

# Retrieval of Transmembrane Proteins to the Endoplasmic Reticulum

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**Abstract.** A COOH-terminal double lysine motif maintains type I transmembrane proteins in the ER. Proteins tagged with this motif, eg., CD8/E19 and CD4/E19, rapidly receive post-translational modifications characteristic of the intermediate compartment and partially colocalized to this organelle. These proteins also received modifications characteristic of the Golgi but much more slowly. Lectin staining localized these Golgi modified proteins to ER indicating that

this motif is a retrieval signal. Differences in the sub-cellular distribution and rate of post-translational modification of CD8 maintained in the ER by sequences derived from a variety of ER resident proteins suggested that the efficiency of retrieval was dependent on the sequence context of the double lysine motif and that retrieval may be initiated from multiple positions along the exocytotic pathway.

SOME proteins remain in the ER as permanent residents, whereas others are exported to the Golgi stack for subsequent distribution to the cell surface, lysosomes, secretory vesicles, et cetera. Current opinion favors the idea that transport of proteins between the organelles occurs by default (Wieland et al., 1987; Pfeffer and Rothman, 1987). Consequently, residency in the ER requires mechanisms that recognize and maintain the proteins in that location. A recognition motif for the retention of soluble ER proteins, the COOH-terminal sequence Lys-Asp-Glu-Leu (KDEL),<sup>1</sup> has been identified (Munroe and Pelham, 1987; Pelham, 1989). Likewise, some ER resident type I transmembrane proteins contain an ER targeting motif (Nilsson et al., 1989) consisting of two lysine residues at the -3 and either the -4 or -5 position from the COOH terminus (Jackson et al., 1990).

ER retention may involve two principally different mechanisms. Either proteins are retained by being excluded from transport vesicles or they enter such vesicles to be subsequently retrieved to the ER. Two putative receptors recognizing the KDEL motif have been described (Lewis and Pelham, 1990; Vaux et al., 1990). Their locations in the Golgi and intermediate compartmentlike structures, respectively (Lewis and Pelham, 1990, 1992; Vaux et al., 1990), argue for retrieval being the mechanism by which KDEL proteins become resident of the ER (see Pelham, 1989; Lewis and Pelham, 1992). Thus, the putative KDEL receptor may recirculate between the ER and the post-ER compartments

(Lewis and Pelham, 1992) as has been shown by morphological analyses for some marker proteins in the intermediate compartment (Lippincott-Schwartz et al., 1989; Schweizer et al., 1990; Saraste and Svensson, 1991) and for class I MHC molecules (Hsu et al., 1991). The existence of a microtubule-dependent retrograde pathway has also been uncovered by treating cells with brefeldin A (Lippincott-Schwartz et al., 1990) although some reservations remain about its physiological significance.

Studies on the retrieval of proteins to the ER or their retention in this or other organelles has been hampered by the lack of biochemical markers permitting kinetic studies. Thus, morphological analyses can only reveal accumulation of proteins as a consequence of a rate limiting step in the transport pathway. For this reason, it is not fully understood from which organelles proteins can be retrieved to the ER, nor is it clear what the requirements for retrieval are.

To address the mechanism by which transmembrane proteins with the double-lysine motif are maintained in the ER, we have examined the post-translational modifications and the subcellular distribution of marker proteins onto which a double lysine motif has been engineered. We now report that such proteins partially localize to the intermediate compartment and provide both biochemical and morphological evidence that they are retrieved to the ER from this and the Golgi compartments.

## Materials and Methods

### Recombinant DNA

Construction of the expression plasmids encoding CD8/E19, CD8/E19-3S, CD8/H25, CD8/HP1, CD8/53kSER, CD8/HMGCoA, CD4/E19, CD4/H25, CD4/HP1, CD4/53kSER and CD4/HMGCoA have been previously described (Nilsson et al., 1989; Jackson et al., 1990). Construction of CD8sol and CD8/KDEL utilized the PCR and oligonucleotides with CD8

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1. *Abbreviations used in this paper:* HPA, *helix pomatia* agglutinin; KDEL, Lys-Asp-Glu-Leu; PNA, *Arachis hypogaea* agglutinin.

