The EMBO Journal vol.5 no.8 pp.1921–1927, 1986

Structural and functional dissection of an MHC class I antigen-binding adenovirus glycoprotein

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Communicated by W.Schaffner

The early transmembrane glycoprotein E19 of adenovirus-2 binds to class I antigens of the major histocompatibility complex (MHC). The association is initiated in the endoplasmic reticulum of infected cells and abrogates the intracellular transport of the class I molecules. To examine which parts of the E19 molecule are responsible for the association with the class I antigens and which parts confine the protein to the endoplasmic reticulum we have constructed a series of mutated E19 genes, which have been expressed in an improved mammalian expression vector. By various manipulations the membrane anchoring and the cytoplasmic domains were removed from the protein. The biosynthesis of the mutant protein was examined. All mutant proteins were secreted from the cells suggesting that the transmembrane and/or cytoplasmic portions of the E19 molecule are responsible for its confinement to the endoplasmic reticulum. The ability to associate with class I antigens was retained by the lumenal domain of the E19 protein.

Key words: adenovirus E19 protein/class I histocompatibility antigens/mammalian vectors/mutants/secretion

Introduction

MHC class I antigens play a crucial role in the cellular immune defense against viral infections. Thus, cytotoxic T lymphocytes, principal effector cells in the elimination of viral infections, are known to be restricted to host-specific class I antigens as well as to viral determinants (Zinkernagel and Doherty, 1974). To evade this immune surveillance some viruses may have developed mechanisms to manipulate the class I antigens of the infected cell such that the susceptibility to class I antigen-restricted cellular cytotoxicity is diminished. One mechanism to accomplish this is provided by the E19 protein of adenovirus-2. This membrane glycoprotein is synthesized at early times of infection (Chow *et al.*, 1979) and binds specifically to MHC class I antigens in the infected cell (Kvist *et al.*, 1978; Signäs *et al.*, 1982; Kämpe *et al.*, 1983) soon after their synthesis. This results in the inhibition of the intracellular transport of the class I antigens so that they are retained in the endoplasmic reticulum and/or the *cis*-Golgi compartment (Severinsson and Peterson, 1985; Burgert and Kvist, 1985; Andersson *et al.*, 1985). Consequently, the amount of cell-surface expressed class I antigens of the virus-infected cell diminishes.

To examine whether the abilities of E19 to bind class I antigens and to be retained intracellularly are endowed in the same or different portions of the molecule we have constructed a series of mutants of the E19 gene in a mammalian expression vector that directs the synthesis of the E19 protein (Pääbo *et al.*, 1983). The mutants, which by various manipulations have their hydrophobic, membrane-spanning domain removed, are all secreted to the medium, albeit at different rates and with a

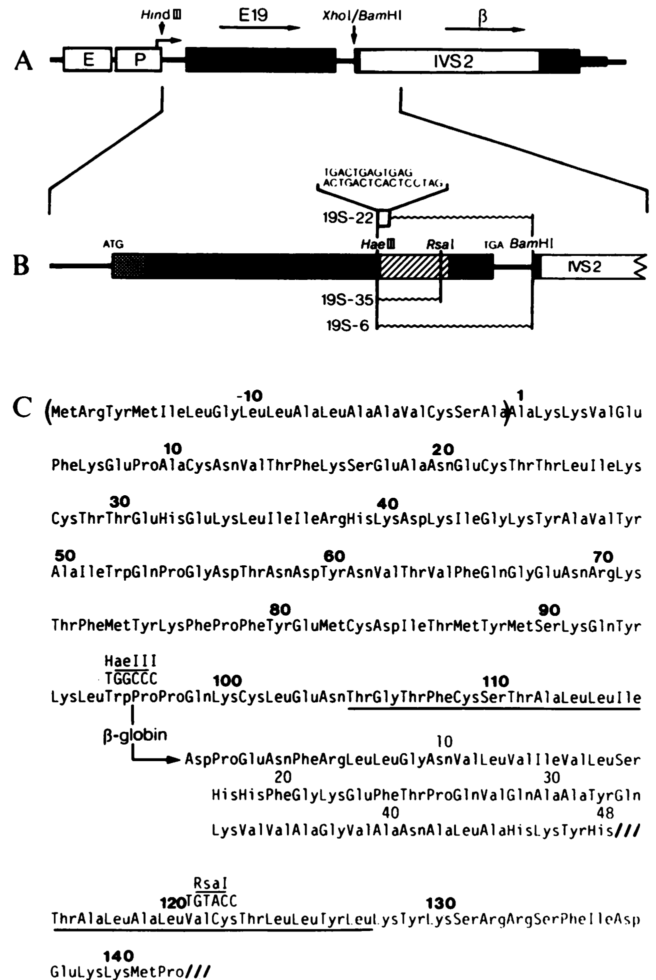

Fig. 1. A. Schematic representation of the recombinant pC81G. The 195-bp enhancer segment of SV40 (E) (Weber *et al.*, 1984) and the human α-globin promoter (P) are indicated. The E19 coding sequence is flanked on its 3′ side by the 3′ half of the rabbit β-globin gene containing the second intron. For further details see Pääbo *et al.*, 1983. **B.** Constructs lacking the transmembrane domain. pC19S-22 was constructed by inserting a synthetic oligonucleotide linker, encoding UGA translation termination signals in all three reading frames, between the *Hae*III and the *Bam*HI sites of pC81G. pC19S-35 has a deletion between the *Hae*III and the *Rsa*I sites. pC19S-6 has a deletion between the *Hae*III and the *Bam*HI site. Sequences coding for the leader peptide are indicated by the dotted area, sequences coding for the hydrophobic membrane anchor domain of the E19 protein are indicated by the hatched area. Deletions are indicated by the wavy lines. **C.** Amino acid sequence of the E19 protein and its mutants. The signal peptide is marked by brackets. The E19 transmembrane domain is underlined. The arrow indicates the fusion point to the 48 amino acids of the C-terminus of rabbit β-globin (amino acids 100–147 of β-globin).

