recognized by CTL.

This mechanism could possibly have given the adenoviruses a selectable advantage during evolution and allowed them to survive and replicate in the host for long periods of time, although a strong antibody response was mounted. Perhaps the non-oncogenic adenoviruses have developed as variants in response to immunological selection. Thus, they were able to interfere with the normal immune mechanism, namely the ability of histocompatibility class I antigens to bind foreign antigens within the α1 and α2 domains and to present those on the cell surface to T lymphocytes.

Materials and methods

Cell culture and DNA transfection of cells

The cell lines 293 (Graham *et al.*, 1977), LTK$^-$ and the mastocytoma P815 were grown in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine and antibiotics. The 293.12 cells were derived from 293 cells (Burgert and Kvist, 1985) by transfection with the Ad-2 *Eco*RI D-fragment (Hérissé *et al.*, 1980; Leff *et al.*, 1984) together with the neophosphotransferase gene (Southern and Berg, 1982). For expression of the H-2 genes and the hybrid genes in 293.12 and 293 cells, the hygromycin selection system (Bernhard *et al.*, 1985) or the neomycin selection system were used. The selection marker gene was co-transfected with the H-2 K^d gene (Kvist *et al.*, 1983), the K^k gene (Arnold *et al.*, 1984b), or plasmids containing the constructs C31, C33, C32 (Arnold *et al.*, 1984a), C23, C24, C25 (Arnold *et al.*, 1985) or C15. The construction of C15 is described below. The transfection was performed essentially as described (Arnold *et al.*, 1984a) except that the selection was carried out in 600 μg/ml G-418 (Gibco, Karlsruhe, FRG) or 180 μg/ml hygromycin (Calbiochem, Lucerne, Switzerland).

Construction of hybrid H-2K genes and the C15 gene

The construction of the hybrid H-2K genes has been described (Arnold *et al.*, 1984a, 1985). Construct C15 was generated in an analogous way. By using the subcloning deletion method of Frischauf *et al.* (1980), a high number of subclones from the K^d and K^k genes were isolated with various deletions of DNA in both the 5′ and 3′ directions. Subclone pK^d-4g contains exons 1–5 and has a length of 5460 nucleotides. Its 3′ end is in the intron between exons 5 and 6 with an introduced *Cla*I site. Clone pK^d-16c has its 5′ end within the original exon 8 of the K^d gene. These two clones were digested with *Cla*I/*Sal*I and *Cla*I/*Hin*dIII respectively, and the appropriate fragments were isolated from a low melting agarose gel and ligated to a *Hin*dIII/*Sal*I vector derived from pBR322 (Figure 6). As pK^d-16c has lost the splice acceptor site normally used, the next downstream occurring was assumed to be used. This acceptor site is just 3 bases upstream of the TGA stop codon. Thus, C15 has alanine as the last amino acid as the normal K^d gene. To ascertain correct splicing, S1 nuclease mapping was performed (data not shown). In addition, the product of the C15 gene has a mol. wt which corresponds to a deletion of ~30 amino acids (Figure 5).

Monoclonal antibodies and antisera

The monoclonal antibody W6/32 reacts with a framework determinant of HLA-A, B and C antigens (Barnstable *et al.*, 1978). The antibody 20-8-4S (Sachs *et al.*, 1981) recognizes the first domain of the H-2 K^d molecule (Arnold *et al.*, 1985). The K9-18 antibody is directed against the third domain of the H-2K^d (Arnold *et al.*, 1985). Two antibodies against the K^k molecule were used, 16-1-11N (Sachs *et al.*, 1981) and H100-30 (Arnold *et al.*, 1985). The rabbit antiserum against the cytoplasmic tail of E3/19K was raised against a synthetic peptide corresponding to the C terminus of the E3/19K protein of Ad-5 (Wold *et al.*, 1985).

Cell labelling, tunicamycin treatment, immunoprecipitation and SDS–PAGE

Labelling of cells with [^{35}S]methionine, immunoprecipitation and SDS–PAGE were carried out as described previously (Kvist *et al.*, 1982; Burgert and Kvist, 1985). Tunicamycin treatment of 293.12 cells was performed by pre-incubating the cells with complete medium containing 7 μg/ml of tunicamycin for 90 min (Calbiochem, Lucerne, Switzerland). The medium was then changed to DMEM–methionine containing the same concentration of tunicamycin. After 45 min incubation, cells were labelled with the same medium containing 150 μCi/ml of [^{35}S]methionine. Tunicamycin was diluted from a stock solution of 3.5 mg/ml in DMSO. Immunoprecipitations under non-reducing conditions were carried out in the presence of 7 mM iodoacetamide in the cell lysate buffer. This concentration was kept throughout the immunoprecipitation and the washing steps. Dithiothreitol (DTT) treatment before SDS–PAGE was omitted.

Other reagents

Protein-A Sepharose was from Pharmacia Fine Chemicals, Uppsala, Sweden. The [^{35}S]methionine was purchased from Amersham, UK. Restriction endonuclease and DNA-modifying enzymes were from Boehringer, Mannheim, FRG.

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