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A Short Sequence in the COOH-Terminus Makes an Adenovirus Membrane Glycoprotein a Resident of the Endoplasmic Reticulum

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Summary

The E19 protein of adenoviruses is a transmembrane protein that abrogates the intracellular transport of class I antigens by forming complexes with them in the ER. We show here that the E19 protein is retained in the ER even in the absence of class I antigens. To define the region conferring residency in the ER, we examined two mutant forms of the viral protein. A 5 amino acid extension of the 15-membered cytoplasmic tail of the protein reduces its interaction with class I antigens but does not change its intracellular distribution. Shortening the tail to 7 amino acids also diminishes the affinity for class I antigens; however, this mutant E19 protein becomes transported to the cell surface. Thus, we concluded that a small stretch of amino acids exposed on the cytoplasmic side of the ER membrane is responsible for the retention of the E19 protein in the ER.

Introduction

The rules governing the targeting of proteins to various subcellular organelles are far from understood. However, defined linear sequences endowed in the structure of the proteins have been implicated as targeting signals for the endoplasmic reticulum (ER) (Blobel and Dobberstein, 1975), the mitochondria (Horwich et al., 1985), and the nucleus (Kalderon et al., 1984). Proteins that have entered the ER may contain additional signals since some proteins occur only transitorily in this organelle en route to other destinations, while other proteins permanently reside in the ER. Two alternative hypotheses have been put forth to account for the sequestration of proteins in the ER. Thus, it is conceivable that putative signals promote the exit of proteins from the ER, while the absence of such signals renders proteins confined to this locality (Fitting and Kabat, 1982). Alternatively, the export of proteins from the ER may be a constitutive process such that signals are needed to retain proteins in the organelle (Kelly, 1985). One way to resolve these possibilities is to examine the effects of various defined structural elements of resident ER proteins on the subcellular distribution of these proteins.

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The E19 protein of the adenovirus family appears to represent a suitable object for such studies. This viral protein appears at early times during an infection (Chow et al., 1979), binds specifically to class I antigens of the major histocompatibility complex (Kvist et al., 1978; Signäs et al., 1982; Pääbo et al., 1983; Kämpe et al., 1983), and, due to its localization in the ER, abrogates the intracellular transport of the class I antigens (Severinsson and Peterson, 1985; Burgert and Kvist, 1985; Andersson et al., 1985).

The primary structure of the E19 protein (Hérissé et al., 1980; Persson et al., 1980) consists of a signal sequence of 17 amino acids (Wold et al., 1985) followed by an intraluminal domain of 104 amino acids. This portion of the E19 protein is separated from a cytoplasmic tail of 15 amino acids by 23 membrane-spanning residues. The intraluminal domain carries two Asn-linked carbohydrate moieties that always occur in the core-glycosylated form (Kornfeld and Wold, 1981). Mutant E19 proteins lacking the membrane-spanning and the cytoplasmic segments are secreted from cells but retain affinity for class I antigens (Pääbo et al., 1986a). This suggests that the signals for retention in the ER might reside in the membrane-spanning and/or cytoplasmic portions of the protein.

In an attempt to identify more specifically the region responsible for the intracellular localization of the E19 protein we have examined two mutant adenoviruses that produce E19 proteins whose cytoplasmic tails have been changed. We show that shortening of the tail by 8 amino acids promotes intracellular transport of the E19 protein, while an extension of the tail by 5 amino acids does not affect its intracellular localization. Thus, a short linear sequence appears responsible for rendering the E19 protein a resident of the ER.