cDNA (Littman et al., 1985) as the template DNA, the polymerase chain reactions were carried out essentially described previously (Nilsson et al., 1989). A 5' primer encoding two Bam HI sites followed by nucleotides 65–82 of the CD8 sequence was used together with a 3' primer encoding the complementary sequence to nucleotides 584–602 in the CD8 sequence followed by a stop codon TGA and two BamHI sites in the PCR to produce the CD8sol cDNA. The COOH terminus of the encoded cDNA was -AVHTRGLDFA. The same 5' primer was used with a 3' primer encoding the complementary sequence to nucleotides 584–602 in the CD8 sequence followed by the sequence AGTGAAAAGATGAGTTGTGAGGATCCGGATCC, the COOH terminus of the encoded protein CD8/KDEL was -AVHTRGLDFASEKDEL. CD8/E19-C/S was constructed using oligonucleotide cassettes, an oligonucleotide coding for nucleotides 606–646 followed by a KpnI site and an Xba I site was annealed with a complementary oligonucleotide and cloned into the CD8/E19 vector which had been cleaved with Eco RV and Xba I. Oligonucleotides encoding the missing portion of CD8/E19 with the codon for amino acid 158 changed from TGC to TCC were directionally cloned into the above plasmid between the Kpn I and XbaI sites. The resulting plasmid encoded CD8/E19 with a single amino acid substitution at amino acid 158 of cysteine to serine.

HLA A2.2 cDNA was obtained from P. Parham (Holmes et al., 1987) and used as a template for a PCR with oligonucleotides that result in a recombinant cDNA encoding the complete extracellular and transmembrane domains of A2.2 and a cytoplasmic tail derived from E19, the carboxy-terminal sequence of the recombinant A2.2/E19 was -GAVVAAVMWKYKSRRSFIDEKMP. The same 5' primer was used with a 3' primer encoding a SEKDEL tail preceded by sequence from the 3' end of the alpha 3 domain, the resulting cDNA encode a soluble A2.2 with a COOH terminus of -PLTYLRWSEKDEL.

Rat prealbumin cDNA (Sundelin et al., 1985) was used as a template in a PCR reaction to produce a recombinant cDNA encoded a prealbumin protein with the amino acid sequence SEKDEL at the COOH terminus, (Melhus, 1990). All of the above recombinant cDNAs were sequenced and cloned into the expression vector pCMU (Nilsson et al., 1989) for transient expression in mammalian cells or into pBS + (Stratagene Inc., La Jolla, CA) for production of mRNA using T7 polymerase for use in an in vitro translation kit (Promega Corp., Madison, WI).

### Cell Culture and Transfection

HeLa and NIH 3T3 cells were grown in DME supplemented with 8% FCS, 2 mM glutamine, 100 U/ml penicillin and streptomycin at 100 µg/ml. For the temperature shift experiments, this media was supplemented with 10 mM Hepes buffer pH 7.3. Transfections were carried out using the calcium phosphate method as described previously (Nilsson et al., 1989), except that 25 µg of DNA were used to transfect  $1 \times 10^5$  cells in a 100-mm petri dish.

### Metabolic Labeling, Immunoprecipitation, Endo H and Neuraminidase Treatment

Metabolic labeling 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 DME (Gibco/BRL, Gaithersburg, MD) before addition of the pulse media. Pulse media contained 0.2 mCi/ml of [<sup>35</sup>S]methionine (*Trans*-label; ICN Biochemicals Inc., Costa Mesa, CA) in methionine deficient DME. Cells were labeled and subsequently chased in the presence of culture media for the times indicated in the individual experiments. Immediately after the chase period cells were rinsed in PBS and lysed in situ with 1% Triton X-100/PBS. Lysates were precleared by centrifugation in a microfuge for 2 × 15 min to remove cell debris, followed by a 2-h incubation with protein A Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The Sepharose was then removed by centrifugation and antibody was added to the lysate. After 2 h, protein A Sepharose was incubated with the lysate for 30 min, and subsequently collected by centrifugation. The Sepharose beads were washed 5 times in PBS containing 0.1% Triton X-100 before analysis by SDS PAGE. Samples were treated for digestion with Endo H (endoglycosidase H, Boehringer Mannheim Corp., Indianapolis, IN) or neuraminidase (Calbiochem Corp., La Jolla, CA) as described by the manufacturers. Preparation of semi-intact cells was as previously described (Beckers et al., 1987) except that cells were scraped off the dish immediately after labeling without osmotic swelling, this resulted in >90% of the cells becoming permeable to trypan blue. The semi-intact cell preparations were used in the standard ER to Golgi in vitro transport assay described by Beckers et al. (1987) in the presence or absence of 150 µM UDP-GalNAc (Sigma Chemical Co., St. Louis, MO). Labeling with

[<sup>3</sup>H]palmitic acid (Amersham Corp., Arlington Heights, IL) was as described by Buss and Sefton (1986). 10–15% acrylamide gradient gels were used for the analysis of CD8 chimeras, 10% gels were used for the analysis of CD4 and HLA A2 chimeras. After electrophoresis, gels were fixed and treated with Amplify (Amersham Corp.). Radioactive protein marker proteins: BSA (67 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD) and lactoglobulin A (18.4 kD) were obtained from New England Nuclear (Boston, MA).

