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Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum

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Several families of transmembrane endoplasmic reticulum (ER) proteins contain retention motifs in their cytoplasmically exposed tails. Mutational analyses demonstrated that two lysines positioned three and four or five residues from the C-terminus represent the retention motif. The introduction of a lysine preceding the lysine that occurs three residues from the terminus of Lyt2 renders this cell surface protein a resident of the ER. Likewise, the appropriate positioning of two lysine residues in a poly-serine sequence confines marker proteins to the ER. Arginines or histidines cannot replace lysines, suggesting that simple charge interactions are not sufficient to explain the retention. The identified consensus motif may serve as a retrieval signal that brings proteins back from a sorting compartment adjacent to the ER.

Key words: Endoplasmic reticulum/ER retention sequence/ protein sorting/protein targeting signal

Introduction

The secretory pathway of eukaryotic cells consists of a series of distinct membrane bound organelles, between which proteins are moved in a vectorial fashion by a series of vesicle budding and fusion events. Access to this conveyor belt is via a signal sequence (Blobel and Dobberstein, 1975), which directs nascent polypeptides to the endoplasmic reticulum (ER), the first organelle of the pathway. Each of the different organelles along the pathway is known to possess resident soluble and membrane proteins that define the unique structural and functional properties of that organelle. Thus, the organelles are both stable and dynamic structures with resident proteins being maintained despite extensive movement of proteins and lipids into and out of them. Current views favour the idea that transport occurs by default (Pfeffer and Rothman, 1987; Wieland *et al.*, 1987) such that proteins inserted into the ER are carried passively to the cell surface. Membrane proteins end up in the plasma membrane whilst soluble proteins are secreted. Consequently, residency of a particular protein in an organelle requires a signal of some kind that retains the protein in that location i.e. a retention sequence.

Such general sequences have recently been identified in both soluble (Munro and Pelham, 1987) and transmembrane (Nilsson *et al.*, 1989) ER resident proteins. Pelham and colleagues noted that three soluble ER resident proteins contain the same C-terminal tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL). Deletion of this sequence or extension of the C-terminus with random amino acids resulted in secretion of the mutated proteins (Munro and Pelham, 1987). In addition, when KDEL was transplanted onto the C-terminus of lysozyme (a protein that is normally secreted), the lysozyme chimera was retained in the ER.

Our interests have focused on sequences responsible for conferring ER residency on type I transmembrane proteins, in particular the adenoviral E3/19K protein. The short cytoplasmic tail of this molecule is critical for the ER retention of this protein (Pääbo *et al.*, 1987). In fact, the last six amino acids (DEKKMP) of the 15-membered cytoplasmic tail are necessary and sufficient for ER retention (Nilsson *et al.*, 1989). These residues can be transplanted onto the cytoplasmic tail of other membrane bound proteins such that ER residency is conferred.

Unlike the highly conserved KDEL motif, which is a characteristic feature of soluble proteins residing in the ER lumen, DEKKMP is not known to occur at the C-terminus of other resident transmembrane ER proteins. Analogous sequences do, however, exist in other proteins such as the C-terminal sequence of a UDP-glucuronosyltransferase. That sequence can functionally replace the E19 retention motif (Nilsson *et al.*, 1989).

In this paper we have tested the C-terminal sequences of additional ER resident proteins for the presence of ER retention motifs. Extensive site-directed mutagenesis has allowed us to formulate a consensus for the ER retention motif which includes two lysines, one of which must be positioned three residues from the C-terminus and the second either four or five residues from the C-terminus.

Results

ER retention sequences of type I transmembrane proteins contain an essential lysine residue

We have previously shown that replacing the cytoplasmic domains of the human T cell surface glycoproteins CD8 and CD4 with that from the adenovirus E3/19K (E19) protein results in the retention of these two chimeric constructs in the ER (Nilsson *et al.*, 1989). CD8 and CD4 are particularly useful marker proteins in this type of study as the former is glycosylated via an O linkage whilst the latter is N-glycosylated. As the composition of these carbohydrates is altered by passage along the secretory pathway, biochemical analyses can be used to determine whether the proteins have been retained in the ER. Transport of CD8 out of the ER to the Golgi is marked by the addition of sialic acid to the O-linked carbohydrate, causing a reduced electrophoretic mobility on SDS-PAGE. Likewise, transport of CD4 from the ER results in the trimming of the core sugars by Golgi enzymes to such an extent that the glycan becomes resistant

to digestion with endoglycosidase H (Endo H). Endo H sensitivity causes a marked change in the electrophoretic mobility of CD4. Thus, Endo H treatment can be used to determine whether CD4 resides in the ER.

Our initial experiments used these assays to screen the C-terminal sequences of several ER resident proteins for the presence of ER retention sequences. Sequences from the following proteins were tested: human UDP-glucuronosyltransferase (UDPGT) HP1 (Harding *et al.*, 1988), human UDPGT H25 (Jackson *et al.*, 1987), human UDPGT H4 (Jackson,M.R. and Burchell,B., unpublished data), rabbit 53 kd sarcoplasmic reticulum (SER) protein (Leberer *et al.*, 1989), human HMG CoA reductase (Chin *et al.*, 1984), human ribophorins I and II (Crimaudo *et al.*, 1987), murine ERp99 (endoplasmin) (Mazzarella and Green, 1987) and a glycoprotein component (SPC22/23) of canine signal peptidase (Shelness *et al.*, 1988). Oligonucleotides encoding the C-terminal 10 residues were directionally cloned into the CD8/E19 expression vector. This vector encodes the signal sequence, extracellular portion and transmembrane sequence of CD8 and the first five residues of the E19 tail. Previous experiments have shown that the five E19 residues do not contribute to the ER residency of this protein. The recombinant plasmids were transfected into HeLa cells by the calcium phosphate method. After 72 h, transfected cells were labelled for 20 min with [^{35}S]methionine and chased for various periods of time in the presence of an excess of unlabelled methionine. Detergent solubilized molecules were subjected to immunoprecipitation with OKT8, a monoclonal antibody recognizing the CD8 protein. SDS−PAGE was used to determine the apparent molecular weight and hence glycosylation state of the expressed proteins. Figure 1 shows that the CD8 chimeras terminating in the sequences from E19, UDPGT HP1, UDPGT H25, UDPGT H4, HMG CoA reductase and the 53 kd SER protein all migrated with an M_r of ~25 000, characteristic of non-sialylated CD8 molecules that are retained in the ER. In contrast, some of the protein expressed from the chimeras terminating in the ribophorin I, ribophorin II, SPC 22/23 and ERp99 sequences migrated with M_r 31 000, indicating that a significant proportion of these proteins had left the ER after 2 h and received sialic acid in the Golgi complex. CD8/ribophorin I and CD8/ERp99 were transported as rapidly as wild-type CD8 (Figure 1, lane 11), whereas the CD8/SPC 22/23 and CD8/ribophorin II proteins leave the ER at a slower rate. The apparent molecular weights of the retained proteins shown in Figure 1 are heterogeneous; in most cases, they migrate as three species. This is the result of post-translational modification of the proteins by the addition of N-acetylgalactosamine (T.Nilsson, M.R.Jackson and P.A.Peterson, unpublished data), the primary O-glycosylation event (see Figure 4).