Results

The E19 Protein Is a Resident of the Endoplasmic Reticulum

Our previous studies have shown that the E19 protein is a resident of the ER (Andersson et al., 1985). Since the E19 protein binds to class I antigens in this locality it could not be ruled out that the retention of the E19 protein in the ER was caused by its complex formation with class I antigens, rather than by an intrinsic property of the E19 protein. To explore whether class I antigens had any role in impeding the intracellular transport of the E19 protein, we infected the murine cell line C57AT1 (Maeta and Hamada, 1979) with adenovirus-2. In agreement with other workers (Tanaka et al., 1985), we were unable to detect any class I antigen transcripts in these cells. While the infection proceeded at a rate slower than in human cells, at 16 hr after the onset of the infection substantial quantities of the E19 protein were present in the C57AT1 cells. This was visualized by indirect immunofluorescence staining of permeabilized cells that had been treated with a monoclonal antibody against the E19 protein (Figure 1). When infected cells that had not been permeabilized were sub-

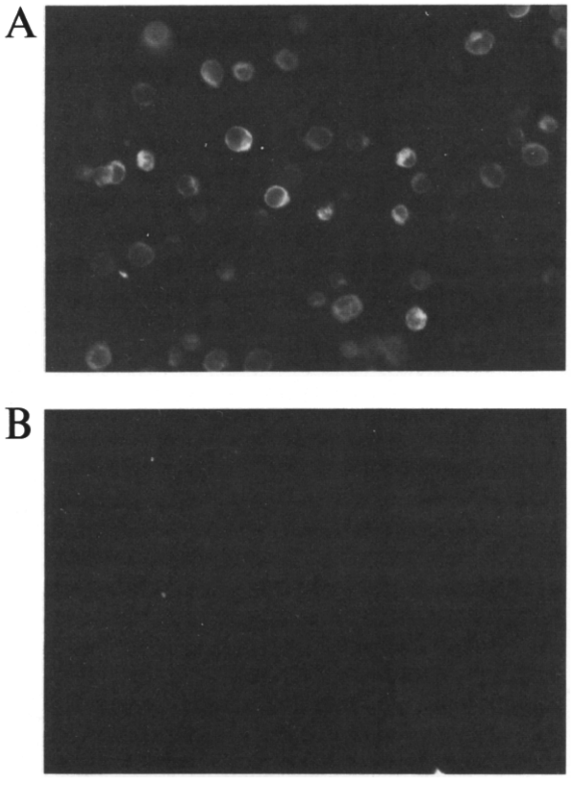

Figure 1. Visualization of the E19 Protein in Adenovirus-2-Infected C57AT1 Cells by Immunofluorescence Microscopy

Cells were infected with adenovirus-2, and 16 hr later they were detergent-permeabilized and fixed (A) or only fixed (B) before being incubated with a monoclonal antibody to the E19 protein. Antibody binding was visualized by staining with fluorescein-conjugated rabbit anti-mouse immunoglobulin antibodies.

jected to the same treatment, no antibody staining could be demonstrated, suggesting that the E19 protein did not exist on the cell surface. Consequently, it seems reasonable to conclude that the E19 protein is also confined to the ER in cells that do not express class I antigens.

Effects of Mutant E19 Proteins on the Binding and Cell-Surface Expression of Class I Antigens

We have previously shown that the intraluminal domain of the E19 protein binds to class I antigens and that this portion of the viral protein, when detached from the intramembranous and cytoplasmic parts, becomes secreted from cells (Pääbo et al., 1986a). In an attempt to localize the region of the E19 protein that confers residency of the viral protein to the ER, we examined some mutant viruses that give rise to E19 proteins with modified cytoplasmic tails (Deutscher et al., 1985).

Figure 2 illustrates the mutant proteins that were used in the present study. Mutant dl712 has a deletion encompassing nucleotides 1691–2122 (for nucleotide numbering, see Cladaras and Wold, 1985). This deletion begins 7 nucleotides downstream of the E19 termination codon at nucleotide 1681. Since this mutant does not affect the structure of the E19 protein, it was used as the control.