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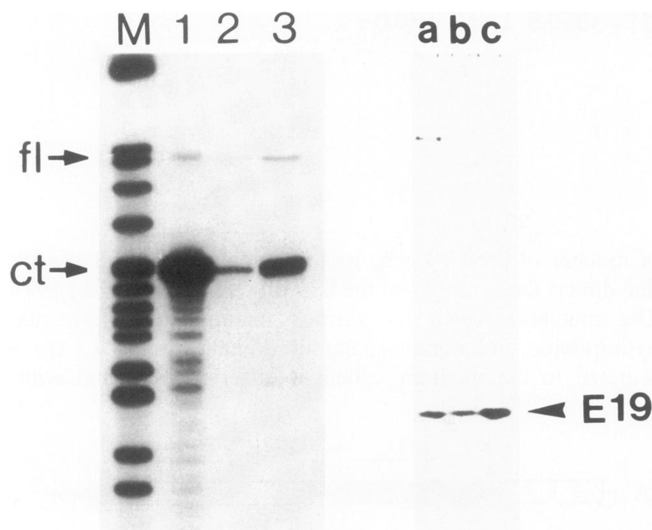

Fig. 2. Comparative analysis of E19 gene expression from various constructs. To the left, analyses of mRNA expression, measured by S1 nuclease assay, are illustrated. A β-globin cDNA was used as the radioactive probe. DNA, 3′ end-labelled at the *Bam*HI site, was hybridized to 20 μg of cytoplasmic RNA from transfected human HeLa cells, digested with S1 nuclease, and then denatured, fractionated by gel electrophoresis, and autoradiographed. The relative amounts of mRNA were quantitated by densitometry. The autoradiography shows the result of hybridization to: **(1)** rabbit reticulocyte RNA (positive control); **(2)** RNA from cells transfected with pA81G; **(3)** RNA from cells transfected with the α-globin promoter clone pC81G. fl, Full length probe; ct, correct terminus; M, marker DNA fragments (plasmid pBR322 cleaved with *Hpa*II). To the right, are analyses of E19 protein expression. HeLa cells were transfected with pA81G, containing the SV40 early promoter (Pääbo *et al.*, 1983) **(lane A)**, pB81G, containing a shortened untranslated leader sequence **(lane B)** and pC81G, containing the α-globin promoter **(lane C)**. Forty-eight hours later the cells were labelled for 4 h with 0.25 mCi of [^{35}S]methinoine and the E19 protein was analyzed by immunoprecipitation and SDS polyacrylamide gel electrophoresis. The relative amounts of proteins were quantitated by densitometric scanning of the autoradiogram. The migration positions of molecular weight markers are given in kilodaltons.

glycosylation-pattern different from that of the membrane-anchored protein. We find that the free, water-soluble lumenal domain of the E19 protein retains the ability to bind specifically to MHC class I antigens.

Results

Construction of a high level expression vector

A series of eucaryotic expression vectors directing the synthesis of the adenoviral E19 protein has been described (Pääbo *et al.*, 1983). In the most efficient of these vectors, pA81G, the E19 gene is under the transcriptional control of the SV40 early promoter and enhancer. In an attempt to optimize the expression from this construct, the unusually long 220-bp leader sequence was shortened by 80 bp. This construct is called pB81G. Another attempt to increase the expression of the E19 protein was made by replacing the SV40 early promoter with the human α-globin promoter. The resulting construct is called pC81G (Figure 1A).

The three constructs pA81G, pB81G and pC81G were transfected into HeLa cells and the transient expression of the E19 protein was examined. The amount of viral protein produced by the α-globin promoter-containing vector pC81G was approximately 4-fold higher than the amounts of E19 generated by the other two vectors (Figure 2). Thus, shortening of the leader sequence did not measurably affect the expression. The higher expression of the E19 protein from the construct containing the

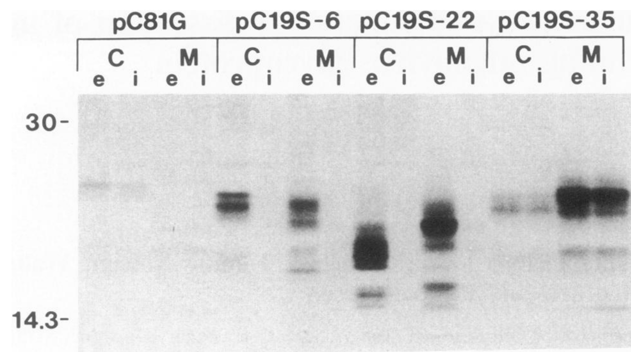

Fig. 3. Expression of the E19 protein and the different mutant proteins in HeLa cells. HeLa cells were separately transfected with 10 μg of the different plasmids and 48 h later labelled for 4 h with [^{35}S]methionine. Immunoprecipitates obtained with a monoclonal antibody against the lumenal domain of E19 (e) and an antiserum against the cytoplasmic domain of E19 (i) were analyzed by SDS-PAGE and visualized by autoradiography. Cells (C) as well as medium (M) from each experiment were analyzed.

α-globin promoter as compared to the construct containing the SV40 early promoter was confirmed by S1 nuclease mapping of transcripts. RNAs were extracted and analyzed 42 h after transfection of HeLa cells with the clones pA81G and pC81G. In complete agreement with the protein measurements, four times more correctly spliced transcripts were recoverd in the cytoplasmic RNA fraction of cells transfected with pC81G as compared to pA81G (Figure 2).

