

Different Fate of a Single Reporter Protein Containing KDEL or KKXX Targeting Signals Stably Expressed in Mammalian Cells*

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In mammalian cells, resident luminal and type I transmembrane proteins of the endoplasmic reticulum usually contain KDEL and KKXX at the carboxyl terminus. These sequences induce retrieval from compartments located downstream in the secretory pathway. It has been suggested that the retrieval may occur from multiple sites, ranging from the intermediate compartment to the trans-Golgi network. To compare the retrieval of luminal and type I membrane proteins, we have used different forms of a single reporter, the human CD8 glycoprotein, stably expressed in FRT cells. Metabolic labeling and oligosaccharide analysis show that the mechanism based on the KDEL signal is leaky. With time, the KDEL-containing CD8 form reaches the trans/trans-Golgi network compartments, where the protein is terminally glycosylated. At this stage, the retrieval mechanism stops being effective and the protein is consequently secreted. Conversely, the mechanism based on the KKXX signal guarantees that most of the KKXX-containing CD8 form resides in the endoplasmic reticulum, little in the Golgi complex and undetectable levels at the plasma membrane. The O-glycosylation of this protein comprises for the vast majority the sole addition of peptide-bound GalNAc that occurs in an early Golgi compartment.

Many endoplasmic reticulum, luminal and type I transmembrane proteins (carboxyl terminus in the cytosol) contain carboxyl-terminal sequences of the prototype KDEL and KKXX, respectively (Munro and Pelham, 1987; Nilsson *et al.*, 1989). These sequences are necessary and sufficient to determine ER¹ localization of modified secretory and plasma membrane proteins (Pelham, 1988; Jackson *et al.*, 1990). This localization is achieved by retrieval mechanisms, understood only in part. Luminal as well as membrane reporter proteins show Golgi post-translational modifications (Pelham, 1988; Pelham *et al.*,

1988; Dean and Pelham, 1990; Jackson *et al.*, 1993; Gaynor *et al.*, 1994; Townsley and Pelham, 1994); a receptor for KDEL-containing proteins, the ERD 2 transmembrane protein, has been identified and localized in the Golgi complex of yeast and mammalian cells (Semenza *et al.*, 1990; Lewis *et al.*, 1990; Lewis and Pelham, 1990; Tang *et al.*, 1993; Griffiths *et al.*, 1994). Binding to the ligand would induce receptor oligomerization and recycling to the ER, where the ligand will be discharged, while the monomeric receptor would return to the Golgi (Lewis and Pelham, 1992; Townsley *et al.*, 1993). Less is known about the retrieval of membrane proteins. *In vitro*, the KKXX sequence binds the coatamer protein complex (Cosson and Letourneur, 1994), and triggers microtubule polymerization (Dahlöf *et al.*, 1991). Yeast coatamer mutants show defective retrieval (Letourneur *et al.*, 1994). How these interesting findings relate to a retrieval function remains to be determined. However, the importance of the cytosolic location of the signal for transmembrane proteins is supported by the observation that some type II proteins (amino terminus in the cytosol), contain an amino-terminal double arginine motif that maintains them in the ER (Schutze *et al.*, 1994).

Current evidence favors the hypothesis that retrieval of luminal and membrane proteins resident in the ER from post ER compartments represents a salvage mechanism for proteins erroneously included in the membrane flow of the secretory pathway (Munro and Pelham, 1987; Mazzarella *et al.*, 1990; Jackson *et al.*, 1993; Pelham and Munro, 1993; Nilsson and Warren, 1994). Resident proteins would be largely excluded from vesicular export from this organelle because their high local concentration favors intermolecular interactions or the presence of retention signals (Sönnichsen *et al.*, 1994).

A number of studies suggests that retrieval may occur from multiple locations, ranging from the IC to the TGN (Pelham 1991; Peter *et al.*, 1992; Jackson *et al.*, 1993; Griffiths *et al.*, 1994; Miesenböck and Rothman, 1995). However, it is difficult to generalize these data and to compare the retrieval of KDEL- and KKXX-containing reporter proteins. Either different proteins have been used in several host cell lines, or transient transfection experiments have been performed, thus greatly limiting the biochemical and morphological analysis. To overcome these problems, we decided to study a single reporter protein, not naturally resident in the ER, in stably transfected clones expressing comparable levels of its modified versions. We chose the human CD8 protein, a uniquely O-glycosylated type I membrane protein, and the FRT cell line (Littman *et al.*, 1985; Nitsch *et al.*, 1985). CD8 has been extensively used to define the sequence of the KKXX signal (Jackson *et al.*, 1990), and the retrieval of KKXX- and KDEL-containing proteins in HeLa and NIH 3T3 cells (Jackson *et al.*, 1993). In addition, much information has been obtained on CD8 structure (Leahy

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¹ The abbreviations used are: ER, endoplasmic reticulum; TGN, trans-Golgi network; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FPLC, fast performance liquid chromatography; mAb, monoclonal antibody; IC, intermediate compartment.

et al., 1992), and on its biosynthesis and *O*-glycosylation in FRT cells (Pascale *et al.*, 1992a; Pascale *et al.*, 1992b). *O*-Glycosylation is a relatively simple multistep process, compartmentalized in the Golgi complex (Roth, 1987; Perez-Vilar *et al.*, 1991; Pascale *et al.*, 1992b). Thus, the analysis of the carbohydrate moiety carried by the different reporter protein forms would contribute significantly to reveal the extent of their penetration in the Golgi complex.