Mutant dl708 lacks nucleotides 1654–2207, such that

dl 712	KYKSRRSFIDEKKMP
dl 708	KYKSRRL
dl 710	KYKSRRSFIDEKKMPGSRTV

Figure 2. Amino Acid Sequences of the Cytoplasmic Segments of the Wild-Type E19 Protein of Adenovirus-2 and of the Mutant Proteins Made from Mutant Viruses dl708 and dl710

The dl712 sequence is identical to that of the wild-type E19 protein of adenovirus-2.

the last 9 codons from the E19 gene are removed. The addition of an XbaI linker introduces a leucine at the carboxy terminus of the protein, which consequently is 8 amino acids shorter than the wild-type molecule.

Mutant dl710 does not contain the normal termination codon since nucleotides 1679–2123 were deleted. Instead, 5 new codons followed by a termination codon were generated by the downstream sequences and the introduction of a BamHI linker. Thus, this mutant gives rise to an E19 protein whose COOH-terminal region is extended by 5 amino acids.

The three mutant viruses were used to infect HeLa cells, and 4 hr after the onset of the infection, the cells were labeled with [^{35}S]methionine for 4 hr. Following the isolation of a detergent-solubilized glycoprotein fraction, aliquots were treated with a monoclonal antibody against the luminal domain of the E19 protein and a rabbit antiserum against class I antigens, respectively. Proteins immunoprecipitated by the rabbit antiserum were subjected to endoglycosaminidase H (Endo H) treatment prior to SDS–PAGE. This enzyme removes Asn-linked carbohydrate moieties that have not been finally trimmed and terminally glycosylated (Tarentino and Maley, 1974).

The autoradiogram depicted in Figure 3 shows that all three mutant viruses gave rise to E19 proteins of the expected sizes. Thus, dl708 manufactured a protein that is slightly smaller than the wild-type product of dl712, while dl710 gave rise to a slightly larger protein. The labeled class I antigens of the virally infected cells were partially sensitive to the enzymatic digestion, while class I antigens of mock-infected cells exhibited almost complete resistance to the enzyme treatment. All three forms of the E19 protein had both their Asn-linked sugar moieties in the high-mannose form (Kornfeld and Wold, 1981) as they were completely sensitive to the Endo H digestion.

It has earlier been shown that when low levels of the E19 protein are expressed in cells infected with wild-type adenovirus-2 the class I antigens segregate into two populations. One population is retained in the ER by the viral protein, while the other becomes transported to the cell surface (Severinsson and Peterson, 1985; Severinsson et al., 1986). Since the mutant viruses express considerably less of the E19 protein than the wild-type virus, the partial resistance to Endo H by the class I antigens might reflect the existence of two such populations. However, the data would also be consistent with the viral mutants allowing the transport of the class I antigens, albeit at a slow rate. To examine these possibilities, pulse-chase experiments were carried out.

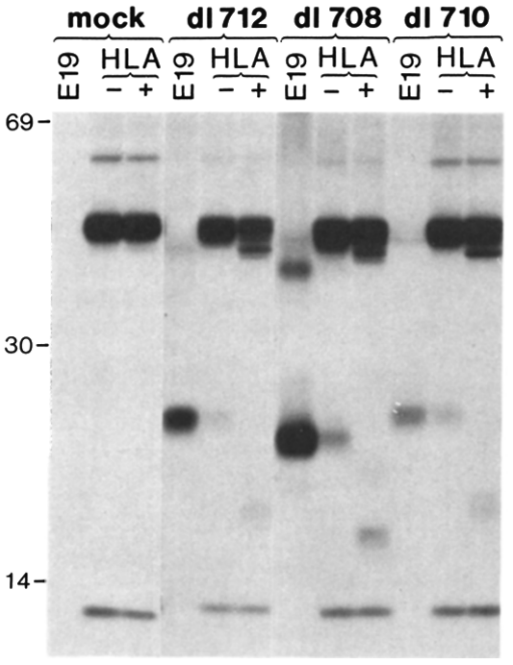

Figure 3. Coprecipitation of Mutant E19 Proteins with MHC Class I Antigens and Partial Inhibition of Class I Antigen Carbohydrate Processing