Expression of E19 mutant proteins

To generate E19 mutants that were devoid of the transmembrane domain three different constructs were made. In one construct, pC19S-6 (Figure 1B), the region encoding the 46 COOH-terminal amino acids of the E19 protein was deleted. The remainder of the gene was fused to the fragment of the rabbit β-globin gene present in construct pC81G. Since the β-globin sequence occurred in the same reading frame as the E19 gene this construct was expected to generate an E19-β-globin fusion protein. To eliminate the possibility that the β-globin portion of the fusion protein affected the conformation of the E19 protein we also introduced an in-frame stop-codon 5′ to the sequence encoding the transmembrane segment of the E19 protein in vector pC81G. The novel construct was called pC19S-22 (Figure 1B). A third construct containing a deletion of the region encoding the E19 transmembrane domain was also made. This vector pC19S-35 was expected to generate a protein in which the extracellular portion of E19 was directly associated with the cytoplasmic tail (Figure 1B).

The various DNA constructs were subjected to transient expression in exponentially growing HeLa cells. Proteins labeled with [^{35}S]methionine were immunoprecipitated with a monoclonal antibody reactive with the extracellular domain of E19 as well as with a rabbit antiserum raised against a synthetic peptide corresponding to the cytoplasmic tail of E19. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography.

Figure 3 summarizes the results. The vector pC81G directed the synthesis of the intact E19 protein. Both types of antibodies precipitated the viral protein from the solubilized cells. As expected, the E19 protein could not be demonstrated in the culture medium. The protein derived from the vector pC19S-6 appeared as a doublet both in the cells and in the medium. Small amounts of low molecular weight components reactive with the monoclonal antibodies were encountered in the medium but not in the

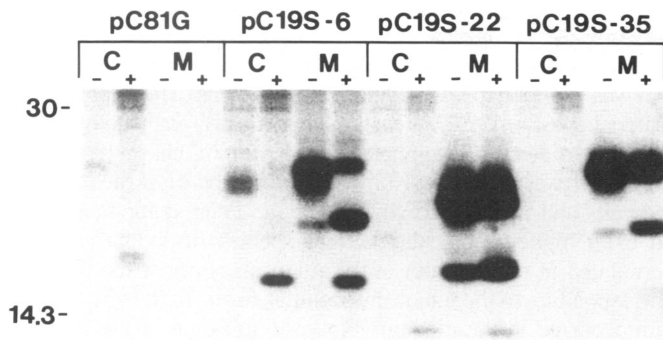

Fig. 4. Endo-H treatment of the E19 protein and its mutants. HeLa cells were transfected with the various DNAs as in Figure 3. Prior to SDS-PAGE analysis immunoprecipitated proteins were treated (+) or mock-incubated (−) with Endo-H. Cells (C) as well as medium (M) were analyzed in each case. Migration positions of molecular weight markers are given in kilodaltons.

cells. As expected, no viral products were recognized by the rabbit antibodies directed against the cytoplasmic domain of the E19 protein. Also the vector pC19S-22 directed the synthesis of molecules that were electrophoretically heterogeneous. Molecules present in the medium had slightly higher apparent molecular weights than these present in the cells. Finally, the construct pC19S-35 gave rise to products that reacted with both types of antibodies. Cells and medium contained molecules that were electrophoretically indistinguishable as well as some additional components of higher and lower apparent molecular weights present in the medium.

These data demonstrate that all three constructs generated viral protein that was recognized by the monoclonal antibodies, while only pC19S-35 generated products reactive with the anti-peptide antibodies. Moreover, all three sets of proteins were secreted into the culture medium.

Glycosylation of the E19 mutant proteins

The electrophoretically heterogeneous behavior of the E19 mutant proteins could have been taken to mean that the vectors gave rise to multiple translation products. Alternatively, it also seemed possible that heterogeneity in the glycosylation of the translation products generated the various electrophoretic species. To examine the latter possibility the immunoprecipitated mutants from cells and medium were subjected to treatment with endoglycosidase H (Endo H) prior to SDS-PAGE analysis. This enzyme removes high-mannose type carbohydrate moieties from glycoproteins but is unable to affect the same molecules when they have been processed to complex type carbohydrate groups in the Golgi complex (Tarentino and Maley, 1974). The results are summarized in Figure 4. Intact E19 protein contains two Asn-linked carbohydrate moieties that always occur in the high-mannose form (Kornfeld and Wold, 1981). Consequently, Endo H-digestion of the viral protein derived from pC81G reduced the apparent molecular weight of the E19 protein by approximately 6000.

The fusion protein derived from the vector pC19S-6 was fully sensitive to Endo H treatment and resolved into one band when isolated from cells. When isolated from medium, the enzyme-treated material resolved into three components, one being resistant to Endo H and two differing in apparent molecular weights by ~3000. Since the β-globin portion of the fusion product does not contain any putative glycosylation site it appears reasonable to conclude that one of the Asn-linked carbohydrate moieties of

