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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., 1965), and the nucleus (Kalderon et al., 1984). Proteins that have entered the ER may contain additional signals since some proteins occur only transiently 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.

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; Anderson et al., 1985). The primary structure of the E19 protein (Héristé 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., 1986). 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 (Anderson 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-
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 (Pålbo 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 d1712 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 d1708 lacks nucleotides 1654-2207, such that 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 d1710 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 [35S]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 antisera against class I antigens, respectively. Proteins immunoprecipitated by the rabbit antiserum were subjected to endoglucosaminidase 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, d1708 manufactured a protein that is slightly smaller than the wild-type product of d1712, while d1710 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.
our studies demonstrated that the luminal domain of the E19 protein, when detached from the membrane-spanning and cytoplasmic domains, becomes secreted
HeLa cells were mock-infected or infected with the indicated mutant viruses. After 16 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 cytometry. The relative fluorescence intensity is plotted on a linear scale against the numbers of cells counted.

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...
The present data are consistent with a linear sequence being responsible for the localization of the El9 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 El glycoprotein of coronavirus (Armstrong et al., 1984), cytochrome P-450 (Ozols et al., 1985), and the rotavirus protein VP7 and NCP5 (Both et al., 1983a, 1983b). In fact, the VP7 glycoprotein has a cytoplasmic tail that consists of the NH2-terminal rather than the COOH-terminal region. However, this protein also becomes secreted upon elimination of a peptide stretch in the NH2-terminal region (Poruchynsky et al., 1985), which suggests that residency in the ER is conferred by the cytoplastically 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 E3 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 El9 gene. After digestion with BamHI, 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 introduced 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 (Pålbo et al., 1986a).

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 (Nilai et al., 1980). 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 Culture and Viral Infections

HeLa cells were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 100 U/ml penicillin, 100 μg/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. Adeno-virus 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 antisem to the hexon protein (Philipson, 1988). 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 (Pålbo 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 Crompton, 1973). Immunoprecipitations and Endo H treatment were carried out as described (Anderson et al., 1985). SDS-PAGE was done according to Blobel and Dobberstein (1974) on 10%-15% gradient slab gels. Labeled proteins were visualized by fluorography (Bonner and Laskey, 1974).

The serological reagents used were a rabbit antisem to human class I antigens (Rank et al., 1979), the W6/32 monoclonal antibody to human class I antigens (Barnstable et al., 1978), a mouse monoclonal antibody to E19 (Severinson and Peterson, 1985), and a rabbit antisem to purified E19 protein (Petersen et al., 1979).

Immunofluorescence and Flow Cytometry

Immunofluorescence staining of cells in suspension was performed as described (Anderson 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 cytometry were performed as described (Anderson et al., 1985).

To measure the elimination of class I antigens from the surface of virally infected cells, flow cytometry was employed. Virally infected cells were cultured with cycloheximide at a final concentration of 100 μM. This concentration reduced the protein synthesis measured as incorporation of labeled methionine into total cellular protein by 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 cytometry.

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 sucrose gradient and centrifuged for 4000 rpm in a Sorval TST 2838/17 rotor for 18 hr. Six fractions were eluted from each gradient, and membranes were pelleted and suspended in the lysis buffer used for immunoprecipitations (Anderson et al., 1985).

The assays for NADPH-cytochrome c reductase (Ohura and Takahashi, 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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