### Nocodazole and Brefeldin A Treatment of Cells

To depolymerize microtubules, cells were incubated in ice cold media for 25 min, the media was then replaced with 37°C DME for the control cells, or with DME containing 10 µg/ml nocodazole (Aldrich Chemical Co., Milwaukee, WI), after 1 h in this media cells were prepared for labeling, nocodazole was maintained in the prelabeling, labeling and chase media. We determined by immunofluorescence microscopy with antitubulin antibodies (Boehringer Mannheim, Corp.) that immediately before labeling, the above pre-treatments left an intact microtubule network in the control cells, while no detectable microtubules could be identified in the nocodazole treated cells. Brefeldin A (Epicentre Technologies) treatment (2 µg/ml) was for 1 h before labeling; the drug was maintained in the media through out the experiment.

### Subcellular Fractionation

Subcellular fractionation was carried out as previously described (Bole et al., 1986). Briefly,  $5 \times 10^7$  HeLa cells transfected with CD8/E19 were biosynthetically labeled for 2 h with [<sup>35</sup>S]translabel as described above and chased for 24 h in the presence of unlabeled methionine. After washing and homogenization the postnuclear supernatant was centrifuged (40,000 rpm for 2 h in a Beckman SW41 rotor) through a discontinuous sucrose gradient. 19 fractions were collected from the gradient, these were analyzed for the presence of galactosyltransferase activity (see Hsu et al., 1991). Anti-CD8 and anti-epoxide hydrolase antibodies were added to each fraction along the Triton X-100 (1%). Immunoprecipitates were analyzed on SDS PAGE and the levels of radioactivity in individual bands quantitated using an Ambis radioanalytic imaging system.

### Antibodies

Ascites fluid containing monoclonal antibodies against CD8 (OKT8) and HLA A2.2 (BB7.2) were prepared by using hybridoma No. CRL 8014 and HB82 from ATCC. Ascites fluid from 19Thy/5D7 a monoclonal antibody against human CD4 was kindly provided by E. Reinherz. Rabbit antisera against the p58 marker protein of the intermediate compartment (Saraste et al., 1987) were provided by J. Saraste (Ludwig Institute for Cancer Research, Stockholm, Sweden), epoxide hydrolase (Bulleid et al., 1986) by J. Craft (University of Glasgow, Glasgow, Scotland), UDP-glucuronosyltransferase (Scragg et al., 1985) by B. Burchell (University of Dundee, Scotland), a 135-kD Golgi protein (mAb 53FC3, Burke et al., 1982) by B. Burke (Harvard Medical School, Boston, MA), and against BiP (Vaux et al., 1990) by S. Fuller (European Molecular Biology Laboratories, Heidelberg, Germany). Antibodies against HPA and PNA lectins were obtained from Bioproducts for Science (Indianapolis, IN). Fluorescein and Texas red labeled affinity purified Goat anti-rabbit or mouse IgG were obtained from Cappel Laboratories (Malvern, PA) and Molecular Probes Inc. (Eugene, OR). Biotinylated antibodies to CD8 and CD4, and Texas red and fluorescein labeled streptavidin were obtained from Becton Dickinson Immunocytometry Systems (Mountain View, CA).