HeLa cells were infected with the mutant viruses indicated or mock-infected as described in Experimental Procedures. Four hours postinfection they were metabolically labeled with [^{35}S]methionine for 4 hr. After solubilization, glycoprotein fractions were isolated and aliquots were subjected to indirect immunoprecipitation using a monoclonal antibody to the E19 protein or a rabbit antiserum to MHC class I antigens. Half of the class I antigens precipitated was in each case subjected to Endo H treatment (+) or mock treatment (–) prior to SDS-PAGE and fluorography. The migration positions of marker proteins are indicated by their molecular weights in kilodaltons.

Class I antigens of cells infected with dl712, which produces the wild-type form of the E19 protein, were only partly resistant to the action of Endo H even after a prolonged chase period. However, in cells infected with the two mutant adenoviruses that gave rise to altered E19 proteins all class I antigens eventually became Endo H–resistant. Concomitant with the terminal glycosylation of the class I antigen heavy chains, the coprecipitation of the E19 mutant proteins diminished despite the fact that the mutant viral proteins were still present in the cell (data not shown).

By densitometric analyses of autoradiographs, it was demonstrated that the half-life of the Endo H–sensitive class I antigen chains is prolonged 3- to 4-fold in cells infected with the mutant virus as compared with mock-infected cells. This diminished rate of intracellular processing of the class I antigen carbohydrate moiety is due to a diminished rate of intracellular transport, since the class I antigen cell surface expression of cells infected with all the mutant viruses could be shown to be drastically reduced, while the rate of elimination of class I antigens from the cell surface in all cases was identical (data not shown).

The Mutant E19 Protein with a Truncated Cytoplasmic Tail Is Transported to the Cell Surface

In view of the observation that class I antigens and the dl708 and dl710 mutant E19 proteins, respectively, are only transiently associated, it seemed possible that one or both of the E19 mutants might become transported to the cell surface. To examine this, cells infected with the three mutant viruses were subjected to flow cytofluorometry after indirect immunofluorescence staining with a monoclonal antibody directed against the luminal domain of the E19 protein. The wild-type E19 protein was never detected at the surface of cells infected with the dl712 mutant (Figure 4). The situation was identical with regard to cells infected with the dl710 mutant, which produced a form of the E19 protein that was extended by 5 amino acids in the COOH-terminal region. In contrast, the dl708 mutant E19 protein, which lacks 8 amino acids in the COOH-terminal region, was detected in increasing amounts on the cell surface as the infection proceeded. Measurable levels of the viral protein were recorded by 4 hr after onset of infection, and substantial amounts of the E19 protein occurred in the plasma membrane by 18 hr (Figure 4). To verify that the documented observations were unrelated to the varying affinities between the different forms of the E19 protein and the class I antigens, identical experiments were also carried out with the C57AT1 cells, which are devoid of class I antigen expression. Results in complete accord with those described above were obtained (data not shown).

To demonstrate that the lack of detectable cell surface expression of the wild type and dl710 mutant E19 proteins was not due to differences in their antigenic determinants as compared with those of the dl708 mutant protein, subcellular fractionation of infected cells was carried out. Four hours after the onset of the infection, the cells were labeled with [^{35}S]methionine for a period of 4 hr. Following subcellular fractionation by gradient centrifugation, the content of labeled E19 protein in the various fractions was assessed by indirect immunoprecipitation and SDS–PAGE. Figure 5 summarizes the results. It can be seen that in cells infected with the dl712 mutant the wild-type E19 protein had a distribution that agreed closely with the cytochrome c reductase activity, confirming that this form of the viral protein is confined to the ER. The mutant E19 protein of dl710, which has a COOH-terminal extension, had an identical subcellular distribution. As expected, the dl708 mutant protein displayed a different distribution such that the greatest amounts were present in the fractions enriched for galactosyltransferase activity and transferrin receptors, respectively. This result is, of course, in complete agreement with this mutant protein being transported through the Golgi complex to the cell surface.