A comparison of the sequences that cause retention reveals a lysine residue positioned three residues from the C-terminus (−3) as a common feature. In order to demonstrate that this lysine residue is important for retention, the appropriate codons in the oligonucleotides encoding four of these sequences were altered from a lysine to a serine and the resulting recombinants assayed as above. Figure 2A shows that in each case, changing the conserved lysine to a serine resulted in the CD8 chimera acquiring sialic acid, indicating that the ER retention motif had been disrupted. The rates of transport of the four CD8−3S mutants were not identical, at the 2 h time point analysed. All the CD8/E19−3S and CD8/HMG CoA−3S protein had acquired sialic acid, indicating that they were rapidly transported out of the ER, whilst the transport rate of the other two mutated proteins was considerably slower. Longer chase times, up to 5 h, are required before all of these more slowly transported proteins acquire sialic acid. It is worth noting that the two rapidly transported proteins contain only one additional basic residue (a lysine at −4) in their C-terminal nine residues, whereas the corresponding sequences of the more slowly transported proteins contain multiple basic residues.

These biochemical data were confirmed by FACS analyses and by immunofluorescence microscopy where cell surface staining of CD8 was found for the serine replacement mutants and for the ERP99, SPC 22/23 and ribophorin I and II tails (data not shown). Immunofluorescence microscopy of detergent-permeabilized cells expressing retained constructs showed characteristic ER staining: strong perinuclear fluorescence and clear staining of a tubular−vesicular lattice at the periphery of the cell.

In order to show that these results were not dependent on the marker protein, the same set of oligonucleotides was cloned into the CD4/E19 expression vector. This gave rise to a set of chimeric CD4 molecules where the cytoplasmic tails were identical to those tested on CD8. The use of CD4 as the marker protein allowed us to determine whether the molecules were actually retained in the ER, as in this location its N-linked sugars should remain sensitive to digestion with Endo H. Figure 2B shows that the N-linked sugars on the

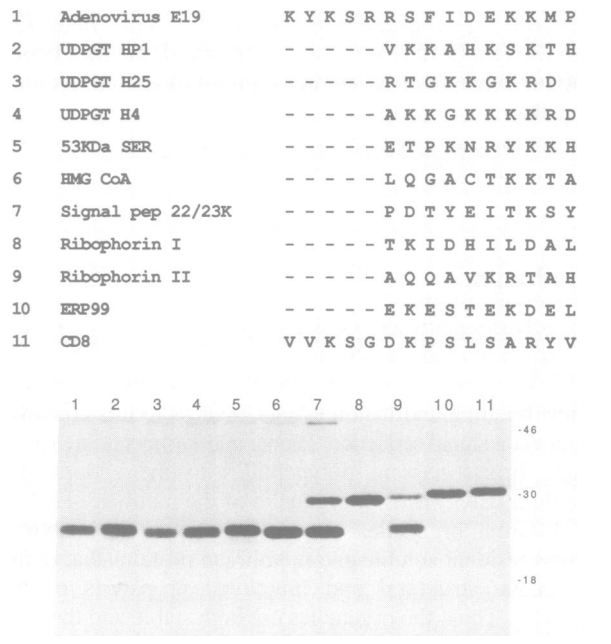

Fig. 1. Screening the C-terminal sequences of ER resident proteins for an ER retention sequence. The C-terminus of the CD8 protein was engineered to encode the sequence of various ER resident proteins (as detailed in the figure). The cDNA constructs were transfected into HeLa cells and 72 h later, the encoded proteins were labelled with a 20 min [^{35}S]methionine pulse, followed by a 2 h chase in the presence of unlabelled methionine. After lysis of the cells, the CD8 protein was collected by immunoprecipitation with OKT8, a monoclonal antibody against the CD8 protein. Figure 1 is an autoradiograph of the immunoprecipitated CD8 chimeras analysed by SDS−PAGE. Sequences 1−11 are the C-terminal tails of the proteins analysed in lanes 1−11 respectively. The migration positions of marker proteins and their M_r are shown on the right of the figure.

CD4 chimeras terminating in the sequences from E19, UDPGT HP1, 53 kd SER protein and HMG CoA reductase were completely Endo H sensitive after a 2 h chase period, indicating that these molecules had been efficiently retained in the ER. As expected, the CD4 chimeras, where the critical (−3) lysine had been mutated to a serine, possessed carbohydrate moieties that were partially Endo H resistant, indicating that these proteins were not retained in the ER. Prolonged chase times show that only one of the two N-linked sugars added to CD4 or the CD4 chimeras becomes Endo H resistant in HeLa cells. Thus after an 8 h chase, one of the sugars on the mature (cell surface) CD4 is completely resistant to digestion with Endo H, while the other is still susceptible. SDS−PAGE analysis of the digested proteins shows that it migrates as the upper of the two bands shown in Figure 2B, lane 9(+). The degree of Endo H resistance of the different chimeras suggests that all four of the CD4−3S mutants and the CD4 wild-type protein (Figure 2B, lane 9) were transported out of the ER at a similar rate. Thus, ~50% of the molecules contained one Endo H resistant N-linked sugar after 2 h, and after a 4 h chase, all of the −3S CD4 chimeras had been transported out of the ER and modified. This finding is in contrast to the markedly different transport rates of the various CD8−3S proteins. This discrepancy probably reflects the overall lower rate of transport of the CD4 wild-type protein compared with the CD8 wild-type protein (compare lane 10, Figure 1 and lane 9, Figure 2B).

The above data provide compelling evidence that a conserved lysine residue positioned three residues from the C-terminus of the protein is a necessary component of the ER retention motif. However, it seems unlikely that it is the only requirement, especially as the CD8/SPC 22/23 protein contains a lysine in the −3 position but is not retained in the ER (Figure 1, lane 7). Thus we conclude that a −3 lysine residue is a necessary but not a sufficient component of an ER retention motif.