### Immunofluorescence Microscopy

Cells were fixed and stained for immunofluorescence as described previously (Jackson et al., 1990). Nocodazole and brefeldin A treatment of cells was as described for the pretreatment in the pulse chase experiments above. To stain with the anti-lectin antibodies cells were pre-incubated in PBS containing the appropriate lectin (Sigma Chemical Co.) at a concentration of 25 µg/ml for 30 min. Blocking of surface PNA binding was carried out by a 30-min pre-incubation with 50 µg/ml *Bauhinia purpurea* agglutinin. Primary antibodies were typically used at a dilution of 1:200 in PBS containing 0.2% gelatin. To be able to double stain cells where both antibodies were of murine origin, standard staining was carried out with one antibody, the cells were then incubated in the presence of an excess of pre-immune mouse IgG with the second antibody which had been conjugated to biotin. Binding of the biotinylated antibody was then detected with streptavidin coupled to

fluorescein or Texas red (Amersham Corp.). Fluorescence microscopy was performed using a Zeiss axiophot microscope using a 63X objective. Kodak Ektachrome 400 film was used for photography. Confocal microscopy was performed on a BioRad MRC-600.

## Results

### *Construction of ER Retained Transmembrane and Soluble Forms of CD8*

Our primary aim was to determine whether the double-lysine ER targeting motif acted by simply retaining (fixing) proteins in the ER or by retrieving proteins from post ER compartments, as suggested for the KDEL motif (Pelham, 1989). For this reason we have compared the post-translational modifications of essentially the same marker protein, CD8, maintained in the ER by either a KDEL or a double-lysine motif. CD8 was chosen for these studies as it receives O-linked carbohydrates. In contrast to the processing of N-linked carbohydrates, which are difficult to monitor until a protein reaches the medial Golgi (Kornfeld and Kornfeld, 1985), much of the biosynthesis of O-linked sugars takes place before the medial Golgi (Piller et al., 1989) and addition of GalNAc, the primary O-glycosylation event, most likely occurs in the intermediate compartment/*cis* Golgi (Tooze et al., 1988; Deschuyteneer et al., 1988; Roth, 1984).

Construction of a CD8 chimera maintained in the ER by the cytoplasmic tail of the adenoviral E3/19K protein, CD8/E19, and a mutated derivative, CD8/E19-3S, where the ER targeting motif has been destroyed by replacing one of the essential lysines with serine, have been previously described (Nilsson et al., 1989; Jackson et al., 1990). cDNAs encoding soluble CD8 (CD8sol) and a KDEL tagged derivative (CD8/KDEL) were constructed using oligonucleotides to introduce a stop codon, or a stop codon preceded by the amino acid sequence SEKDEL into the CD8 cDNA at the junction between the extracellular and transmembrane domains. Expression of these recombinant cDNAs in HeLa cells followed by pulse chase analysis (Fig. 1 A) showed that after 30 min of labeling, CD8 sol and CD8/KDEL migrated with approximately their predicted electrophoretic mobilities, although some heterogeneity in their  $M_r$  was observed (see below). After 2 h of chase, a significant proportion of the CD8sol migrated very much more slowly than the CD8/KDEL, this species was also present in the medium but only of the CD8sol cultures. Neuraminidase treatment (not shown) indicated that the reduced migration of CD8sol was primarily the result of sialic acid addition confirming that after 2 h, CD8sol but not CD8/KDEL, had passed through the Golgi complex. These results were entirely analogous to those previously obtained for the ER maintained transmembrane protein CD8/E19 and its cell surface expressed counterpart CD8/E19-3S (Jackson et al., 1990; see also Fig. 1 C).