EXPERIMENTAL PROCEDURES

Materials—All culture reagents were supplied by Life Technologies, Inc. Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milano, Italy), and Serva Feinbiochemica (Heidelberg, Germany). SDS was purchased from BDH (Poole, United Kingdom). [³⁵S]Cysteine (specific activity 1000 Ci/mmol) was obtained from DuPont NEN; [³H]GlcN (specific activity 20 Ci/mmol) and [³H]Gal (specific activity 30 Ci/mmol) were from Amersham (Buckinghamshire, United Kingdom). Mouse monoclonal OKT8 was supplied by Ortho (Raritan, NJ). Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Lectin from *Arachis hypogaea* was from Sigma. Rabbit anti-*A. hypogaea* was purchased from Serotec (Kidlington, United Kingdom). Rhodamine and fluorescein-conjugated goat anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). The reduced glycans GalNAcOH, Galβ1,3GalNAcOH, GlcNAcβ1,6(Galβ1,3)GalNAcOH, and Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcOH used as markers in HPLC analysis were prepared as described by Pascale *et al.* (1992a).

Cell Culture and Transfection—Parental FRT cells, and the clones isolated after transfection, were cultured in Coon's modified Ham's F-12 medium containing 10% fetal calf serum and maintained in a 95% air, 5% CO₂ incubator. The plasmids carrying the cDNAs coding for human CD8 (Migliaccio *et al.*, 1990), the 146 amino-terminal residues (CD8-S) (Leahy *et al.*, 1992), the ectodomain with the carboxyl-terminal sequence SEKDEL (CD8-K) (Jackson *et al.*, 1993), and the ectodomain and transmembrane domain linked to E19 protein carboxyl terminus (CD8-E19) (Jackson *et al.*, 1990), were manipulated and co-transfected with a plasmid carrying the bacterial *neo* gene as described previously (Migliaccio *et al.*, 1990). Stable transformants were selected in the presence of G418 and positive clones screened by immunofluorescence.

Radioactive Labeling, Sample Preparation, and Analysis—Radioactive labeling, preparation of cell extracts, immunoprecipitation, SDS-PAGE and fluorography, and indirect immunofluorescence were performed as detailed previously (Bonatti *et al.*, 1989; Migliaccio *et al.*, 1989; Pascale *et al.*, 1992a). Immunofluorescence with anti-p23 KDEL receptor mAb was as described (Tang *et al.*, 1994). CD8 and CD8-derived forms were detected by immunofluorescence and immunoprecipitation with the mouse monoclonal OKT8 antibody; Western blot analysis was performed with a mouse monoclonal antibody raised against the first 11 amino-terminal residues of human CD8 conjugated to keyhole limpet hemocyanin.

Analysis of [³H]GlcN-labeled *O*-Glycans—All analysis were performed on CD8-derived proteins immunoprecipitated after 16 h of labeling with [³H]GlcN, in order to ensure that the specific activity of [³H]GlcNAc and [³H]GalNAc nucleotide donors was at equilibrium. Areas of polyacrylamide-gels corresponding to bands visualized by fluorography were excised and digested overnight with Pronase at 60 °C as detailed elsewhere (Pascale *et al.*, 1992a). The radioactivity released by Pronase digestion from each band ranged from 25,000 to 230,000 cpm. The glycopeptides isolated by Bio-Gel P-4 (400-mesh) column (1 × 75 cm) were analyzed for the radioactive amino-sugar composition and subjected to the mild alkaline borohydride treatment as described previously (Pascale *et al.*, 1992a). Reduced *O*-glycans generated by the treatment were isolated by Bio-Gel P-4 filtration and analyzed by HPLC as described by Malagolini *et al.* (1994). The fractionation of neutral, mono-, and disialylated *O*-glycans was conducted on a DEAE-Sephacel column eluted with a gradient (0.05–0.2 M) of pyridine/acetate buffer, pH 5, as described previously (Pascale *et al.*, 1992a).

RESULTS

Experimental Strategy—A schematic view of the different CD8-derived proteins, stably expressed by FRT cell lines, is shown in Fig. 1. Several clones were isolated for each construct; each showed almost identical doubling time and capacity to form epithelial monolayers as the parental cell line. In this paper we present the data derived from a detailed analysis of a

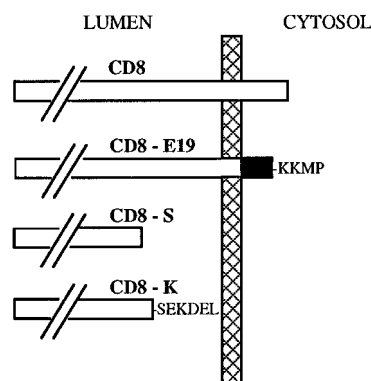


FIG. 1. Schematic drawing of the different CD8 forms used. Human CD8 (white bar) has 162 amino acids in the ectodomain (114 in the NH₂-terminal immunoglobulin domain and 48 in the linker), followed by 26 residues in the transmembrane and in the cytosolic domains (Littman *et al.*, 1985). The cytosolic domain of CD8-E19 protein (black bar) consists of the 15 residues of the corresponding domain of E19 protein, the last 4 amino acids being KKMP (Nilsson *et al.*, 1989). CD8-S has the first 146 residues of CD8 ectodomain (Leahy *et al.*, 1992). CD8-K consists of the first 159 residues of CD8 ectodomain followed by SEKDEL (Jackson *et al.*, 1993).

single clone for each construct; however, the main results were all reproduced in separate clones.