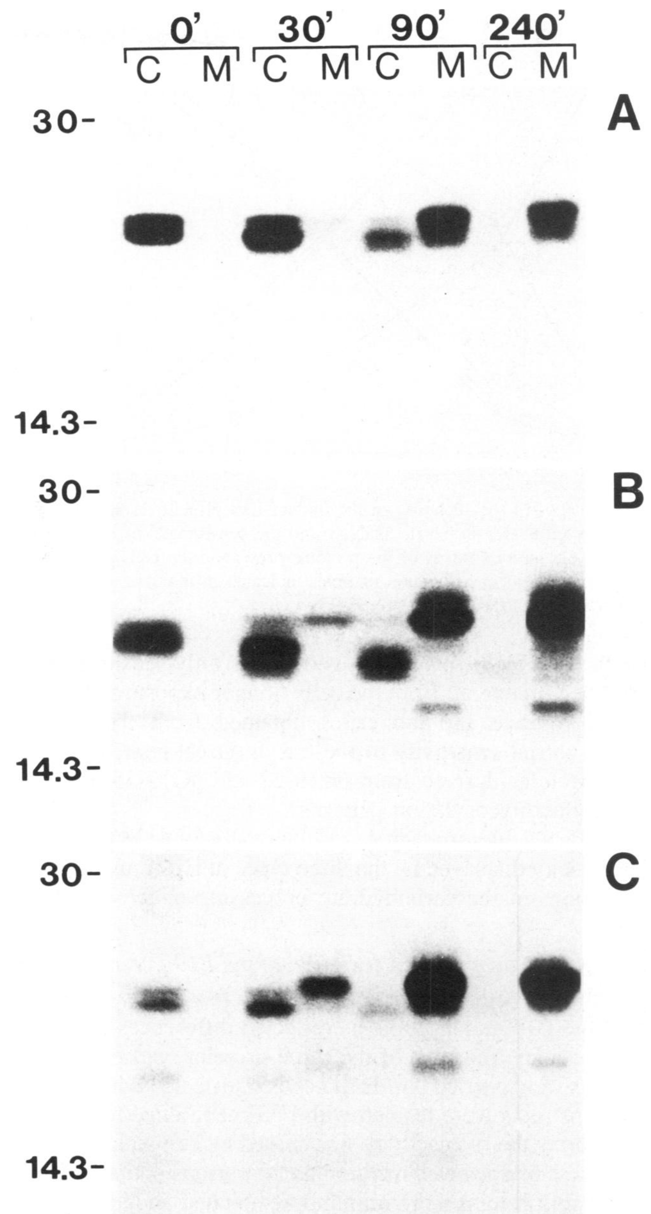

Fig. 5. Biosynthesis of mutant proteins. Pulse-labelled HeLa cells, transfected with the different constructs were chased for 0, 30, 90 and 240 min, respectively. Cells (C) and medium (M) were collected at the times indicated. The migration positions of molecular weight markers are given in kilodaltons. A, pC19S-5; B, pC19S-22; C, pC19S-35.

E19 had undergone terminal glycosylation in the largest fraction of this mutant protein.

The E19 mutant recovered from the solubilized cells after transfection with the vector pC19S-22 was fully sensitive to the Endo H treatment. After digestion the protein gave rise to one apparently homogeneous species (Figure 4). The difference in apparent molecular weights between the untreated mutant protein and the enzymatically digested component is consistent with the elimination of two high-mannose sugar moieties. In contrast, the same protein recovered from the medium displayed only partial sensitivity to the enzyme treatment and the digested product displayed an apparent molecular weight, which suggested that only one of the carbohydrate moieties had been removed by the digestion. Figure 4 also shows that the viral protein, whose synthesis had

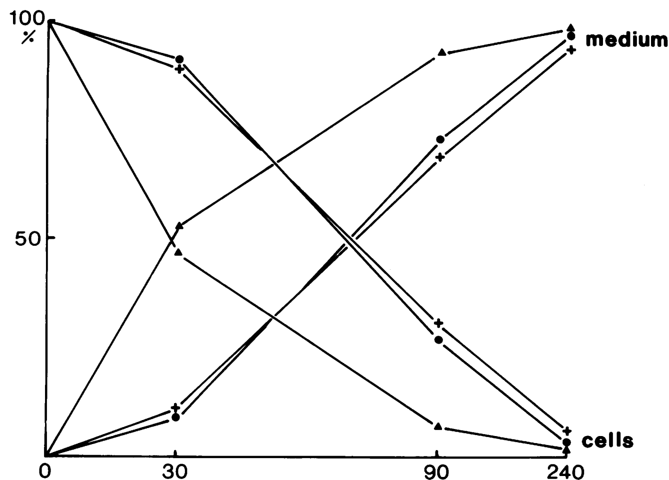

Fig. 6. Kinetics of the secretion of the mutant E19 protein. Fluorographs were subjected to densitometric analysis and the percentages of the total amount of the various forms of the proteins present in the cells and in the medium are plotted as a function of time, indicated in minutes. (+) pC19S-6; (●) pC19S-22; (▲) pC19S-35.

been directed by the pC19S-35 vector was fully sensitive to Endo H when recovered from the cells (longer exposure of gel not shown), whereas the molecules obtained from the medium dislayed partial sensitivity to the enzyme treatment. Thus, the mutant proteins derived from pC19-22 and pC19-35 displayed very similar glycosylation patterns.

These data demonstrate that most if not all of the electrophoretic heterogeneity displayed by the three types of E19 mutants is due to variations in the carbohydrate processing of the translation products.

Intracellular transport and secretion of the E19 mutant proteins

To examine whether the heterogeneity in the glycosylation pattern of the E19 mutant proteins reflected differences in the rate of intracellular transport of the three proteins, pulse-chase experiments were carried out. HeLa cells transfected with the three DNA constructs were labeled with [^{35}S]methionine for 20 min. Subsequently the radioactivity was chased by cultivating the cells in an excess of unlabeled methionine for various periods of time. E19 mutant proteins were immunoprecipitated and analyzed by SDS-PAGE. Figure 5 summarizes the results.

The construct pC19S-6 initially gave rise to two electrophoretically resolved E19 components, which exclusively occurred intracellularly. After 30 min of chase the major portion of the intracellular radioactive material occurred as a doublet with diminished apparent molecular weights as compared to the material present at the end of the labeling period. This reduction in the apparent molecular weight is consistent with the trimming of the core sugar moieties, as has previously been described for the intact E19 protein (Kornfeld and Wold, 1981). However, a novel species with slightly higher apparent molecular weight occurred and this form of the protein was also present in the culture medium. As the chase period was prolonged the latter form of the E19-β-globin fusion protein accumulated in the medium but substantial amounts of the lower molecular weight material, which remained as an electrophoretic doublet, also emerged extracellularly. Separate experiments (not shown) suggested that the highest molecular weight species of the E19-β-globin fusion protein contained one carbohydrate moiety sensitive to Endo H digestion while the material in the doublet contained two such carbohydrate moieties (cf. Figure 4).