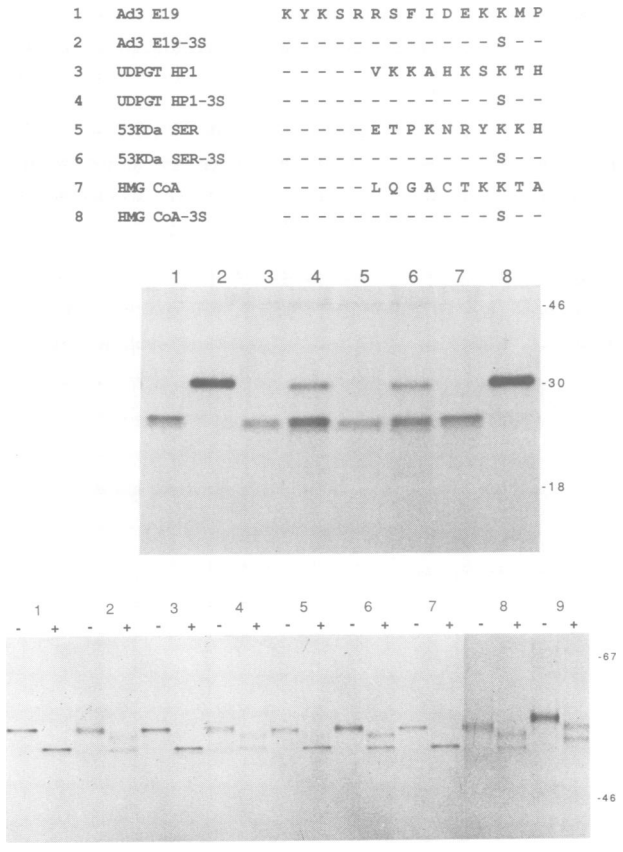

Fig. 2. Retention of CD8 and CD4 in the ER by transplanted C-terminal sequences from resident ER proteins is dependent on a −3 positioned lysine residue. CD8 or CD4 cDNAs, engineered to encode the different C-terminal tails detailed in the figure, were transfected into HeLa cells and labelled as described in Figure 1. After lysis of the cells, CD8 and CD4 proteins were collected by immunoprecipitation with OKT8 and 19Thy/5D7 antibodies respectively. (**A**) An autoradiograph of an SDS−PAGE analysis of the precipitated CD8 chimeras; the sequences 1−8 are the cytoplasmic tails of the proteins shown in lanes 1−8 respectively. (**B**) A similar analysis to (A) of the precipitated CD4 chimeras, where the lane number indicates the sequence of the cytoplasmic tail (as in Figure 1A), except for lane 9, which is the wild-type CD4 protein. Each CD4 immunoprecipitate is shown with (+) or without (−) prior digestion with endoglycosidase H. The migration positions of marker proteins and their M_r are shown on the right of the figures.

Addition or deletion of residues from the C-terminus of the E19 sequence disrupts the retention motif

In view of the above findings, it seemed that the position of specific residues relative to the C-terminus might be critical in defining the ER retention structure. To test this, oligonucleotides encoding the E19 tail plus either one or two additional serine residues were cloned into the CD8/E19 expression vector. SDS−PAGE analysis of these addition mutants (Figure 3, lanes 1 and 2) showed that both proteins were sialylated and therefore inefficiently retained in the ER. The mutant with two additional serines acquired sialic acid more rapidly than that with a single serine. Figure 3 also shows the effect of deleting C-terminal residues from the E19 tail (Nilsson *et al.*, 1989). It can be seen that the greater the deletion, the more rapidly the protein acquires sialic acid. The CD8/E19D1 construct is of particular interest, since although a lysine residue still occupies the −3 position, the protein is transported out of the ER. The position of the motif at the C-terminus is thus critical, and the further it is placed from this optimal position the less effective it is at retaining the protein.

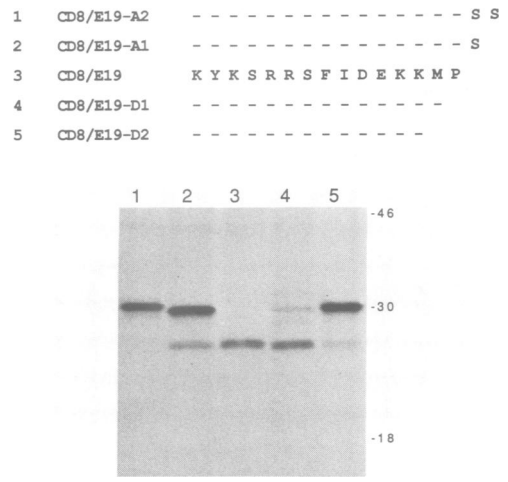

Fig. 3. Addition of residues to the C-terminus of CD8/E19 inactivates the ER retention sequence. HeLa cells transfected with cDNA encoding CD8/E19 A2-D2 were labelled and analysed as described in the legend to Figure 1. The C-terminal sequences of these proteins are shown; the adjacent number indicates the lane position on the gel. Molecular weight standards are shown in kd on the right of the figure.

The E19 ER retention motif contains two critical lysine residues neither of which can be substituted by arginine or histidine

In order to identify other important features of the ER retention motif, a series of replacement mutants in the E19 tail was made. For each position the appropriate codon was altered such that the mutant encoded a serine residue at this position, leaving the rest of the E19 sequence intact. SDS−PAGE analysis of the constructs expressed in HeLa cells showed that only two of the replacement mutants were sialylated (Figure 4, lanes 6 and 7). In addition to the critical −3 lysine already identified, replacement of the adjacent lysine positioned four amino acids from the C-terminus (−4) completely disrupted the retention motif. Efficient retention of all the other replacement mutants in the ER was confirmed by immunofluorescence microscopy of detergent permeabilized cells (data not shown). From these data we can conclude that two lysine residues positioned at −3 and −4 are the important features of the E19 retention sequence.

One obvious question arising from the above conclusion is whether conservative substitutions of these lysine residues would disrupt the retention motif. Oligonucleotides replacing these residues with either arginine or histidine were cloned into the CD8/E19 expression vector. SDS−PAGE analysis of the encoded proteins (Figure 5) showed that none of these substitutions were able to maintain the CD8 protein in the ER. Indeed, these substitutions were as disruptive to the ER retention motif as the serine replacements, with one exception. Replacement of the −4 lysine with arginine resulted in a protein which acquired sialic acid at a rate that was significantly lower than the corresponding serine replacement, indicating that this sequence caused partial retention in the ER. From the above experiments we have established that it is the lysine residues and their position with respect to the C-terminus that are the essential components of the E19 ER retention motif.

The consensus sequence for an ER retention motif consists of two lysine residues close to the C-terminus

Inspection of other ER retention sequences showed that only the HMG CoA reductase retention sequence contains a second lysine residue at the −4 position (see Figure 1). All the other sequences do, however, contain a second lysine residue positioned either five (−5) or two (−2) residues from the C-terminus. This feature becomes more apparent on inspecting an alignment of the C-terminal sequences of several E19 proteins encoded by different adenoviruses, UDPGT isoforms from different species, HMG CoA reductase from hamster and man and the 53 kd SER protein (see Table I). In addition to the conserved −3 lysine in these sequences, it is worth noting that all of the UDPGT proteins have a common lysine at −5, despite little homology between many of these sequences. Furthermore, the E19 protein encoded by the adenovirus 3 contains a second lysine at position −5 rather than −4.