Throughout this work, the CD8 forms bearing the KDEL and KKXX sequences have been compared to the 146-residue anchorless form (CD8-S) and to the wild-type transmembrane form of CD8, respectively. Apart from the localization signals, these two control proteins also differ slightly from their tagged counterpart (see Fig. 1). However, previous experiments have shown that the anchorless form of CD8, which matches CD8-K but lacks the KDEL sequence, and the CD8-E19 form mutagenized in either one of the two crucial lysine residues are glycosylated and transported in the same way as the shorter CD8-S and unmodified CD8 used in this study (Jackson *et al.*, 1990, 1993). We chose the latter forms, due to the extensive information available concerning their structure, biosynthesis, glycosylation, and intracellular transport (Leahy *et al.*, 1992; Pascale *et al.*, 1992a, 1992b).

Immunofluorescence Localization—Double indirect immunofluorescence was performed to establish the intracellular localization of the different CD8 proteins. Fig. 2 (panels a and b) shows that CD8-S was clearly detectable in the Golgi complex, as revealed by the extent of colocalization with mannosidase II (Moremen and Touster, 1985). Conversely, CD8-K showed a very prominent ER labeling (Fig. 2, c–f), as confirmed by the large overlap with an ER marker, the SSRP (Migliaccio *et al.*, 1992). Although difficult to detect in the highly stained perinuclear region, a relatively minor Golgi labeling was present also in CD8-K-expressing cells. As expected, CD8-S and CD8-K were not detected on the cell surface. In CD8-E19-expressing cells, CD8-E19 was present in the ER and Golgi complex (Fig. 3, a–c), as revealed by the strong staining of the nuclear envelope and by the comparison with the respective markers, but completely undetectable on the cell surface (Fig. 4). Conversely, unmodified CD8 protein accumulated in the Golgi complex and on the plasma membrane, but was not detectable in the ER (Fig. 3, e and f). In conclusion, both CD8-K and CD8-E19 appeared localized in the first segment of the secretory pathway, *i.e.* mostly in the ER but also in Golgi complex. Instead, CD8-S and unmodified CD8 behaved as normal secretory and surface membrane protein, concentrating in the Golgi complex and in the plasma membrane, respectively.

The markers of the ER and Golgi complex gave the same results when tested in the parental cell line or in the transfected clones (data not shown). However, it has been reported

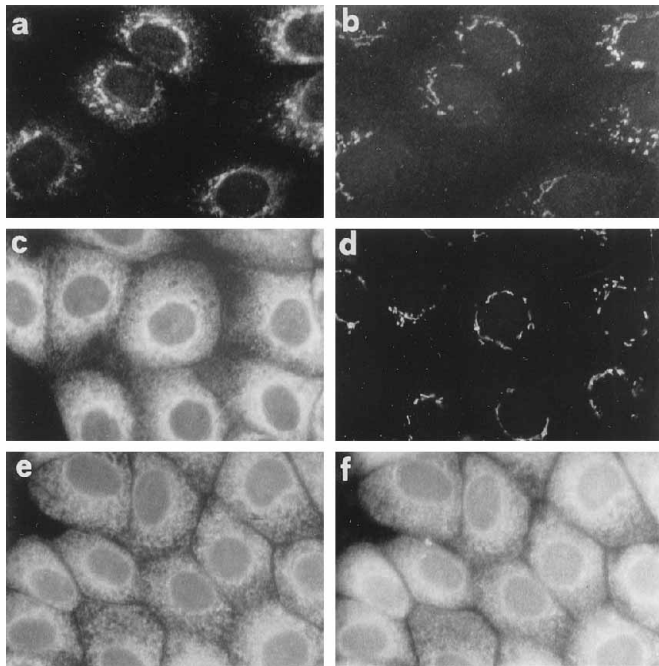


FIG. 2. **Intracellular localization of CD8-S and CD8-K proteins.** Cells were treated for double indirect immunofluorescence light microscopy as indicated under "Experimental Procedures." *a* and *b*, CD8-S-expressing cells; *c*–*f*, CD8-K-expressing cells. *a*, *c*, and *e*, anti-CD8 OKT8 mAb; *b* and *d*, anti-mannosidase II polyclonal antibody; *f*, anti-SSRP polyclonal antibody.

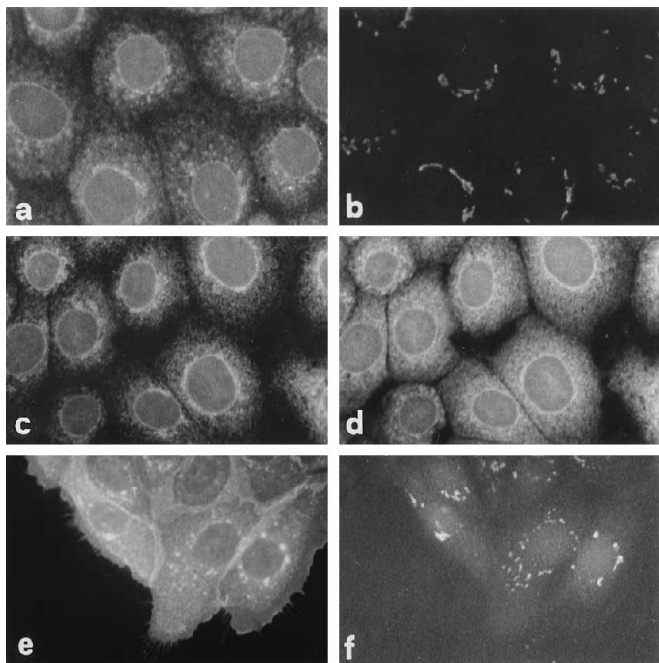


FIG. 3. **Intracellular localization of CD8-E19 and CD8 proteins.** *a*–*d*, CD8-E19-expressing cells; *e* and *f*, CD8-expressing cells. *a*, *c*, and *e*, anti-CD8 OKT8 mAb; *b* and *f*, anti-mannosidase II polyclonal antibody; *d*, anti-SSRp polyclonal antibody.