Discussion

Our previous studies demonstrated that the luminal domain of the E19 protein, when detached from the membrane-spanning and cytoplasmic domains, becomes secreted

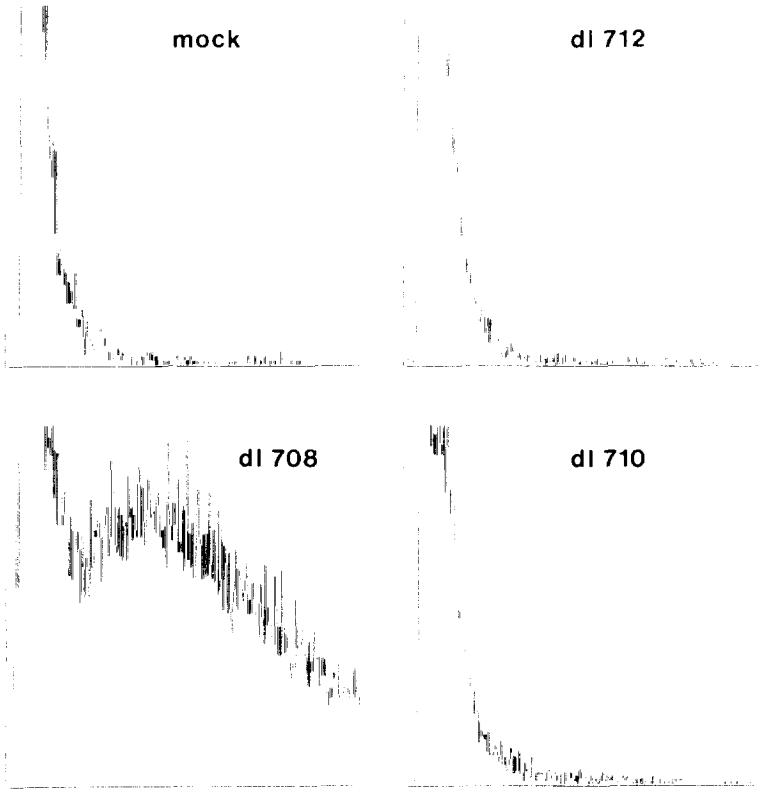

Figure 4. Flow Cytofluorometric Analysis of Cell-Surface Expression of the Various E19 Proteins in HeLa Cells Infected with the Virus Mutants

HeLa cells were mock-infected or infected with the indicated mutant viruses. After 18 hr, the cells were incubated with a monoclonal antibody to the E19 protein. Fluorescein-conjugated rabbit anti-mouse immunoglobulin antibodies were used for the secondary staining, and approximately 50,000 cells were analyzed by flow cytofluorometry. The relative fluorescence intensity is plotted on a linear scale against the numbers of cells counted.

via transport from the ER through the Golgi complex to the outside of the cell (Pääbo et al., 1986a). This information would suggest that portions of the intact viral protein other than the luminal domain are responsible for its retention in the ER. However, the detached luminal domain exhibits only weak affinity for class I antigens, so it could not be ruled out that it is the complex formation with class I antigens rather than any unique portion of the E19 protein that abolishes the exit from the ER. In the present study these alternatives were examined by expressing the E19 protein in cells that do not produce any class I antigens. Immunofluorescence staining of such cells clearly demonstrated that the intact E19 protein resides intracellularly, so it can be concluded that the viral protein is indeed endowed with the trait to be a resident ER protein.