From these observations, we speculated that the consensus for an ER retention motif includes two lysines, one positioned at −3 and the other at either −5, −4 or −2. Our initial test of this hypothesis was to move the −4 lysine in the adenovirus-2 E19 sequence to the −5 position and determine whether it could still retain the CD8/E19 chimera in the ER. SDS−PAGE analysis of this molecule expressed in HeLa cells (Figure 6, lane 2) showed that the protein did not acquire sialic acid, indicating that this sequence was able to retain CD8 in the ER as efficiently as the wild-type sequence (Figure 6, lane 1). Furthermore, replacement of the −5 lysine in the UDPGT HP1 sequence with serine resulted in disruption of this ER retention motif (Figure 6,

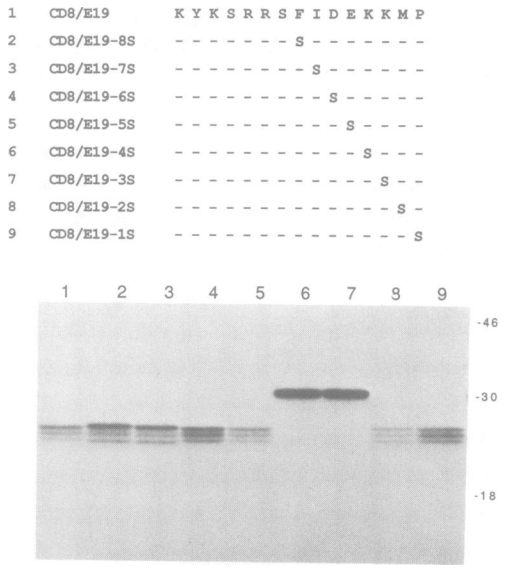

Fig. 4. Two lysine residues in the C-terminal sequence of CD8/E19 are critical for a functional ER retention motif. HeLa cells transfected with cDNA encoding a systematic series of serine replacement mutants of CD8/E19 (−1S to −8S) were pulse−chase labelled and processed as described in the legend to Figure 1. The C-terminal sequences of these replacement mutants are shown; the adjacent number indicates the lane position on the gel. Molecular weight standards are shown in kd on the right of the figure.

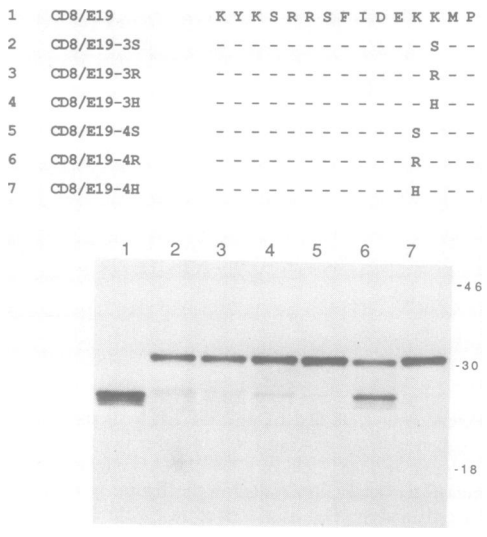

Fig. 5. The critical lysine residues in the E19 ER retention motif cannot be substituted by argine or histidine. cDNAs encoding arginine or histidine replacements of the −3 or −4 lysine residues in the CD8/E19 C-terminal sequence were transfected into HeLa cells and the expressed constructs pulse−chase labelled and processed as described in Figure 1. The sequence of the cytoplasmic tails of these mutants are shown; the adjacent number indicates the lane position on the gel. Molecular weight standards are shown in kd on the right of the figure.

Table I. Conserved lysine residues are found in the C-terminal sequences of a subset of ER resident proteins

Human Ad2 E19	-	K	Y	K	S	R	R	S	F	I	D	E	K	K	M P
Human Ad5 E19	-	K	Y	K	S	R	R	S	F	I	E	E	K	K	M P
Human Ad3 E19	-	V	I	R	P	R	Q	S	N	E	E	K	E	K	M P
Human UDPGT H25	-	C	V	W	K	F	Y	R	T	G	K	K	G	K	R D
Human UDPGT H4	-	C	F	R	K	L	A	K	K	G	K	K	K	K	R D
Human UDPGT HP1	-	G	K	K	G	R	V	K	K	A	H	K	S	K	T H
Rat UDPGT R38	-	I	Y	R	L	F	V	K	K	E	K	K	M	K	N E
Rat UDPGT R23	-	M	Y	R	F	F	V	K	K	E	K	K	M	K	N E
Rat UDPGT R1	-	V	Y	R	F	F	V	K	K	E	K	K	T	K	N E
Rat UDPGT 2F	-	F	C	C	R	K	T	A	N	M	G	K	K	K	K E
Rat UDPGT K39	-	G	G	K	G	R	V	K	K	G	H	K	S	K	T H
Mouse UDPGT	-	I	Y	R	F	F	V	K	K	E	N	K	M	K	N E
Human HMG CoA	-	I	N	L	Q	D	L	Q	G	T	C	T	K	K	S A
Hamster HMG CoA	-	I	N	L	Q	D	L	Q	G	T	C	T	K	K	S A
SER 53Kd	-	D	K	T	G	C	G	T	P	K	N	R	Y	K	K H

An alignment of the C-terminal sequences of E19 proteins from adenovirus Ad2 (Hérissé *et al.*, 1980), Ad3 (Signäs *et al.*, 1986) and Ad5 (Cladaras and Wold, 1985), UDPGT isoforms from different species: human H25 (Jackson *et al.*, 1987), human H4 (Jackson,M.R. and Burchell,B., unpublished data), human HP1 (Harding *et al.*, 1988), rat rlug 38 (Harding *et al.*, 1987), rat rlug 23 and rat rlug 1 (Jackson and Burchell, 1986) rat 2F (Mackenzie, 1986), rat 4-NP (Iyanagi *et al.*, 1986) and mouse m-1 (Kimura and Owens, 1987). HMG CoA reductase from human (Luskey and Stevens, 1985) and hamster (Chin *et al.*, 1984). Rabbit 53 kd SER protein (Leberer *et al.*, 1989). The conserved −3 lysine residue is highlighted.

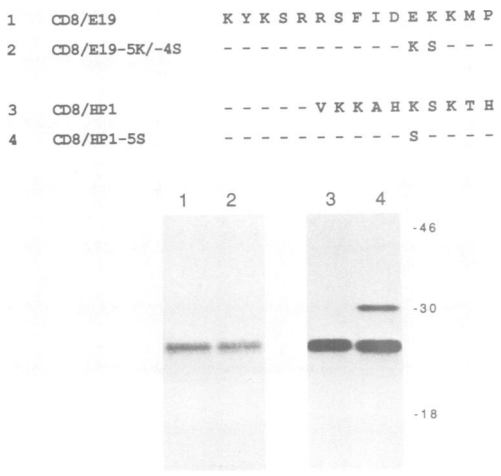

Fig. 6. Disruption or creation of ER retention sequences by a lysine residue positioned five residues from the C-terminus. HeLa cells transfected with cDNAs encoding the indicated mutations of CD8/E19 and CD8/HP1 were pulse−chase labelled and processed as described in Figure 1. The sequences of the cytoplasmic tails of the proteins analysed are shown above the figure; the adjacent number indicates the lane position on the gel. Molecular weight standards are shown in kd on the right of the figure.

lane 4). Thus these two experiments are consistent with the view that a second lysine at position −4 or −5 forms part of the consensus retention motif.

Creation of ER retention sequences

Apart from the lysine content of the retention sequences shown in Table I, no other common feature was obvious. It seemed important to determine whether the consensus proposed above could function in any context or whether the surrounding amino acids create a special environment in which it must be presented.