that overexpression of KDEL-bearing proteins may result in the transfer of the KDEL receptor from the Golgi complex to the ER (Lewis and Pelham, 1992). Thus we controlled the distribution of the mammalian equivalent of yeast ERD-2 receptor, the p23 protein (Tang *et al.*, 1993), in FRT-, CD8-K-, and CD8-E19-expressing cells. In all cell lines, p23 was mostly localized in the Golgi complex (data not shown).

Biosynthesis and Maturation of the Different CD8-derived

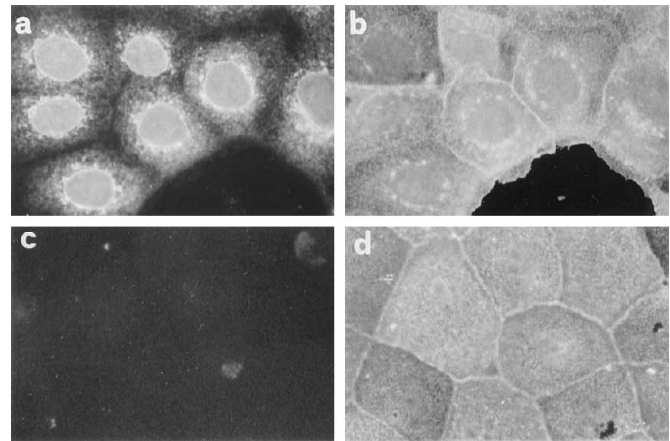


FIG. 4. **CD8-E19 is not present on the surface of the cell.** *a*–*d*, CD8-E19-expressing cells. *a* and *b*, permeabilized cells; *c* and *d*, non-permeabilized cells. *a* and *c*, anti-CD8 OKT8 mAb; *b* and *d*, anti-A. hypogaea polyclonal antibody. To detect Gal exposing glycans (Goldstein and Hayes, 1978), cells were incubated for 20 min with 1 mg/ml A. hypogaea before exposure to the primary and secondary antibodies.

Proteins—Extensive work has been previously performed on the biosynthesis and glycosylation of unmodified CD8 stably expressed by FRT cells (Pascale *et al.*, 1992a, 1992b). In summary, SDS-PAGE analysis of labeling experiments performed either with [³⁵S]cysteine or [³H]sugar precursors showed that CD8 is synthesized as a 27-kDa form, which is quickly converted to a transient and initially glycosylated 29-kDa form, before the full maturation to a completely glycosylated 32–34-kDa doublet. The intermediate form is generated in an early Golgi compartment, while the mature doublet in the trans-Golgi/TGN region. The entire maturation and transport process are quite fast; the 27-kDa form is modified with a half-time of 10 min, whereas the mature doublet is generated and reaches the plasma membrane with half-times of 15 and 30 min, respectively.

On the basis of this information, we first performed pulse-chase analysis on the clones expressing CD8-S and CD8-K protein. As shown in Fig. 5A, [³⁵S]cysteine labeling revealed that CD8-S is synthesized as a 19-kDa precursor that is converted to a 29-kDa mature form. Intermediate forms in the maturation are detected at low level, and only the mature form is secreted. Conversely, CD8-K is synthesized initially as a 20-kDa form (Fig. 5B), which is slowly and progressively converted to several intermediate forms; little of the 30-kDa mature form is detectable intracellularly at all chase time points, but it is clearly present in the extracellular medium (Fig. 5B). Densitometric analysis of the autoradiograms of several pulse-chase experiments showed that the half-times of the disappearance of newly synthesized form, and of secretion, were approximately 1 and 2 h for CD8-S, and 16 and 20 h for CD8-K, respectively (data not shown). These results suggested that the delay in processing and secretion time of CD8-K is not proportionally distributed, but more severe for the early events of the secretory pathway.

To better evaluate this hypothesis, we moved to labeling experiments with [³H]GlcN, which is incorporated in O-glycans as GalNAc, GlcNAc, and NeuNAc, and [³H]Gal. As shown in Fig. 6, only the intermediate and mature forms were visualized. The intracellular mature forms were labeled with both sugar precursors, the intermediate forms of CD8-S were detectable with [³H]GlcN but not with [³H]Gal, and the intermediate forms of CD8-K were labeled anyhow, but [³H]GlcN revealed more discrete bands. Finally, both labeling conditions confirmed that only the mature forms of CD8-S and CD8-K were secreted. Next, we performed pulse-chase labeling experiments

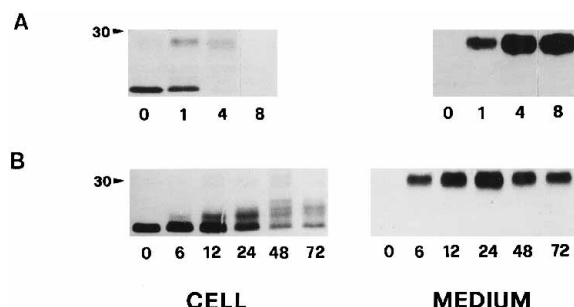


FIG. 5. Biosynthesis and secretion of CD8-S and CD8-K proteins. SDS-PAGE analysis of the immunoprecipitated products after pulse-chase labeling with [35 S]cysteine. *A*, CD8-S-expressing cells, pulse time 20 min. *B*, CD8-K-expressing cells, pulse time 1 h. The decrease in the amount of the mature form of CD8-K in the extracellular medium after 48 h of chase is a random event. *Numbers* on the bottom indicate the chase times in h. *Numbers* on the left indicate the position on the same gel of a 30-kDa marker protein. Only the relevant portion of the gel is shown.