During the pulse-chase experiment the E19 mutant protein derived from the vector pC19S-22 exhibited electrophoretic characteristics very similar to those of the fusion protein. Thus, Figure 5B shows that at the end of the labeling period the mutant protein was confined to intracellular compartments. The doublet band material on SDS-PAGE occurred intracellularly with a diminished apparent molecular weight after 30 min of chase. A higher molecular weight species, however, could be discerned both in the cells and in the medium. While the latter components and an even higher molecular weight species of the protein accumulated in the medium, only minor amounts of the material corresponding to the major intracellular forms of the mutant protein occurred in the medium. Endo H-digestion of the secreted products demonstrated that only the forms corresponding to the major intracellular species comprised core-glycosylated molecules (not shown). However, most molecules seemed to have at least one of their carbohydrate moieties in terminally glycosylated form.

The major portion of the E19 mutant protein generated from the vector pC19S-35 emerged as an electrophoretic doublet by the end of the labeling period, and its localization was exclusively intracellular (Figure 5C). After 30 min of chase the doublet seemed to display a somewhat increased electrophoretic mobility. Concomitantly, the medium contained substantial amounts of the most slowly migrating form of the E19 mutant protein species. On prolonging the chase period the highest molecular weight species was accompanied in the medium by molecules indistinguishable from the intracellular doublet. Enzymatic treatment with Endo H revealed that virtually all of the extracellular E19 components had their carbohydrate moieties in terminally glycosylated form (not shown). However, small amounts of material, corresponding in electrophoretic mobility to the intracellular species, displayed one core-glycosylated carbohydrate moiety.

All three DNA constructs gave rise to low molecular weight material reactive with the anti-E19 antibodies (see Figures 3 and 5). The nature of this material was not examined but previous studies have shown that the intact E19 protein occurs in two forms, one of which contains but a single carbohydrate moiety (Storch and Maizel, 1980).

Autoradiograms similar to those of Figure 5 were analyzed by densitometry and the relative amounts of the E19 mutant proteins present intracellularly and in the medium were calculated. Figure 6 shows that the rate of transport was identical for the E19 $-\beta$-globin fusion protein and the E19 mutant corresponding to the lumenal domain. However, the rate of intracellular transport was considerably higher for the mutant containing the cytoplasmic tail.

Binding of the E19 mutant proteins to human class I MHC antigens

It has previously been suggested that intact E19 protein and class I major histocompatibility antigens may interact via the transmembrane and intracytoplasmic domains (Signäs *et al.*, 1982). To test this proposal we made use of an affinity system where highly purified, papain-solubilized class I antigens were covalently coupled to CNBr-activated Sepharose 4B. As controls bovine serum albumin, retinol-binding protein and β_2-microglobulin, the small subunit of class I antigens, were separately coupled to Sepharose 4B. Adenovirus 2-infected cells were labelled with [^{35}S]methionine and lysed. The isolated glycoprotein fraction was divided into aliquots that were incubated overnight at +4°C with the various Sepharose-coupled proteins. After desorption, proteins were subjected to indirect immunoprecipitation followed by SDS-PAGE. Figure 7A shows that no radioactive protein reactive with the antibodies was recovered from the Sepharose con-

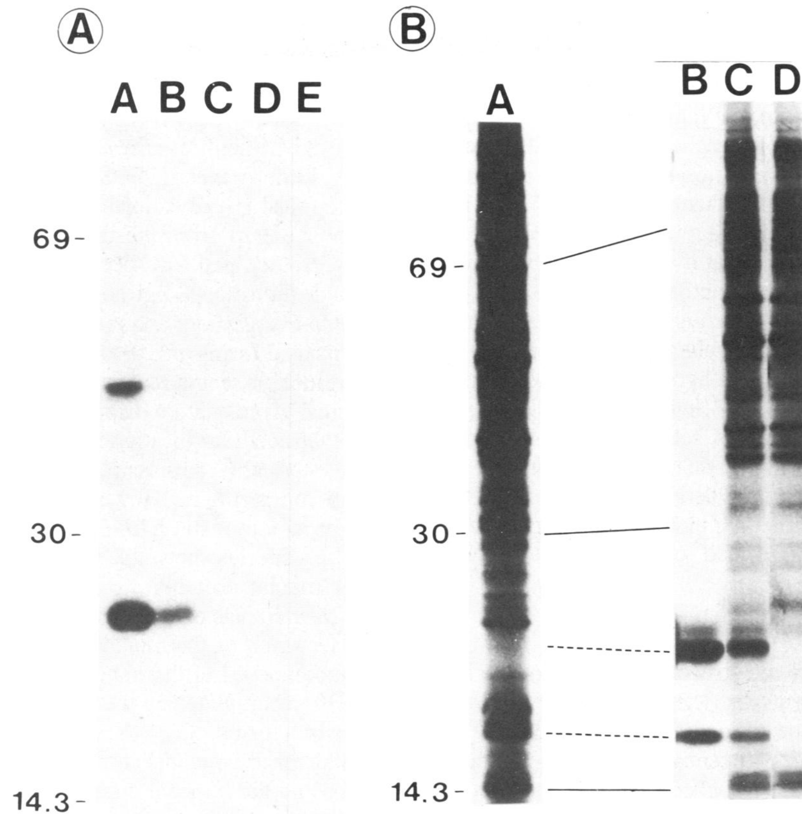

Fig. 7. A. Binding of the native E19 protein to MHC class I antigens. HeLa cells were infected with adenovirus 2 and labelled for 4 h with [^{35}S]methionine. From an aliquot of a glycoprotein fraction of the lysed cells, the E19 protein was isolated by indirect immunoprecipitation **(lane A)**. Other aliquots of the glycoprotein fraction were incubated with Sepharose-coupled papain solubilized class I antigens **(lane B)**, bovine serum albumin **(lane C)**, β_2-microglobulin **(lane D)** and the retinol-binding protein **(lane E)** in the presence of 1% Triton X-100. After washing in phosphate-buffered saline containing 1% Triton X-100, proteins were eluted with 0.1 M glycine–HCl, pH 2.5, also containing detergent. The eluate was neutralized by an equal volume of 1 M Tris–Cl, pH 8.0, and immunoprecipitations were performed with a rabbit antiserum to the E19 protein. Precipitated proteins were analyzed by SDS-PAGE and autoradiographed. **B.** Binding of mutant E19 protein to MHC class I antigens. Medium from cells transfected with pC19S-22 and labelled for 4 h with [^{35}S]methionine **(lane A)** was separately incubated with Sepharose-coupled class I antigens **(lane C)** and β_2-microglobulin **(lane D)**. Proteins were eluted as described above and directly analyzed by SDS-PAGE and autoradiography. **Lane B** illustrates the mutant E19 protein isolated from the medium by indirect immunoprecipitation. Migration positions of molecular weight markers are given in kilodaltons and indicated by solid lines. The migration positions of the two major forms of the E19 mutant protein are indicated by dashed lines.