To address this issue, we took advantage of the availability of the sequence for Lyt2, the murine homologue of human CD8. This transmembrane protein contains three Asn-linked carbohydrate moieties and is normally expressed on the cell surface despite the fact that it contained a lysine residue in the −3 position (Nakauchi *et al.*, 1985). To create the consensus motif for ER retention in the C-terminal tail of the Lyt2 molecule, we mutated the codon for glutamic acid in the −4 position such that it would encode a lysine. The wild-type and the mutated Lyt2 sequences were appended onto the CD8 marker protein construct. SDS−PAGE analysis of the expressed proteins (Figure 7A) shows that the CD8/Lyt2 chimera was transported out of the ER rapidly acquiring sialic acid (Figure 7A, lane 1). In contrast, the CD8/Lyt2-4K mutant protein (Figure 7A, lane 2) was completely retained in the ER and did not acquire sialic acid.

To verify further that a point mutation might be sufficient for creating an ER retention signal, the wild-type mouse Lyt2 cDNA was mutated in a similar manner by use of the PCR technique. The resulting cDNA encoded a complete Lyt2 sequence with the −4 residue altered to encode a lysine. The proteins encoded by the wild-type and mutated cDNAs were expressed in HeLa cells. As can be seen in Figure 7B, only the mutant protein contained sugars sensitive to digestion with Endo H after an appropriate chase period (lane 7). In keeping with this observation, only the wild-type Lyt2 protein contained carbohydrate sensitive to digestion with neuraminidase (lane 5). This provided further evidence that the Lyt2-4K protein was unable to reach the Golgi complex, the site of sialic acid addition. FACS analysis of cells expressing the two molecules with anti-Lyt2 antibodies confirmed that only the wild-type molecule was expressed on the cell surface (Figure 7C). The ER location of the mutated Lyt2 protein was further verified by immunofluorescence microscopy (Figure 7D). Thus, we were able to create an ER retention sequence by simply satisfying the consensus motif described above. Furthermore, these data suggest that there are no stringent requirements for the context in which the ER retention motif must be presented.

Confirmation of the consensus sequence of ER retention in the poly-serine background

To test further the hypothesis that the requirements for ER retention are simply two correctly positioned lysine residues, we constructed CD8 chimeras whose C-terminal sequences contained either one or two lysine residues surrounded by serine residues. Figure 8A shows that CD8 molecules with a poly-serine tail or with a poly-serine tail containing a single lysine residue positioned at −4 were not retained in the ER. However, introduction of a second lysine residue into this sequence at the −3 position caused complete retention of the marker protein in the ER (Figure 8A). We wished to use this model system to analyse the ER retention motif in

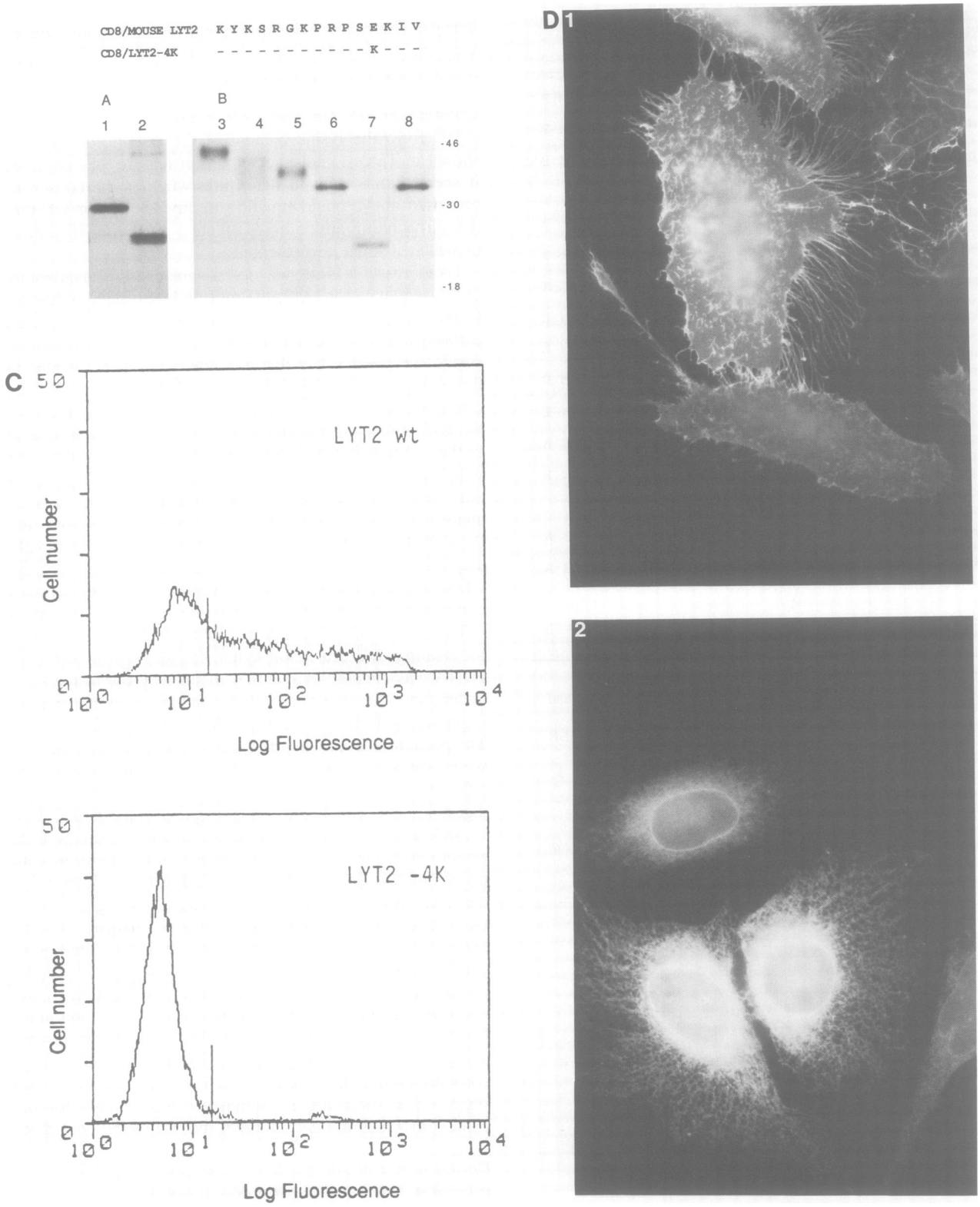

Fig. 7. Creation of an ER retention sequence in the C-terminus of mouse Lyt2a by a point mutation. (**A**) HeLa cells transfected with CD8/Lyt2 and CD8/Lyt2-4K cDNA were pulse−chase labelled and processed as described in Figure 1. Lane 1 and 2 show SDS−PAGE analysis of protein expressed by constructs CD8/Lyt2 and CD8/Lyt2-4K respectively. (**B**) HeLa cells transfected with cDNA encoding mouse Lyt2 and Lyt2-4K were labelled with methionine and lysed as described in the legend to Figure 1. Proteins encoded by Lyt2 (lanes 3−5) or Lyt2-4K (lanes 4−6) were immunoprecipitated from the lysates with a rat anti-mouse Lyt2 antibody. Immunoprecipitates were then analysed directly by SDS−PAGE (lanes 3 and 6), or after digestion with Endo H (lanes 4 and 7) or neuraminadase (lanes 5 and 8). (**C**) HeLa cells transfected with Lyt2 or Lyt2-4K cDNA (as in Figure 7B) were stained for surface expression of Lyt2 using a rat anti-mouse Lyt2 antibody and a fluorescein-conjugated goat anti-rat IgG antibody. Cells were analysed by a fluorescence activated cell sorter as described in Materials and methods. (**D**) HeLa cells transfected with Lyt2 (1) or Lyt2-4K (2) cDNA were fixed using paraformaldehyde, permeabilized using 0.1% Triton X-100 and stained as described in Figure 7C with anti-mouse LYT2 antibody and a fluorescein conjugated secondary antibody prior to fluorescence microscopy.