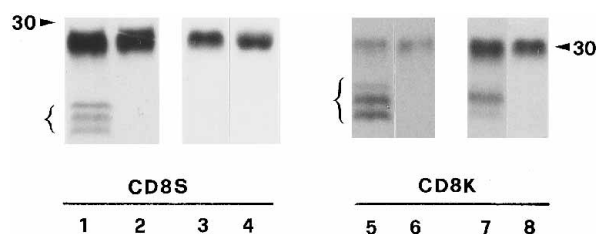


FIG. 6. Intracellular accumulation of the initially glycosylated, intermediate forms of CD8-K. SDS-PAGE analysis of the immunoprecipitated products after 3 h of labeling with [3 H]GlcN (lanes 1, 2, 5, and 6) or [3 H]Gal (lanes 3, 4, 7, and 8) from CD8-S- and CD8-K-expressing cells. *Lanes* 1, 3, 5, and 7, intracellular products; *lanes* 2, 4, 6, and 8, extracellular products. *Brackets* on the left indicate the initially glycosylated, intermediate forms of CD8-S and CD8-K. *Numbers* on the left indicate the position on the same gel of a 30-kDa marker protein. Only the relevant portion of the gel is shown.

with these sugar precursors. With [3 H]Gal, the mature forms of both CD8-S and CD8-K were quickly secreted, whereas the intermediate forms of CD8-K lasted intracellularly for long time (data not shown). To amplify this result, we labeled CD8-K-expressing cells with both [3 H]GlcN and [3 H]Gal. As shown in Fig. 7 (panels *A* and *B*), the mature form of CD8-K moved quickly from the cell to the medium (some mature form is already secreted in the pulse time). Conversely, the intermediate forms lasted even 48 h after the pulse, showing a slow decrease in electrophoretic mobility (Fig. 7A). That these intermediates do not represent a dead end product is shown in Fig. 7C. The bulk of secretion of the mature form did occur during the first 6 h of chase, but significant secretion took place up to 48 h. Clearly, new mature form is generated from the intermediate forms during the chase, and it is not detectable intracellularly because it is rapidly secreted.

In conclusion, the labeling experiments performed with [35 S]cysteine or [3 H]sugars indicated that processing and secretion of CD8-K is much slower than CD8-S. In addition, it appeared that most of the delay was due to a slow arrival of newly synthesized CD8-K to the late Golgi compartments.

Next we performed pulse-chase labeling experiments on CD8-E19-expressing cells. As shown in Fig. 8, CD8-E19 is initially synthesized as a 24/25-kDa doublet form, which is slowly converted to a discrete 28-kDa form. The doublet does not represent an initial glycosylation step (see below), but it is due to an unknown reversible modification (Jackson *et al.*, 1993). In spite of the low increment of the apparent molecular mass, the 28-kDa form was the final maturation product of CD8-E19 visualized by long labeling with [3 H]GlcN and less

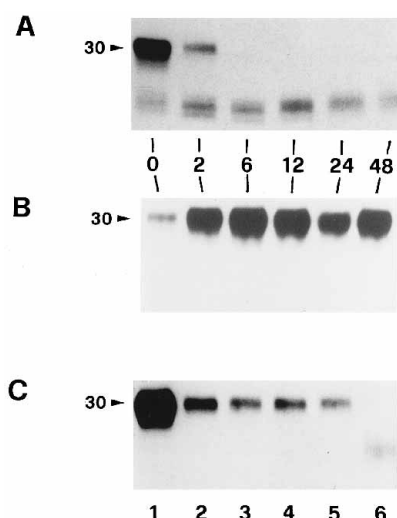


FIG. 7. Delayed terminal glycosylation, but subsequent rapid secretion, of CD8-K protein. SDS-PAGE analysis of the immunoprecipitated products after pulse-chase labeling with both [3 H]GlcN and [3 H]Gal of CD8-K-expressing cells. *A*, intracellular products. *B*, extracellular products. Pulse time 1 h; numbers indicate the hours of chase. *C*, the extracellular products that accumulate in the following time intervals (in h) after 1 h of labeling: 0–6 (lane 1), 6–12 (lane 2), 12–24 (lane 3), 24–36 (lane 4), 36–48 (lane 5). In lane 6, the intracellular products after 48 h of chase. *Numbers* on the left indicate the position on the same gel of a 30-kDa marker protein. Only the relevant portion of the gel is shown.