The free luminal domain of the E19 protein does not associate with class I antigens during their intracellular transport (Pääbo et al., 1986a). This observation may be accounted for by the assumption that the intact viral protein, by being membrane-anchored, is confined to a more limited space and thereby occurs in a higher local concentration than the free luminal domain. However, in this study using two other mutant forms of the E19 protein, which are membrane-integrated but whose COOH-terminal regions have been changed, we observe that the cytoplasmic tail of the viral protein also seems to contribute to the association with the class I antigens. Thus, the E19 protein of the mutant dl710, which is not transported out of the ER, associates only transiently with the class I antigens. This is shown by the lack of coprecipitation of the two proteins at later times during the pulse-chase experiments and by the fact that all class I antigens of cells expressing the mutant E19 proteins eventually become resistant to the enzyme Endo H. The situation is similar with regard to the mutant dl708 E19 protein, which has a truncated cytoplasmic tail. In the latter case, however, it cannot be ruled out that the dissociation between the viral protein and the class I antigens occur in a compartment other than the ER, since this mutant E19 protein becomes transported to the cell surface (see below).

The intracellular transport of the class I antigens could be monitored by examining their glycosylation state. Conversion of their carbohydrate moiety from the core-glycosylated form to the complex form indicated that transport had occurred from the ER to the Golgi complex. Likewise, the intracellular transport of the free luminal domain of the E19 protein could be followed by similar means (Pääbo et al., 1986a). Such analyses did not prove informative as regards the membrane-anchored E19 mutant proteins, since their Asn-linked carbohydrate moieties always remained in the high-mannose form. However, subcellular fractionation and immunofluorescence staining experiments unequivocally demonstrated that the dl708 mutant produced an E19 protein that was transported to the cell surface. The analyses also revealed that the dl710 mutant E19 protein, like the wild-type protein, was retained in the ER. Consequently, a shortening of the cytoplasmic tail by 8 amino acids obviously eliminates the signal for residency in the ER while an extension of the tail by 5 amino acids leaves the signal intact. Furthermore, a chimeric

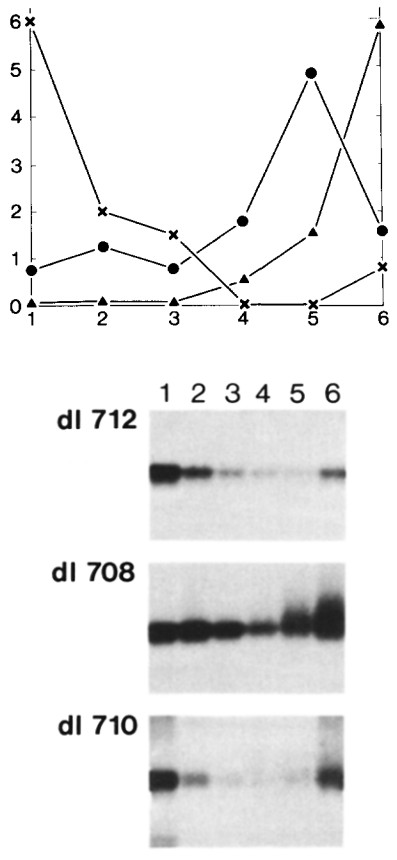

Figure 5. Subcellular Fractionation of HeLa Cells Infected with the Various Mutant Viruses

HeLa cells were infected and labeled 4 hr later with [^{35}S]methionine. After a labeling period of 4 hr, the cells were homogenized and fractionated as described in Experimental Procedures. Numbers 1 to 6 represent the fractions from the sucrose gradients from the bottom to the top. A representative result of the various assays for the subcellular markers is shown. All quantitations are recorded in arbitrary units. (x) NADPH cytochrome C reductase, (●) galactosyltransferase, (▲) transferrin receptor.

protein composed of the extracellular domain of the human interleukin-2 receptor β-chain (Leonard et al., 1984; Nikaido et al., 1984) and the cytoplasmic domain of the E19 protein remains confined to the ER (S. Broome, personal communication). Thus, a signal sufficient for the localization of integral membrane proteins to the ER exists in the COOH-terminus of the E19 protein.