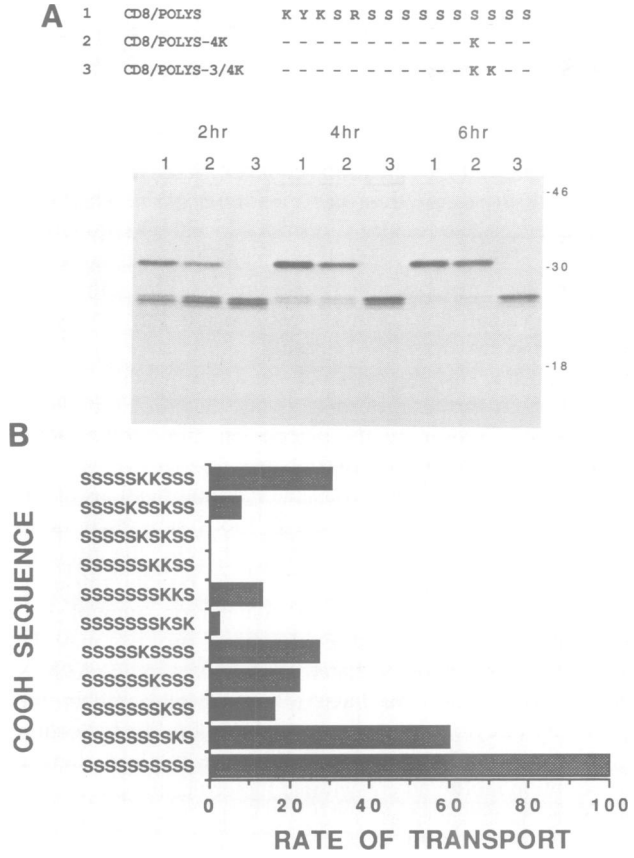

Fig. 8. The consensus sequence for an ER retention sequence defined in a poly-serine background. (**A**) HeLa cells were transfected with three of the eleven CD8/poly-serine constructs. The expressed proteins were labelled for 20 min and then chased in the presence of unlabelled methionine for 2, 4 or 6 h as indicated on the Figure. CD8 protein was then processed as described in the legend to Figure 1. The number adjacent to the sequence indicates the gel lane for that construct. The migration positions of molecular weight marker proteins are shown in kd. (**B**) Each of the eleven CD8/poly-serine constructs was transfected into HeLa cells, labelled as above, and chased for 2 h before immunoprecipitation. Following SDS–PAGE, autoradiographs were evaluated using a laser scanner and the ratio of the amount of CD8 protein migrating at 31 kd to the total amount of CD8 protein present was calculated for each construct. This figure was then expressed as a percentage of the value obtained for the CD8/poly-serine construct, giving rise to a rate of transport value, which is shown plotted against the corresponding C-terminal sequence.

a defined background. A set of CD8 chimeras was made that contained either one or two lysine residues in the poly-serine background. The constructs were analysed as in Figure 8A. For each construct, the fraction of the molecules receiving sialic acid after 2 h was calculated and used as a measure of the rate of transport for each protein. Figure 8B shows these values expressed as a percentage of a rate of transport of the parent poly-serine construct. Figure 8B shows that complete retention of the marker protein was achieved only when a lysine was at position −3 if a second lysine residue occupied the −4 or −5 position. Locating the second lysine at either −1, −2, or −6 did not achieve this, nor did positioning of two lysine residues at −4 and −5. The constructs containing a single lysine residue in the poly-serine background were transported at significantly different rates. Interestingly, a single lysine at the −3 position causes the greatest reduction in the transport rate, which is consistent with its critical role in the retention of transmembrane ER proteins. The ER retention motif defined in the poly-serine background is in total agreement with the rest of this work. It clearly highlights the importance of a lysine residue at the −3 position acting in conjunction with a second lysine positioned at either −4 or −5.

Discussion

Several distinct mechanisms may be responsible for the retention of proteins in the ER. It is conceivable that proteins of the translocation complex form oligomeric assemblies that are excluded from the exocytotic pathway by being fixed to submembranous structures (Horst and Meyer, 1985). Such proteins may not need any specific retention signal to become localized in the ER. It is less likely that soluble and membrane proteins, which are uniformly distributed in the organelle, might be retained by the same means. The uniform distribution suggests that membrane proteins can move in the lateral plane of the membrane. Such movement could formally be saltatory between subunits of cytoskeletal elements but in such a case, retention in the ER would probably never be complete. A more attractive mechanism has been suggested by Pelham, based on his studies of soluble ER resident proteins (Pelham, 1988). Such proteins, displaying the C-terminal sequence KDEL (Munro and Pelham, 1987) probably leave the ER but are rapidly retrieved by a membrane-bound receptor from an adjacent sorting compartment (Warren, 1987). Such a retrograde flow of proteins might occur since transport from the Golgi compartment to the ER is apparent in cells treated with Brefeldin A (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). It is tempting to suggest that proteins like E19 are similarly retrieved to the ER. Such proteins obviously need a structural motif that can be recognized by the putative retrieval machinery.

Membrane proteins confined to sub-regions such as the smooth ER may be retained by forming oligomeric assemblies (Chin *et al.*, 1982; Orci *et al.*, 1984) or other types of aggregates (Copeland *et al.*, 1986; Bergmann and Fusco, 1990, see Rose and Doms, 1989) that are too large to be accommodated in transport vesicles. Consequently, such proteins may not need structural retention motifs. However, during transport from the translocation complex to their final destination, smooth ER proteins may leave the ER randomly, so it is conceivable that they need a retrieval signal to ascertain that oligomeric assembly can take place. Additional mechanisms for retaining in the ER membrane proteins of other topologies, e.g. proteins with uncleaved signal sequences or type II membrane proteins, must also exist. Some viruses may have evolved unique retention strategies as exemplified by the ER retention sequences for the VP7 protein of rotavirus and for the PreS1 surface glycoprotein of hepatitis B virus (Stirzaker and Both, 1989; Kuroki *et al.*, 1989).