taining albumin, retinol-binding protein or β_2-microglobulin (lanes C–E). However, the papain-solubilized class I antigens bound a substantial amount of the E19 protein (cf. lanes A and B). These data demonstrate that the affinity system reproduces the *in vivo* specificity of the interaction between class I antigens and the E19 protein (Kämpe *et al.*, 1983).

To examine whether the mutant E19 proteins displayed affinity for class I antigens, the medium of [^{35}S]methionine-labelled HeLa cells, which had separately been transfected with the three vectors, was divided into aliquots that were incubated with Sepharose containing covalently bound proteins as described above. Equal amounts of desorbed material were subjected to SDS-PAGE with and without prior immunoprecipitation of E19-reactive molecules. Figure 7B summarizes a typical experiment carried out with the vector pC19S-22. It can be seen that the mutant E19 protein in the culture medium is present in trace amounts (lane A). However, the mutant protein is efficiently bound by the immobilized class I antigens (lane C) but not by β_2-microglobulin (lane D) or any of the other proteins (not shown) despite the fact that a larger number of unrelated proteins bound non-specifically to the matrices. The bound mutant protein was enriched greater than 500-fold.

The binding of the three mutant E19 proteins to immobilized

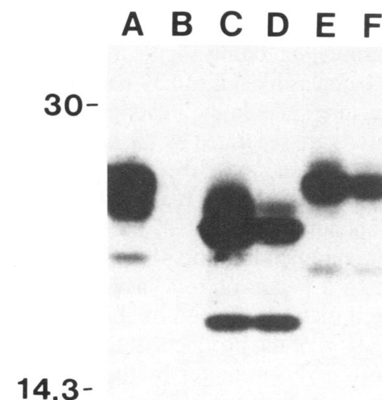

Fig. 8. Binding of the various E19 protein mutants to MHC class I antigens. Aliquots of medium from HeLa cells transfected with pC19S-6 **(lanes A** and **B)**, pC19S-22 **(lanes C** and **D)** and pC19S-35 **(lanes E** and **F)** were directly subjected to indirect immunoprecipitation using a rabbit antiserum to the E19 protein **(lanes A, C** and **E)**. Alternatively, the medium was incubated with Sepharose-coupled class I antigens as described in Figure 7 and the mutant proteins were isolated by indirect immunoprecipitation from the eluates. Precipitated proteins were analyzed by SDS-PAGE and autoradiography. Migration positions of molecular weight markers are indicated in kilodaltons.

class I antigens was assessed by incubating aliquots of the culture medium of cells transfected with the vectors and labelled with [^{35}S]methionine with Sepharose containing covalently bound class I antigens, bovine serum albumin, the retinol-binding protein and β_2-microglobulin, respectively. In each case, labelled proteins desorbed from the Sepharose were immunoprecipitated with an anti-E19 serum. For comparison, mutant proteins were also immunoprecipitated directly from an aliquot of the culture medium. Analyses by SDS-PAGE revealed that no mutant proteins bound to Sepharose containing the three control proteins (not shown) while the mutant proteins derived from vectors pC19S-22 and pC19S-35 bound efficiently to the Sepharose-coupled class I antigens (Figure 8, lanes D and F). The hybrid E19$-\beta$-globin protein, however, did not measurably interact with the class I antigens (Figure 8, lane B). Thus, it seems reasonable to conclude that the mutant E19 proteins that were truncated in the COOH-terminal region retained considerable affinity for the class I antigens, while the hybrid protein, although containing an identical segment of the E19 protein, had lost its ability to interact with class I molecules.

Discussion

As a first attempt to delineate the structural features of the E19 protein that are responsible for its restricted intracellular transport, on the one hand, and its association with class I molecules, on the other, we have mutated the E19 gene such that it should direct the synthesis of non-membrane attached forms of the protein. Three modifications were made but the point of intervention in the E19 gene was the same in all constructs. Thus, the sequence encoding the lumenal domain of the protein was terminated immediately prior to the two proline codons (positions 97 and 98 in Figure 1C). Such adjacent prolines frequently occur in flexible peptide segments that connect the extracellular portion of integral membrane proteins with the transmembrane domain (see e.g. Kimball and Coligan, 1983; Larhammar *et al.*, 1983). Mutations introduced at this point may consequently leave the conformation of the lumenal domain intact. Thus, construct pC19S-22 encodes only the glycosylated portion of E19 that is normally exposed on the lumenal side of the membrane. In another construct, pC19S-35, this part of the protein was directly fused to the cytoplasmic tail by deleting all but four codons of the transmembrane-encoding segment of the gene. It is conceivable that the cytoplasmic tail of E19 has an independent three-dimensional structure since in the intact protein the lumenal and cytoplasmic portions are separated by the transmembrane domain. An attempt was also made to deliberately perturb the folding of the lumenal portion of E19. We reasoned that in frame joining of the lumenal-encoding segment of the E19 gene with the β-globin gene segment encoding the 48 COOH-terminal residues should give rise to a fusion protein in which the conformation of the E19 protein may be affected by the β-globin part. To increase the expression of the products of the three constructs, we replaced the SV40 early promoter by the human α-globin promoter in all three vectors. The SV40 early promoter is not very efficient due to a relatively 'weak' TATA-box (Vigneron *et al.*, 1984) which unlike other TATA-boxes is not flanked by GC-rich sequences. In contrast, the human α-globin gene has been reported to be transcribed efficiently in some constructions even in the absence of a linked viral enhancer (Humphries *et al.*, 1982).