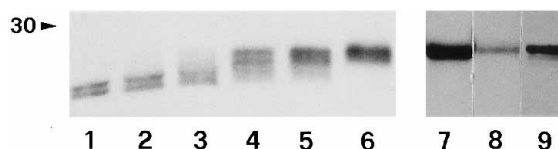


FIG. 8. Biosynthesis and partial maturation of CD8-E19 protein. SDS-PAGE analysis of the immunoprecipitated products after pulse-chase labeling of CD8-E19-expressing cells with [35 S]cysteine. Pulse time 20 min (lane 1); chase times of 1, 2, 4, 6, and 24 h (lanes 2–6, respectively). In *lanes* 7 and 8, the immunoprecipitated products after 16 h of labeling with [3 H]GlcN or 16 h labeling with [3 H]Gal. In lane 9, the intracellular products detected by Western blot analysis. The number on the left indicates the position on the same gel of a 30-kDa marker protein. Only the relevant portion of the gel is shown.

efficiently with [3 H]Gal (compare *lanes* 7 and 8 of Fig. 8), and the only one detectable by Western blot analysis (Fig. 8, lane 9). As expected, CD8-E19 was not found in the extracellular medium (data not shown). Interestingly, very prolonged chase time showed that the 28-kDa form is remarkably stable, having a half-time for degradation greater than 3 days. All together, these results strongly suggested that CD8-E19 undergoes a slow and only partial maturation process.

O-Glycosylation of the Different CD8-derived Proteins—We have previously described the O-glycosylation process of CD8 expressed in FRT cells (Pascale *et al.*, 1992a). In these cells, the maturation process produces neutral and sialylated O-glycans with the branched core-2 structure. Such a structure is formed by the addition of one GlcNAc residue in β 1,6-linkage to the peptide-bound GalNAc that is usually already elongated at the 3-hydroxyl with one Gal residue (Brockhausen *et al.*, 1985). We found that the major glycan carried by mature CD8 is the neutral Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc tetrasaccharide. A monosialylated homolog of the tetrasaccharide was also found to be present in a significant amount, whereas disialylated glycans were identified in a very low proportion. These carbohydrate structures correlate very well with the glycosyltransferase pattern of FRT cells, namely with the high expression of the core-2 glycosyltransferase (β 1,6-N-acetylglucosami-

TABLE I
Ratio between [^3H]GlcNAc and [^3H]GalNAc in intermediate and mature forms of CD8 and CD8-derived proteins from [^3H]GlcN-labeled cells

CD8 forms were immunoprecipitated from the corresponding cells labeled for 16 h with [^3H]GlcN and subjected to SDS-PAGE. The bands migrating as intermediate or mature forms (see Figs. 6 and 8) were excised from the gel, digested with Pronase, and subjected to Bio-Gel P-4 filtration as detailed under "Experimental Procedures." In all the cases, 80–85% of total radioactivity applied to the column was recovered in fractions eluted in the void volume. An aliquot (about 4,000 cpm) of pooled void-volume fractions was hydrolyzed with 4 N HCl for 4 h at 100 °C and then analyzed for radiolabeled amino-sugar composition as described in the text. Under the hydrolysis condition GalNAc and GlcNAc were converted and recovered as the corresponding deacetylated sugar. Values are mean \pm S.D. of three independent preparations of CD8 and CD8-S and of two preparations of CD8-K and CD8-E19.

CD8 form	Apparent molecular mass	[^3H]GlcNAc/[^3H]GalNAc
<i>kDa</i>		
CD8i	29	0.17 \pm 0.02
CD8m	32–34	0.61 \pm 0.03
CD8-Si	22–25	0.17 \pm 0.006
CD8-Sm	29	0.74 \pm 0.11
CD8-Ki	22–25	0.11 \pm 0.018
CD8-Km	30	0.89 \pm 0.5
CD8-E19	28	0.07 \pm 0.008

nyltransferase) and a low activity of sialyltransferases (Pascale *et al.*, 1992b). Our previous results indicated also that the various steps of carbohydrate assembly correspond to the transit of CD8 in specific compartment of the Golgi complex (Pascale *et al.*, 1992b). The addition of the peptide-bound GalNAc occurs in an early compartment and results in the formation of the intermediate form that carries predominantly non elongated-GalNAc; most of the branching and galactosylation takes place in a late Golgi region, and subsequently the addition of sialic acid completes the conversion into the mature doublet form of 32–34 kDa.

We decided to characterize the carbohydrate chains of the intermediate and mature forms of all CD8-derived proteins to ascertain if the modified peptide moiety changed the general pattern of *O*-glycosylation, and at which stage the glycan processing was arrested by the KDEL and KKXX motifs with a consequent intracellular accumulation of forms that are not fully processed.

As mentioned before, the branching by GlcNAc represents a crucial step in the *O*-glycan processing, which very likely coincides with the routing to a late compartment of the Golgi complex. Therefore, we compared the amounts of [^3H]GlcNAc and [^3H]GalNAc incorporated in the intermediate and mature forms of CD8, CD8-S and CD8-K, and the single glycosylated form of CD8-E19. As expected, the ratio between [^3H]GlcNAc and [^3H]GalNAc was very low in the intermediate forms and much higher in the mature forms, indicating that the branching does parallel the maturation process (Table I). The relative proportion of [^3H]GlcNAc in CD8i and CD8-Si was the same, whereas less [^3H]GlcNAc was found in CD8-Ki and even less in CD8-E19. To characterize the carbohydrate carried by CD8-Ki and CD8-E19, the reduced *O*-glycans, generated by mild alkaline borohydride treatment of Pronase-glycopeptides, were analyzed by HPLC. Table II shows that in both cases the predominant carbohydrate was, by far, non-elongated GalNAc; it represented 85 and 67% of the [^3H]GlcNAc-derived radioactivity incorporated in CD8-E19 and CD8-Ki, respectively. Very similar results have been obtained with a separate preparation. The relative heterogeneity in the elongated *O*-glycans of CD8-Ki and CD8-E19 may account for their migration pattern,