In the present study we have examined the possible existence of structural motifs for the retention of several transmembrane proteins in the ER. The C-terminal sequences of ten resident ER proteins were appended to the protein CD8, and six of these sequences caused complete retention of this marker protein. In addition to the E19 sequence, the C-terminal sequences of HMG CoA reductase, a 53 kd SER protein, and three UDP-glucuronosyltransferases cause retention. The fact that each of these sequences contains a lysine three residues from the C-terminus and that mutation of this residue to serine abolishes retention, suggests that

all sequences operate by the same mechanism. Identical results were obtained with another marker protein, establishing that the C-terminal sequences represented the necessary and sufficient retention motifs.

The four proteins whose C-terminal sequences failed to retain the marker proteins are ERP99, ribophorins I and II and SPC 22/23. In contrast to previous suggestions (Mazzarella and Green, 1987), ERP99 is a soluble ER protein (Koch *et al.*, 1988) retained by its C-terminal KDEL sequence (Munro and Pelham, 1987). The membrane topology of the SPC 22/23 is also in doubt (Shelness *et al.*, 1988). SPC 22/23, like ribophorins I and II are part of the translocation machinery and form a subdomain in the ER which is resistant to high salt extraction procedures (Hortsch and Meyer, 1985). As discussed above, none of these proteins may depend on a discrete structural motif for ER retention.

The lysine positioned three residues from the C-terminus is obviously a necessary component of the E19 retention sequence. Further mutation analyses indicated that a second lysine in the −4 position is the only other essential residue. However, the −4 lysine could be moved to the −5 position without affecting the retention. This observation was particularly informative as the putative retention sequences of 14 out of 15 ER proteins display lysines in positions −4 or −5 in addition to the −3 lysine (Table I). The fact that the 53 kd SER protein has arginine in the −5 position and lysine in the −2 position may compensate for the lack of lysine in the −4 or −5 position (see below).

The critical positional effect of the lysines was established by analysing addition and deletion mutants. While all such changes resulted in cell surface expression of the marker protein, the rate of transport seemed to be directly proportional to the extent of the deletion from, or addition to, the E19 sequence. Presumably, these slowly transported constructs contain a suboptimal retention sequence and the further the sequence is altered from the optimum the less effective it is.

The observation that two lysine residues in defined positions represent the critical elements of the retention sequence was further supported by our ability to create a retention motif in a novel sequence context. Thus, the introduction of a lysine adjacent to the −4 lysine in the T cell surface protein Lyt2 rendered this molecule a resident of the ER. This result prompted us to test further the retention motif in a defined sequence context. Using a poly-serine tail as the neutral background, lysines were introduced in various positions. The incorporation of a lysine diminished the transport rate by various extents depending on the position, and as expected, lysine in the −3 position had the greatest effect followed by lysines in the −4 and −5 positions. The simultaneous introduction of two lysine residues reduced the transport rate of the marker protein considerably, but only when the lysines occupied positions −3 and −4 or −5 did complete ER retention occur. However, it is conceivable that other combinations of two lysines in a more favourable sequence context might cause complete retention as is suggested by the 53 kd SER sequence.

The consensus ER retention sequence we have identified has the following features: firstly, there is an absolute requirement for two lysine residues. Secondly, the position of these lysines with respect to the C-terminus is critical. Thirdly, mechanistically the retention motif seems to be involved in a non-saturable process as some transfected cells express and accumulate high levels of the retained proteins without measurable leakage to the cell surface. The ER retention motif shows similarities to the peroxisomal targeting sequence -SKL (Gould *et al.*, 1989; Miyazawa *et al.*, 1989) and the retention sequence -KDEL present on soluble ER proteins (Munro and Pelham, 1987). The latter two sequences also require C-terminal locations and involve a lysine residue. However, what makes the targeting sequence we have described different from the other two sequences is that substitutions of either one of the two lysines for arginine disrupts the retention motif, indicating that lysines do not simply participate in a non-specific charge interaction. A further difference is that there are apparently no stringent requirements placed on the amino acids immediately flanking the two lysines.

Examination of the post-translational modifications of ER proteins displaying the consensus motif described here, argues for a retention mechanism whereby the proteins actually leave the ER only to become rapidly retrieved (T.Nilsson, M.R.Jackson and P.A.Peterson, unpublished observations). Such a retrieval/sorting process may be analogous to receptor-mediated endocytosis in as much as the retention motif we have identified may reflect the specificity of cytoplasmically located adaptin-like molecules (Glickman *et al.*, 1989). Such molecules might recognize the retention motif and concentrate molecules to be retrieved into retrograde transport vesicles in much the same way as endocytosis of the LDL receptor is mediated by the sequence motif NPXY in its cytoplasmic tail (Chen *et al.*, 1990). Such a mechanism would account for the partial retention of some constructs with suboptimal retention sequences since retrieval would depend on the affinity of the ER retention sequence for a putative adaptin-like molecule. The recent finding that clatherin deficient mutants of yeast mislocalize a resident *trans*-Golgi enzyme (Payne and Schekman, 1989) sets a precedent for this type of mechanism.

Alternative models which propose that ER resident proteins never leave this organelle require some mechanism whereby these molecules are excluded from the transport vesicles. It is of course conceivable that the retention motif we have identified serves as the substrate for some post-translational modification e.g. acylation, which may target the molecules to the ER in the same way as some lipids accumulate in specific organelles (see Pagano and Sleight, 1985). Indeed, lysine residues are amongst the most frequently modified of all amino acids (Wold, 1981) but we have no evidence of such modifications of the two critical lysines. Another alternative is that the lysine residues might interact directly with the lipid head groups of the membrane such that the protein is unable to enter transport vesicles. Interaction of basic residues with the cytoplasmic face of the ER membrane has been shown to be a major factor influencing the transmembrane topology of proteins (von Heinje, 1989). However the fact that lysines cannot be replaced by arginines argues against this possibility.

The finding that the consensus sequence defined in this study for the retention of transmembrane ER proteins can be appended to a variety of proteins should serve as a useful tool in dissecting the detailed molecular mechanism responsible for retention. Armed with synthetic peptides of and antibodies against such sequences, it should be possible to identify molecules participating in the retention machinery.