When transfected into HeLa cells the vectors generated the expected proteins which were all efficiently secreted to the medium. Each protein appeared heterogeneous on SDS-PAGE analysis. However, Endo-H treatment of the translation products strongly suggested that the heterogeneity was due to variations in the carbohydrate processing. All proteins secreted to the medium displayed a greater extent of Endo-H resistance than the intracellular forms. This observation in conjunction with the pulse-chase experiments confirm earlier reports (Rose and Bergmann, 1983; Sambrook *et al.*, 1985; Doyle *et al.*, 1985) demonstrating that terminal glycosylation of secreted proteins is rapidly followed by their export from the cell and that the rate-limiting step in the secretory pathway occurs prior to transit of the proteins through the trans-Golgi compartment.

Endo-H digestions and pulse-chase experiments established that the mutated forms of E19 could become terminally glycosylated. Therefore, it seems reasonable to conclude that the absence of terminal glycosylation displayed by the intact E19 protein is due to its intracellular location rather than its carbohydrate moieties being sterically inaccessible to the glycosyl transferases of the Golgi compartment. However, steric factors and/or a changed conformation of the E19$-\alpha$-globin fusion protein may explain why this species showed less terminal glycosylation than the two other mutant proteins.

Measurements of the relative rates of the intracellular transport and secretion of the mutant E19 proteins demonstrated that the form composed of the joined lumenal and cytoplasmic domains of E19 accumulated in the medium 2- to 3-fold faster than the other two forms. Since the degree of terminal glycosylation was similar for the mutants composed of the lumenal-cytolasmic domains and the lumenal domain, respectively, it can be concluded that the different rates of intracellular transport of the E19 mutants is not due to variations in the carbohydrate moieties. Instead, it may be suggested that the mutant forms of E19 interact differently with putative adaptor molecules responsible for the exit from the endoplasmic reticulum (see Blobel, 1980; Fries *et al.*, 1984). It is conceivable that such interactions may be the rate-limiting step in the intracellular transport.

In contrast to a previous report (Signäs *et al.*, 1982), which concluded that the interaction between E19 and class I antigens occurs through the transmembrane and cytoplasmic domains, our findings document that the interaction engages the lumenal portion of E19. This conclusion is born out by the demonstration that the mutant form of E19 comprising the lumenal portion of the molecule only binds to papain-solubilized class I antigens. Moreover, the failure of the E19$-\beta$-globin fusion protein to measurably bind to the class I molecules may be interpreted to mean that the conformation of the lumenal part of E19 is of importance of the interaction. However, it cannot be excluded from our data that the membrane-spanning and intracellular part of E19 in a specific or non-specific way contributes to the binding of the class I antigens. In fact, the interaction between the class I antigens and the lumenal form of E19 is obviously weaker than the interaction between class I antigens and the intact form of the protein, since the mutant proteins are not retained on the cell-surface in conjunction with the class I molecules . However, this observation may just reflect the fact that membrane-anchored E19 molecules as well as class I molecules are confined to the same extremely small volume, which is defined by the phospholipid bilayer of the endoplasmic reticulum, and allows mobility of the molecules in only two dimensions (Cohen and Eisen, 1977). In addition, none of the mutant E19 proteins occur as dimers while this is the case for the intact protein (unpublished observations). Dimerization may accordingly allow multi-point association between E19 and class I molecules and hence strengthen the interaction.

The present data suggest that the properties of the intact E19

protein to bind class I antigens and to be confined to intracellular compartments are endowed in different parts of the molecule. While the structures responsible for the association of E19 with class I antigens preferentially occur in the lumenal portion of the molecule, the regions conferring an intracellular localization of E19 seem to be confined to the transmembrane and possibly the cytoplasmic segments of the protein.

Materials and methods

Recombinant DNAs

The eukaryotic expression vector pA81G, which directs the synthesis of the adenoviral E19 protein, has been previously described (Pääbo *et al.*, 1983). The 220-bp-long leader sequences of this vector were shortened by deleting an 80 bp *Hin*dIII to *Dde*I fragment (nt 1286 – 1366 of the sequence published by Hérissé *et al.*, 1980). This construct is called pB81G.

A *Hin*fI fragment of the α-globin gene, spanning the region from nt -112 to $+19$ respective to the site of transcription initiation, was subcloned into the *Pst*I site of *pUC9* and the SV40 72-bp repeat enhancer was inserted upstream of the promoter. This promoter – enhancer element was first tested by cloning it in front of the SV40 T-antigen coding region and the resulting construct was transfected into HeLa cells. The cells were fixed after 2 days and stained for T-antigen by indirect immunofluorescence. A large number of stained nuclei was found, indicating strong promoter activity of the α-globin fragment (not shown). The SV40 promoter in vector pB81G was then replaced by this efficient promoter element leading to construct pC81G.

The E19 gene was cut by endonuclease *Hae*III at nt 1778, removing the region coding for 46 amino acids at the carboxy terminus. This gene fragment was fused to the filled-in *Bam*HI site of the rabbit β-globin gene contained in the plasmid pC81G. Since the β-globin sequences can be translated in the normal reading frame, an E19 – β-globin fusion protein is expected to be expressed from this construct, called pC19S-6 (Figure 1B).

An oligonucleotide linker was chemically synthesized. This molecule encodes UGA triplets for translation termination in all three possible reading frames (Figure 1B) and has at its 3′ end a protruding 5′ end complementary to the *Bam*HI cohesive end. This stop-linker was ligated between the *Hae*III site, used for the previous construct, and the *Bam*HI site of the rabbit β-globin gene leading to construct pC19S-22 (Figure 1B).

Finally, an E19 internal fragment from the *Hae*III site (1778) to the *Rsa*I site at nucleotide position 1856, which encodes 26 amino acids, was deleted, yielding construct pC19S-35 (Figure 1B).