### *Post-translational Modifications of CD8/E19*

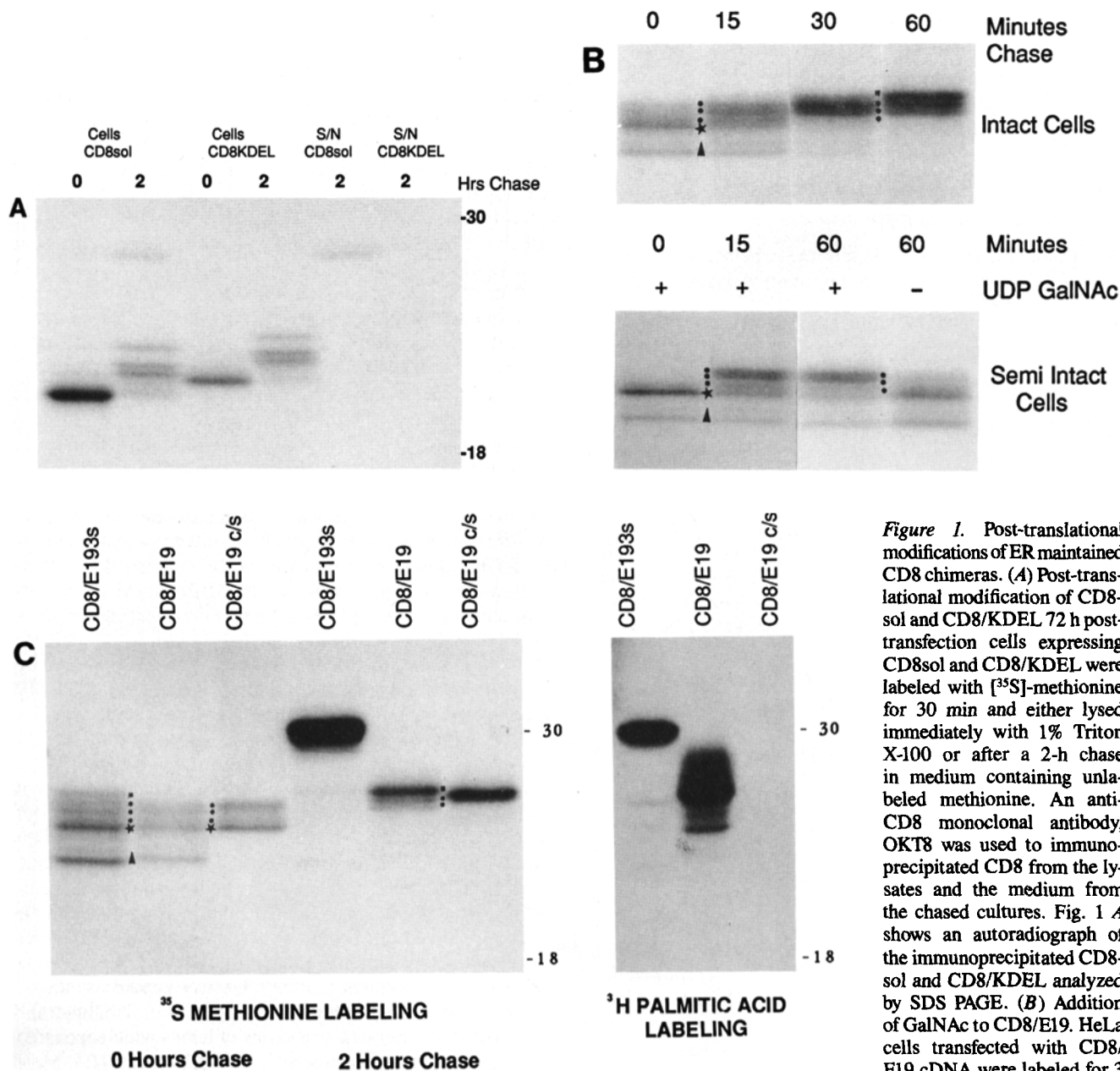
Pulse chase analysis of CD8/E19 showed (Fig. 1 B) that, like CD8/KDEL, it gradually received post-translational modification(s). As wild-type CD8 is O-glycosylated we have previously suggested that these modifications may be the addition of multiple GalNAc moieties (Jackson et al., 1990). However, immediately after a short labeling (see Fig. 1 B, 0 time point, semi-intact cells) CD8/E19 migrated on SDS

PAGE as predominantly two bands, in contrast to a single band for CD8/KDEL. Nascent CD8/E19, determined by translating CD8/E19 in a rabbit reticulocyte lysate in the presence of dog pancreas microsomes, co-migrated with the slower of the two bands (marked with an asterisk in Fig. 1 B, data not shown). This suggested that immediately after synthesis, a portion of CD8/E19 receives a modification which causes it to migrate more rapidly (species marked with a triangle) on SDS PAGE. The series of CD8/E19 species observed after labeling (Fig. 1 B, see Fig. 6 A) might then be accounted for by the simultaneous existence of molecules containing 0, 1 and more GalNAc residues. Each form differing in carbohydrate content showed further heterogeneity as not all molecules contained the modification which results in more rapid migration on SDS-PAGE.

To examine the contribution of GalNAc addition to the post-translational modification of CD8/E19 we utilized semi-intact HeLa cells transfected with CD8/E19 in an *in vitro* ER to Golgi transport assay (Beckers et al., 1987). It can be seen from Fig. 1 B that if UDP GalNAc was added to the incubations, nascent CD8/E19 rapidly became modified such that after 15 min it migrated in a manner similar to that observed in intact cells. If however, the incubations were not supplemented with UDPGalNAc much less modification occurred suggesting that the intracellular pools of UDPGalNAc had been depleted. Addition of multiple GalNAc moieties to nascent CD8/E19 therefore seems to be the primary cause of the multiple species of CD8/E19 marked by dots in Fig. 1 B, but do not account for the species of CD8/E19 marked with a square in Fig. 1 B or the rapidly migrating form of CD8/E19 marked with a triangle.