TABLE II
Relative distribution of reduced *O*-glycans in [^3H]GlcN-labeled CD8-E19 and intermediate forms of CD8-K

[^3H]GlcN-labeled glycopeptides, obtained as described in the legend of Table I, were subjected to mild alkaline borohydride treatment. The released reduced *O*-glycans were isolated by a Bio-Gel P-4 column and then subjected to HPLC analysis. Fractions eluted at the retention times of the following glycan markers: GalNAcOH, Gal β 1,3GalNAcOH, GlcNAc β 1,6(Gal β 1,3)GalNAcOH and Gal β 1,4GlcNAc β 1,6(Gal β 1,3)-GalNAcOH, were pooled and counted for the radioactivity.

Glycan	[^3H]GlcN-derived radioactivity			
	CD8-E19		CD8-Ki	
	<i>cpm</i>	<i>%</i>	<i>cpm</i>	<i>%</i>
GalNAcOH	4701	85	2388	67
Gal β 1,3GalNAcOH	166	3	356	10
GlcNAc β 1,6(Gal β 1,3)GalNAcOH	221	4	285	8
Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAcOH	442	8	535	15

i.e. for the discrete bands visualized by SDS-PAGE (see Figs. 7 and 8).

Finally, we measured the relative amounts of neutral *versus* sialylated mature forms. No significant difference was found in the degree of sialylation of mature forms of CD8, CD8-S and CD8-K, whereas exclusively neutral glycans were found in CD8-E19, as well as sialic acid (data not shown).

On the basis of these results, we concluded that (i) the *O*-glycosylation process of CD8-S and CD8-K is similar to that of CD8, despite the difference in the intracellular transit rate; (ii) the intracellular accumulation of CD8-Ki corresponds to the arrest of *O*-glycosylation at a stage in which the peptide-bound GalNAc is predominantly non elongated (this arrest is not complete, so that CD8-Ki is eventually processed to CD8-Km by the addition of GlcNAc and Gal and by sialylation); (iii) the *O*-glycosylation of CD8-E19 is more drastically restricted, such that the vast majority of *O*-linked carbohydrate on CD8-E19 consists of a single GalNAc residue, with only traces of branched core-2 glycans. Remarkably, no sialylated glycans were found.

DISCUSSION

The main outcome of this study is summarized in Fig. 9. The retrieval mechanism based on the KDEL signal is leaky. With time, CD8-K reaches the trans-Golgi/TGN region, from where retrieval is no longer effective. As a result, the protein is secreted as a fully glycosylated form. Conversely, the mechanism based on the KKXX signal is "leak-proof"; it retains the bulk of CD8-E19 in the ER as a partially processed glycoprotein. The principal results in support of our conclusions are as follows. (a) The mature form of CD8-K is secreted with a 10-fold delay with respect to CD8-S. Almost all the delay is due to events occurring before CD8-K arrives in the trans-Golgi/TGN region. Both the unglycosylated and the initially glycosylated forms of CD8-K are intracellularly located longer than the respective CD8-S forms. Eventually, CD8-K is partially routed out of the ER/cis-medial Golgi compartments; it is terminally glycosylated in the trans/TGN Golgi region similarly to unmodified CD8 and CD8-S, and is rapidly secreted. Indeed, CD8-K and CD8-S proteins isolated from cell media carry as major *O*-glycan the same core-2 branched tetrasaccharide found in unmodified CD8 (Serafini-Cessi *et al.*, 1995). (b) CD8-E19 accumulates mostly in the ER, but carries nascent *O*-linked GalNAc acquired in the cis/medial Golgi compartments. The typical core-2 branching structure of CD8 *O*-glycans manufactured by FRT cells is practically absent in the intracellularly accumulated CD8-E19. This protein is not detectable at the cell surface, although it lasts several days within the cell, whereas unmodified CD8 is terminally glycosylated and reaches the

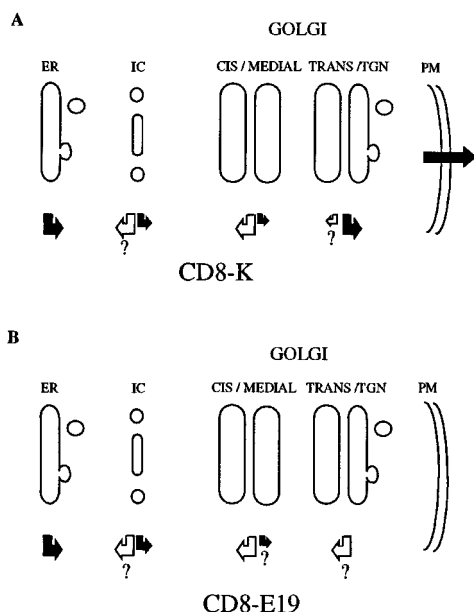


FIG. 9. Schematic drawing of the intracellular routing of CD8-K and CD8-E19. Rightward pointing black arrows indicate anterograde transport; leftward pointing white arrows indicate retrograde transport toward the ER. Question marks hypothesize transport steps of the two reporter proteins that are not demonstrated by the present work. The size of the arrows reflects the balance of anterograde versus retrograde movement of the proteins.

plasma membrane with a half-time of 30 min (Pascale *et al.*, 1992a).