All these DNA constructs were made using standard recombinant DNA techniques (Maniatis *et al.*, 1982). For verification, the nucleotide sequences of the mutant regions were determined in pC19S-22 and pC19S-35 (not shown).

Cell culture and DNA transfection

Cell culture of HeLa cells has been described (Pääbo *et al.*, 1983). Transfections were performed as described by Banerji *et al.* (1981) with the following modifications. Cells were trypsinized 4 h before transfection. The pH of the transfection solutions was carefully titrated in steps of ~ 0.01 around pH 6.80 and each step was tested in a transfection assay. The pH yielding the highest transient expression of the SV40 large T-antigen in HeLa cells transfected with SV40 DNA was determined. Under these conditions 60 – 70% of the cells were positive when stained for large T-antigen by indirect immunofluorescence 48 h after transfection.

Antisera

The antiserum against the E19 protein has been described previously (Persson *et al.*, 1979) as has the monoclonal antibody, directed against the lumenal portion of the E19 protein (Severinsson and Peterson, 1985) and the antiserum, reactive with the intracellular domain of the E19 protein (Andersson *et al.*, 1985).

Other procedures

S1 nuclease mapping, using a 3′ specific probe labelled at the *Bam*HI site of the rabbit β-globin cDNA, was done according to the method of Weber and Schaffner (1985).

Metabolic labeling of cells, immunoprecipitations, Endo H digestions and SDS-PAGE have been described (Pääbo *et al.*, 1983; Severinsson and Peterson, 1985). Kodak XAR film was used throughout. For quantitation of protein bands, fluorographs were scanned using a Bio-Rad densitometer. Coupling of proteins to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed according to the instructions of the manufacturer.

Acknowledgements

We thank Peter Lind for synthesis of the stop-linker and Christina Plöen for manoeuvring the word processor. This work was supported by the Swedish Cancer Society, The Swedish Natural Research Council, the Kanton of Zürich, the Swiss National Research Foundation (grant No. 3.603.84) and an EMBO short-term fellowship to F.W.

References

Andersson,M., Pääbo,S., Nilsson,T. and Peterson,P.A. (1985) *Cell*, **43**, 215 – 222.

Banerji,J., Rusconi,S. and Schaffner,W. (1981) *Cell*, **27**, 299 – 300.

Blobel,G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1496 – 1500.

Burgert,H.-G. and Kvist,S. (1985) *Cell*, **41**, 987 – 997.

Chow,L.T., Broker,T.R. and Lewis,J.B. (1979) *J. Mol. Biol.*, **134**, 265 – 303.

Cohen,R.J. and Eisen,H.N. (1977) *Cell Immunol.*, **32**, 1 – 9.

Doyle,C., Roth,M.G., Sambrook,J. and Gething,M.-J. (1985) *J. Cell Biol.*, **100**, 704 – 714.

Fries,E., Gustafsson,L. and Peterson,P.A. (1984) *EMBO J.*, **3**, 147 – 152.

Herisse,J., Courtois,G. and Galivert,F. (1980) *Nucleic Acids Res.*, **8**, 2173 – 2192.

Humphries,R.K., Ley,T., Turner,P., Moulton,A.D. and Nienhuis,A.W. (1982) *Cell*, **30**, 173 – 183.

Kämpe,O., Bellgrau,D., Hammerling,U., Lind,P., Pääbo,S., Severinsson,L. and Peterson,P.A. (1983) *J. Biol. Chem.*, **258**, 10594 – 10598.

Kimball,E.S. and Coligan,J.E. (1983) In *Contemporary Topics in Molecular Immunology*, Vol. 9, Plenum Press, New York and London, pp. 1 – 63.

Kornfeld,R. and Wold,W.S.M. (1981) *J. Virol.*, **40**, 440 – 449.

Kvist,S., Oestberg,L., Persson,H., Philipson,L. and Peterson,P.A. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5674 – 5678.

Larhammar,D., Andersson,G., Andersson,M., Bill,P., Böhme,J., Claesson,L., Denaro,M., Emmoth,E., Gustafsson,K., Hammerling,U., Heldin,E., Hyldig-Nielsen,J.J., Lind,P., Schenning,L., Servenius,B., Widmark,E., Rask,L. and Peterson,P.A. (1983) *Hum. Immunol.*, **8**, 95 – 103.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning*. Cold Spring Harbor Publications, Cold Spring Harbor, New York.

Pääbo,S., Weber,F., Kämpe,O., Schaffner,W. and Peterson,P.A. (1983) *Cell*, **33**, 445 – 453.

Persson,H., Signäs,C. and Philipson,L. (1979) *J. Virol.*, **29**, 938 – 948.

Rose,J.K. and Bergmann,J.E. (1983) *Cell*, **34**, 513 – 524.

Sambrook,J., Rodgers,L., White,J. and Gething,M.-J. (1985) *EMBO J.*, **4**, 91 – 103.

Severinsson,L. and Peterson,P.A. (1985) *J. Cell Biol.*, **101**, 540 – 547.

Signäs,C., Katze,M.G., Persson,H. and Philipson,L. (1982) *Nature*, **299**, 175 – 178.

Storch,T.G. and Maizel,J.V.,Jr. (1980) *Virology*, **103**, 54 – 67.

Tarentino,A.L. and Maley,F. (1974) *J. Biol. Chem.*, **294**, 811 – 817.

Vigneron,M., Barrera-Saldana,J.A., Baty,D., Everett,R.E. and Chambon,P. (1984) *EMBO J.*, **3**, 2373 – 2382.

Weber,F., de Villiers,J. and Schaffner,W. (1984) *Cell*, **36**, 983 – 992.

Weber,F. and Schaffner,W. (1985) *Nature*, **315**, 75 – 77.

Zinkernagel,R.M. and Doherty,P.C. (1974) *Nature*, **248**, 701 – 702.

Received on 23 December 1985; revised on 2 May 1986