At early chase times, the post-translational modification of CD8/E19 appeared to be more extensive than that of CD8/KDEL (see Fig. 1, A and B). Such differences might be due to palmitoylation of CD8/E19, as a cysteine residue (Cys-185) located at the junction between the transmembrane domain of CD8 and the E19 tail is a potential site for such a modification (Schultz et al., 1988). Oligonucleotides were used to mutate this cysteine to a serine residue giving rise to the replacement mutant CD8/E19-C/S. Biosynthetic pulse chase analysis showed that just after labeling, the CD8/E19-C/S migrated as a series of bands which appeared to be a subset of those present in CD8/E19 (Fig. 1 C). Most noticeable was the absence in CD8/E19-C/S of the species which migrated more rapidly than nascent CD8/E19. After a chase period of 2 h, CD8/E19 migrated as a triplet, where the slowest migrating form was the dominant species, whereas the CD8/E19-C/S consisted of the middle species of the three bands seen in CD8/E19. The dominant species observed for CD8/E19 (marked with a square) and CD8/E19-C/S at this time point presumably both contain a full complement of GalNAc and differ in their electrophoretic properties due to palmitoylation of only CD8/E19.

Recent studies have shown that palmitoylation of proteins takes place after exit from the ER (Bonatti et al., 1989). It was, thus, important to demonstrate that cysteine-185 received palmitate. Fig. 1 C shows that both CD8/E19 and CD8/E19-3S were readily labeled by overnight incubation with [ $^3$ H]palmitic acid, whereas CD8/E19-C/S did not display any labeling. Parallel cultures labeled with [ $^{35}$ S]methionine confirmed that all three proteins were expressed at comparable levels (not shown). We propose that soon after synthesis, CD8/E19 receives an unknown addition, depen-



**Figure 1.** Post-translational modifications of ER maintained CD8 chimeras. (A) Post-translational modification of CD8sol and CD8/KDEL 72 h post-transfection cells expressing CD8sol and CD8/KDEL were labeled with [<sup>35</sup>S]-methionine for 30 min and either lysed immediately with 1% Triton X-100 or after a 2-h chase in medium containing unlabeled methionine. An anti-CD8 monoclonal antibody, OKT8 was used to immunoprecipitated CD8 from the lysates and the medium from the chased cultures. Fig. 1 A shows an autoradiograph of the immunoprecipitated CD8sol and CD8/KDEL analyzed by SDS PAGE. (B) Addition of GalNAc to CD8/E19. HeLa cells transfected with CD8/E19 cDNA were labeled for 3 min with [<sup>35</sup>S]methionine, and