In an attempt to define further the critical region for retention in the ER, a comparison of the sequences of the E19 proteins of adenovirus-2 (Hérissé et al., 1980), adenovirus-3 (Signäs et al., 1987), and adenovirus-5 (Wold et al., 1985) was made. Only the last 3 amino acids of the truncated region are identical in all three E19 proteins. Therefore, it is tempting to speculate that this sequence, Lys-Met-Pro, is crucial for the confinement of the E19 proteins to the ER.

Membrane proteins specific for the ER are efficiently retained in this organelle after their synthesis (Lewis et al., 1985; Brands et al., 1985). A general mechanism by which this retention occurs has not yet been identified, although an energy-sensitive translocation step within the ER has been implicated (Kabcenell and Atkinson, 1985). The present data are consistent with a linear sequence being responsible for the localization of the E19 protein to the ER. Whether this will turn out to be a feature as universal for ER proteins as the signal sequence is for the entry of proteins into the ER is a matter of conjecture only. Such a putative signal for residency in the ER cannot consist of an identical sequence in all proteins. This can be inferred from the fact that there are no obvious common sequence motifs among the ER proteins 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (Chin et al., 1984), the E1 glycoprotein of corona virus (Armstrong et al., 1984), cytochrome P-450 (Ozols et al., 1985), and the rotavirus proteins VP7 and NCVP5 (Both et al., 1983a, 1983b). In fact, the VP7 glycoprotein has a cytoplasmic tail that consists of the NH_2-terminal rather than the COOH-terminal region. However, this protein also becomes secreted upon elimination of a peptide stretch in the NH_2-terminal region (Poruchynsky et al., 1985), which suggests that residency in the ER is conferred by the cytoplasmically exposed portion of the molecule.

The observation that two viral membrane proteins that are residents of the ER can exit this organelle upon removal of short sequence stretches provides compelling evidence for the transport out of the ER being constitutive (Kelly, 1985). Furthermore, the recent observation that soluble ER proteins display a short sequence in their COOH-termini that is both necessary and sufficient for the retention of such proteins in the ER (Munro and Pelham, 1987) demonstrates that soluble as well as transmembrane ER proteins may be prevented from leaving the ER due to short sequence motifs. However, the cellular components recognizing these two classes of sequence motifs must be distinct, since they interact with luminal signals in the former case and with signals present in cytoplasmic domains of the proteins in the latter case.

Experimental Procedures

Adenovirus Mutants

The construction of the three mutants of the adenovirus-5 used in the present study has been outlined elsewhere (Deutscher et al., 1985). Briefly, the subcloned E19-coding region of the adenovirus-2 genome was cleaved at a restriction site downstream of the E19 gene. After digestion with Bal31, a BamHI or XbaI linker was ligated to the plasmid as it was recircularized. In all cases, the extent of the deletion and the number of linkers introduced were verified by sequencing. The entire mutated DNA fragment was then used to replace the corresponding fragment in the adenovirus-5 genome, the DNA was transfected into KB cells, and virus stocks were prepared. Adenovirus-2 and adenovirus-5 are very similar in sequence (Wold et al., 1985) and have been shown to affect the cell-surface expression of class I antigens identically (Pääbo et al., 1986b).

All three mutant viruses have been shown to cause changes in the splice pattern of the E3 region of the viral genome such that the coding region for the E19 protein is removed from a large proportion of the primary transcripts (Bhat et al., 1986). Consequently, the mutant viruses generate only 5%–15% as much E19 protein as the wild-type virus (Deutscher et al., 1985). However, to some extent this can be compensated for by increasing the multiplicity of the infection (see below).