Materials and methods

Recombinant DNAs

Construction of the recombinant CD8/E19 has been described previously (Nilsson *et al.*, 1989); briefly, two oligonucleotides were used as primers in a PCR reaction with CD8 cDNA (Littman *et al.*, 1985), the 5′ primer encoded a *Bam*HI site followed by nucleotides 65–82 of the CD8 sequence (Littman *et al.*, 1985) and the 3′ primer consisted of nucleotides 666–686 of the CD8 sequence and nucleotides 1872–1919 of the E3/19k sequence (Hérissé *et al.*, 1980), followed by a *Bam*HI site. The PCR product CD8/E19 encoded the complete extracellular and transmembrane domain of CD8 followed by the 15 amino acids that constitute the E19 tail. An *Xba*I site was introduced between nucleotides 1881–1886 of the E3/19k sequence without altering the resulting amino acid sequence. This PCR fragment was digested with *Bam*HI and subcloned between the *Bgl*II and *Bam*HI sites of the expression vector pCMU IV (Nilsson *et al.*, 1989), thereby destroying the 5′ *Bam*HI site of the insert. The same strategy was used to generate the CD4/E19 protein (Nilsson *et al.*, 1989) from the CD4 cDNA (Maddon *et al.*, 1985). Construction of expression plasmids encoding CD8, CD8/E19D1 and D2, CD8/H25 and CD4 have been described previously (Nilsson *et al.*, 1989). All further CD8/E19 or CD4/E19 tail mutants were constructed by directional cloning of oligonucleotides between the unique *Xba*I and *Bam*HI sites in these plasmids. The sequences for the oligonucleotides used to construct CD8/HP1, CD8/H4, CD8/SER 53K, CD8/HMG CoA, CD8/SRP 22/23, CD8/Ribophorins I and II, CD8/ERp99 and CD8/Lyt2 were derived from the C-terminal (last 10 amino acids) sequences of the published cDNAs (see text for references). Replacement mutations in these constructs used the codon requiring the least number of nucleotide changes. The synthetic poly-serine tail background was encoded by the following oligonucleotide and its complementary strand:

Poly-serine: CTAGATCTTCAAGCTCTAGTTCTAGTAGTTCTAGT-TGAATTCG

Lysine replacements in this sequence were encoded by AAG in the −3 position and by AAA at all other locations. All constructs were sequenced directly in the expression plasmid using a primer specific for sequences downstream of the *Bam*HI site in pCMU.

The Lyt2 cDNA isolated by Nakauchi *et al.* (1985) was used in PCR reactions (Saiki *et al.*, 1988) to produce the Lyt2 and Lyt2-4K cDNAs. Both reactions used the same 5′ primer consisting of nucleotides 295–315 of the Lyt2 sequence; this oligonucleotide spans the single *Eco*RI site in this cDNA. The 3′ primer for the Lyt2 wild-type cDNA consisted of nucleotides 728–756 of the Lyt2 sequence, followed by a *Bam*HI site; the 3′ primer for the Lyt2-4K sequence was the same except for a point mutation at position 742 which replaced an A in the original sequence with a G. Amplification was carried out in a DNA thermal cycler (Cetus) for 25 cycles using the following conditions: 1 min of primer annealing at 58°C, 1 min of chain elongation at 60°C and 40 s of denaturation at 92°C. Reaction products were extracted once with phenol, the DNA was precipitated with ethanol and digested with *Eco*RI and *Bam*HI. The *Eco*RI/*Bam*HI fragments were subsequently subcloned into the polylinker of pBluescript (Stratagene) and sequenced into the polylinker of pBluescript (Stratagene) and sequenced. The remaining 5′ sequence of Lyt2 cDNA was replaced by subcloning an *Xho*I–*Eco*RI fragment from the original cDNA between the pBluescript polylinker *Xho*I site, and the 5′ *Eco*RI site of the PCR fragment. The complete Lyt2 and Lyt2-4K cDNAs were then directionally cloned as a *Xho*I–*Bam*HI fragment into the vector pCMU II for expression in HeLa cells. This vector is a derivative of pC81G (Pääbo *et al.*, 1986) and was constructed by digesting pC81G with *Hin*dIII and *Bam*HI and inserting a complementary pair of oligonucleotides encoding a polylinker sequence.

Oligonucleotides were synthesized on a Gene Assembler (Pharmacia LKB) and purified on FPLC using a PreRPC/HR5/5 column (Pharmacia LKB).

Cell culture and transfection

HeLa cells (ATCC CCL185) were grown in Dulbecco's modified Eagles medium (DMEM; Gibco) supplemented with 8% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin and streptomycin at 100 μg/ml. Transfections were carried out using the calcium phosphate method as described previously (Pääbo *et al.*, 1986), except that 25 μg of DNA were used to transfect 1×10^5 cells in a 100 mm Petri dish.

Metabolic labelling, immunoprecipitation, Endo H and neuraminadase treatment

Metabolic labelling of cells was carried out as follows, 60–72 h post-transfection, cells were rinsed in PBS three times and then incubated for 20 min in methionine deficient DMEM (Gibco) before addition of the pulse media. Pulse media contained 0.2 mCi/ml of [^{35}S]methionine (Trans-label; ICN Biochemicals Inc.) in methionine deficient DMEM. Cells were routinely labelled for 20 min followed by chase in the presence of culture media for 2 h. Cells were then rinsed in PBS and lysed *in situ* with 1% Triton X-100/PBS. Cell lysates were precleared by centrifugation in a microfuge for 2 × 15 min to remove cell debris, followed by an incubation with rabbit preimmune serum and finally Protein A–sepharose. Antibody was then added to the lysate and incubated on ice for 2–4 h. Protein A–Sepharose (Pharmacia), or Protein A–Sepharose preincubated with rabbit anti-rat IgG (Cappell) for collecting rat anti-mouse Lyt2 IgG, was used to collect the antibody complexes which were then washed five times with 0.1% Triton X-100/PBS. Where necessary, samples were then treated with Endo H (endoglycosidase H, Boehringer Mannheim) or neuraminadase (Calbiochem) as described by the manufacturers, before analysis of the immunoprecipitates by SDS–PAGE. For analysis of CD8 and Lyt2, 10–15% acrylamide gradient gels were routinely used, although 12% gels were also employed. 10% gels were used to analyse the CD4 protein. Following electrophoresis, gels were fixed and treated with Amplify (Amersham). To quantify radioactivity incorporated into protein, band intensities on the fluorograph were determined by scanning densitometry using an LKB Ultroscan XL. Radioactive protein marker proteins, bovine serum albumin (67 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd) and lactoglobulin A (18.4 kd), were obtained from New England Nuclear.

Antibodies

OKT8 ascites were prepared by using hybridoma no. CRL 8014 from ATCC (Hoffman *et al.*, 1980). Ascites fluid from 19Thy/5D7, a monoclonal antibody against human CD4, was kindly provided by E.Reinherz (Hussey *et al.*, 1988). Rat anti-mouse Lyt2.2 monoclonal antibody, 2.43, (Sarmiento *et al.*, 1980) was prepared using hybridoma no. TIB 210.

Immunofluorescence microscopy and cytofluorometry

Cells were fixed and stained for immunofluorescence as described previously (Nilsson *et al.*, 1989) with the following modifications. 48 h after transfection, cells were trypsinized and plated onto cover slips treated with Celltack (Collaborative research) as described by the manufacturer. 12 h later, cells were fixed in 4% paraformaldehyde/PBS for 20 min, followed by three washes in 50 mM ammonium chloride/PBS. Cells were permeabilized with 0.1% Triton X-100/PBS for 3 min before incubation with the specific antibody. Fluorescein labelled secondary antibodies were from Cappell. Fluorescence microscopy was performed using a Zeiss axiophot microscope using a 63× objective. Kodak Tri-X PAN film was used for photography.

Cells were prepared for FACS analysis as described previously (Nilsson *et al.*, 1989), except that cells were removed 72 h post-transfection from the dish using PBS containing 2 mM EDTA. Labelled cells were analysed using a Beckton and Dickinson FACS440.

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