Several other observations reinforce these results. The clones analyzed in detail were indistinguishable from the parental cells for all the criteria examined (doubling time, expression of epithelial phenotype, ER and Golgi complex ultrastructure, distribution of marker proteins, Golgi localization of the p23 KDEL receptor). Furthermore, different clones confirmed the slow secretion of CD8-K and the tight ER retention of CD8-E19. Thus, we had no indication that the expression of significant amounts of the different CD8 forms altered the normal physiology or the retrieval functions of FRT cells.

Numerous reports confirm part of the results we have obtained and largely support their general interpretation. Transient transfection experiments revealed a delayed secretion of KDEL-containing rat growth hormone and human chorionic gonadotrophin in COS and HeLa cells (Zagouras and Rose, 1989). Similarly, a small amount of CD8-K protein is secreted at the end of a 24-h chase in transiently transfected HeLa cells (Jackson *et al.*, 1993). Some KDEL-containing proteins are secreted in exocrine pancreatic cells (Takemoto *et al.*, 1992). The distribution in the Golgi complex of the p23 KDEL receptor has been studied by immunoelectron microscopy. Under physiological conditions, the receptor was mostly present in the IC and in the cis- and medial Golgi; under stress conditions a shift to the trans/TGN regions was observed (Griffiths *et al.*, 1994). None of these data contradict the hypothesis that the efficiency of the retrieval process for KDEL-containing luminal protein decreases from the cis/medial to the trans/TGN region of the Golgi complex. This effect could be due to a decreasing concentration gradient of the receptor, or to a decreased affinity of ligand binding. Minor changes in the luminal environment of the Golgi compartments may affect the affinity of binding.

Less is known about KKXX-containing membrane proteins. CD8-E19 and CD4-E19 transiently transfected in HeLa and NIH 3T3 cells appeared localized mainly in the ER by immunofluorescence microscopy (Jackson *et al.*, 1990). By cell fractionation of transiently transfected HeLa cells, the bulk of

sialylated as well as only GalNAc modified CD8-E19 was seen cosedimenting with an ER marker (Jackson *et al.*, 1993). This finding suggests retrieval from both the cis- and the trans-Golgi compartment. We did not detect sialylation of stably expressed CD8-E19, and the amount of fully galactosylated oligosaccharide chains was very limited. Our data are then insufficient to indicate the routing of CD8-E19 to the trans/trans-Golgi network area (see below). An immunoelectron microscopical analysis of CD8-E19-expressing cells could provide the answer to this question. In conclusion, the evidence available suggests that KKXX-containing reporter proteins are restricted to the ER by means of an efficient retrieval mechanism. This efficiency could be the result of a cytosol-based mechanism, either peculiarly able to operate from the cis/medial Golgi, as our data may indicate, or equally able to interact with KKXX-exposing proteins in all Golgi compartments.

It could be argued that the results obtained with CD8-K and CD8-E19 are due to a severe impairment in the folding and oligomerization of the newly synthesized proteins. Indeed, it is well known that only properly folded/oligomerized secretory and plasma membrane proteins are exported from the ER (Doms *et al.*, 1993; Helenius, 1994), but once exported they proceed toward the surface of the cell without other identified "quality control" steps. Clearly, this hypothesis would explain only the delay in the initial glycosylation events of CD8-K and CD8-E19, not the behavior of CD8-K intermediate forms or the accumulation in the ER of glycosylated CD8-E19. In newly synthesized CD8, proper folding can be easily monitored from the expression of the epitope recognized by OKT8 mAb (a conformation-specific antibody), and by the formation of disulfide-bond homodimers (Verde *et al.*, 1995). Newly synthesized CD8-K and CD8-E19 (as well as CD8-S) are detected by OKT8 mAb, and we have evidence that CD8-E19 and CD8-S rapidly form disulfide-linked homodimers (data not shown). It is difficult to ascertain the oligomerization status of CD8-K. The entire anchorless ectodomain of CD8 forms homodimers not linked by disulfide bridge, even in the absence of the KDEL sequence (Leahy *et al.*, 1992). Preliminary results obtained by FPLC analysis showed that the secreted form of CD8-K was homodimeric, whereas part of the intracellular forms migrated as multimeric complexes. Interestingly, it has been suggested that occupied KDEL receptors oligomerize, forming protein patches that migrated to the ER (Townsend *et al.*, 1993).

It has been suggested that the IC between ER and Golgi complex is a site of retrieval to the ER (Lewis and Pelham, 1990; Jackson *et al.*, 1993; Griffiths *et al.*, 1994). This hypothesis can not be tested by biochemical assays because of the lack of a specific post-translational modification marker for the IC. The immunofluorescence pattern we observed with CD8-K, p23 KDEL receptor, and CD8-E19 do not exclude their presence in the IC. However, these patterns are largely different from the ones given by proteins enriched in the IC (Saraste and Svensson, 1991; Schweizer *et al.*, 1990; Schweizer *et al.*, 1993; Lotti *et al.*, 1992). We have observed that newly synthesized CD8-K and CD8-E19, in contrast to CD8-S and unmodified CD8, require several hours to undergo the initial *O*-glycosylation events. There is compelling evidence that these events occur in the cis/medial Golgi compartments (Roth *et al.*, 1994; Piller *et al.*, 1989; Verde *et al.*, 1995; Vassard *et al.*, 1995). This finding may thus support the view of efficient retrieval occurring upstream of these compartments. A cell fractionation-based approach could help to resolve this issue.

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