Cell Cultures and Viral Infections

HeLa cells were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 100 U/ml penicillin, 100 μl/ml streptomycin, 4 mM glutamine, 2.5% fetal calf serum, and 2.5% newborn calf serum. Mouse L cells and C57AT1 cells (Maeta and Hamada, 1979) were grown in the same medium containing 10% fetal calf serum. Adenovirus-2 and the mutant viruses were grown in HeLa cells and purified as described by Green and Wold (1979). The amounts of infectious particles were determined by titration of fluorescence forming units (FFU) in HeLa cells, using a rabbit antiserum to the hexon protein (Philipson, 1968). C57AT1 cells and L cells were infected with 5 FFU per cell, while HeLa cells were infected with 25 FFU of the mutant viruses per cell in order to allow for more production of the E19 protein. In all cases, the virus was allowed to adsorb on the cells for 1 hr in serum-free medium before normal growth medium was added and the cells were cultivated for the times indicated.

Radioactive Labeling of Cells, Antibodies, Immunoprecipitations, Endo H Treatment, and SDS-PAGE

Continuous metabolic labeling of cells was carried out essentially as described (Pääbo et al., 1983). The protocol for the pulse-chase experiments is described by Andersson et al. (1985). After detergent lysis of the labeled cells, glycoprotein fractions were isolated by Lens culinaris hemagglutinin-Sepharose-4B affinity chromatography (Hayman and Crumpton, 1972). Immunoprecipitations and Endo H treatment were carried out as described (Andersson et al., 1985). SDS-PAGE was done according to Blobel and Dobberstein (1975) on 10%–15% gradient slab gels, and labeled proteins were visualized by fluorography (Bonner and Laskey, 1974).

The serological reagents used were a rabbit antiserum to human class I antigens (Rask et al., 1976), the W6/32 monoclonal antibody to human class I antigens (Barnstable et al., 1978), a mouse monoclonal antibody to E19 (Severinsson and Peterson, 1985), and a rabbit antiserum to purified E19 protein (Persson et al., 1979).

Immunofluorescence and Flow Cytofluorometry

Immunofluorescence staining of cells in suspension was performed as described (Andersson et al., 1985). When indicated, cells were permeabilized prior to staining by incubation in 0.1% Triton X-100 in PBS for 5 min at room temperature. After washing, the cells were fixed by incubation in a 4% solution of formaldehyde in PBS for 5 min at 4°C. The formaldehyde was quenched by incubating the cells in 0.1 M glycine in PBS for 10 min. Immunofluorescence staining and flow cytofluorometry were performed as described (Andersson et al., 1985).

To measure the elimination of class I antigens from the surface of virally infected cells, flow cytofluorometry was employed. Virally infected cells were treated with cycloheximide at a final concentration of 100 μl/ml. This concentration reduced the protein synthesis measured as incorporation of labeled methionine into total cellular protein to less than 10%. At various times after the addition of the protein synthesis inhibitor the cells were incubated with rabbit antibodies against class I antigens and subjected to flow cytofluorometry.

Subcellular Fractionations

Subcellular fractionations were performed essentially as described (Fries et al., 1984). Briefly, approximately 1×10^7 cells were sequentially washed once in the following solutions: 136 mM NaCl, 4.7 mM HEPES (pH 7.4); 250 mM sucrose; 50 mM sucrose. The cells were then suspended in 1 ml of 50 mM sucrose and homogenized by 15 strokes in a homogenizer (Contes, B pestle), 90 μl of a 65% sucrose solution was added, and the homogenate was centrifuged for 10 min at 3300 rpm. The supernatant was applied at the bottom of a continuous 45%–30% sucrose gradient and centrifuged at 2500 rpm in a Sorvall TST 28.38/17 rotor for 18 hr. Six fractions were eluted from each gradient, and membranes were pelleted and suspended in the lysis buffer used for immunoprecipitations (Andersson et al., 1985).

The assays for NADPH-cytochrome c reductase (Omura and Takesue, 1970) and galactosyltransferase (Rothman and Fries, 1981) were as described. The fractions containing plasma membranes were identified by metabolically labeling cells for 2 hr followed by a 2 hr chase period in an excess of unlabeled methionine and then immunoprecipitating the transferrin receptor from fractions with the OKT 9 antibody (Reinherz et al., 1980).

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