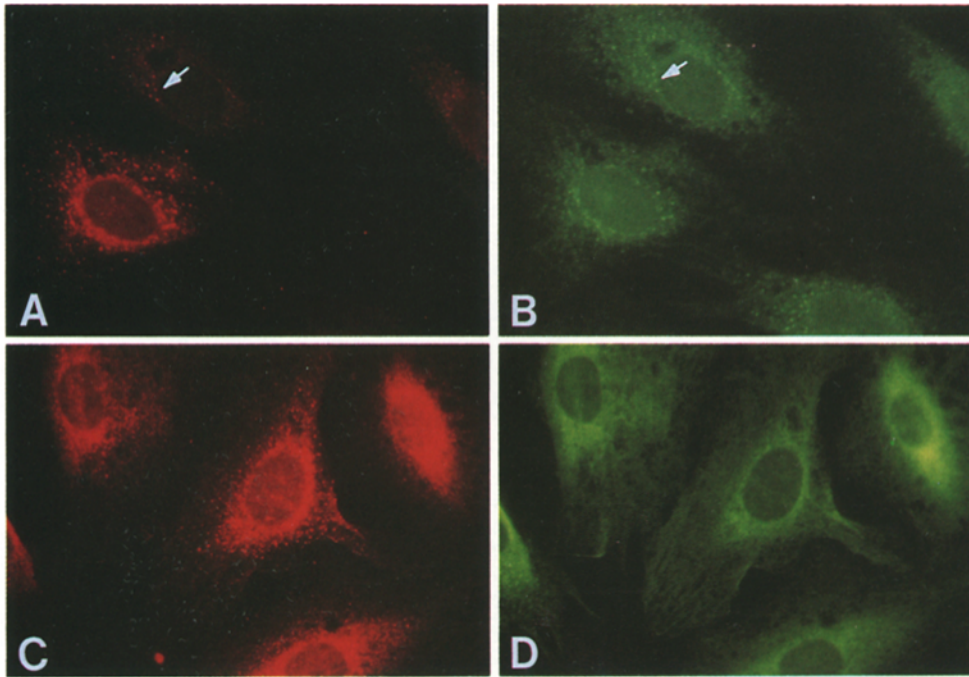
then either analyzed after different chase times (as described in Fig. 1 A) or, washed with ice cold buffer, scraped off the dish with a rubber policeman, pelleted by centrifugation and subsequently washed twice with ice cold buffer before use in the *in vitro* transport assay as described by Beckers et al. (1987). UDPGalNAc (150 μM) was added to the assay mixture as indicated in the figure. The assay was started by transfer to a 37°C water bath and stopped at the times indicated by addition of 0.5 ml of 1% Triton X-100 in PBS. Fig. 1 B shows an autoradiograph of CD8/E19 immunoprecipitated from the intact and semi-intact cells, analyzed by SDS PAGE. The asterisk indicates the migration position of nascent CD8/E19 translated in a rabbit reticulocyte lysate. The dots indicate species of CD8/E19 that have received GalNAc, and the triangle indicates the species of CD8/E19 that migrates more rapidly on SDS due to some unknown modification. (C) Palmitoylation of CD8/E19. HeLa cells transfected with CD8/E19 or CD8/E19-3S or CD8/E19C/S cDNA were labeled with [<sup>35</sup>S]methionine for 30 min and subsequently chased in medium containing unlabeled methionine for the times indicated, or labeled overnight in the presence of 3H palmitic acid. CD8 protein was collected by immunoprecipitation and analyzed by SDS PAGE. The asterisk and dots on the figure indicate the migration position of nascent CD8/E19 and GalNAc modified forms of this protein, respectively (see Fig. 1 B). The asterisk, dots and triangle indicate the species of CD8/E19 described in Fig. 1 B. The square symbol is positioned adjacent to the species of CD8/E19 which we suggest has a full complement of GalNAc and palmitate.

dependent upon cysteine 185, which results in a more rapid migration of CD8/E19 on SDS PAGE (band marked with a triangle in Fig. 1 C). As suggested above, the modifications of cysteine-185 and the carbohydrate addition to CD8 (marked by dots), are independent but temporally overlapping events. After ~20 min of chase, the unknown modification is reversed, perhaps by the addition of palmitate, such that

electrophoretic mobility became mainly dependent on the carbohydrate additions.

#### Identification of CD8/E19 and CD4/E19 in a Post ER Compartment

The above biochemical data in conjunction with other reports (see Pelham, 1989) support the idea that proteins



**Figure 2.** Immunofluorescence localization of CD8/E19 and CD4/E19 to the intermediate compartment/*cis* Golgi. Transfected 3T3 cells expressing CD4/E19 were fixed with 4% formaldehyde and permeabilized using 0.1% Triton X-100. Cells were stained with mouse anti-CD4 and rabbit anti-p58 antibodies (A and B) or mouse anti-CD4 and rabbit anti-epoxide hydrolase antibodies (C and D). Fluorescein anti-rabbit or Texas red anti-mouse secondary reagents were used to identify binding of the primary antibodies. An arrow in Fig. 2, A and B, indicates a cluster of vesicles that clearly co-stain for CD4E/19 and p58. (E) Sequences of the cytoplasmic tails of the various CD8 and CD4 chimeras. The sequences shown (see Jackson et al., 1990) are the complete cytoplasmic tail sequences of the chimeras, based on the predicted location of the transmembrane domains of CD4 (Maddon et al., 1985) and CD8 (Littman et al., 1985).

## E

### Cytoplasmic Tail Sequences of CD8 and CD4 Chimeras

Ad2	E19	K Y K S R R S F I D E K K M P
UDPGT	H25	- - - - - R T G K K G K R D
UDPGT	HP1	- - - - - V K K A H K S K T H
HMGCoA		- - - - - L Q G A C T K K T A
53kSER		- - - - - E T P K N R Y K K H

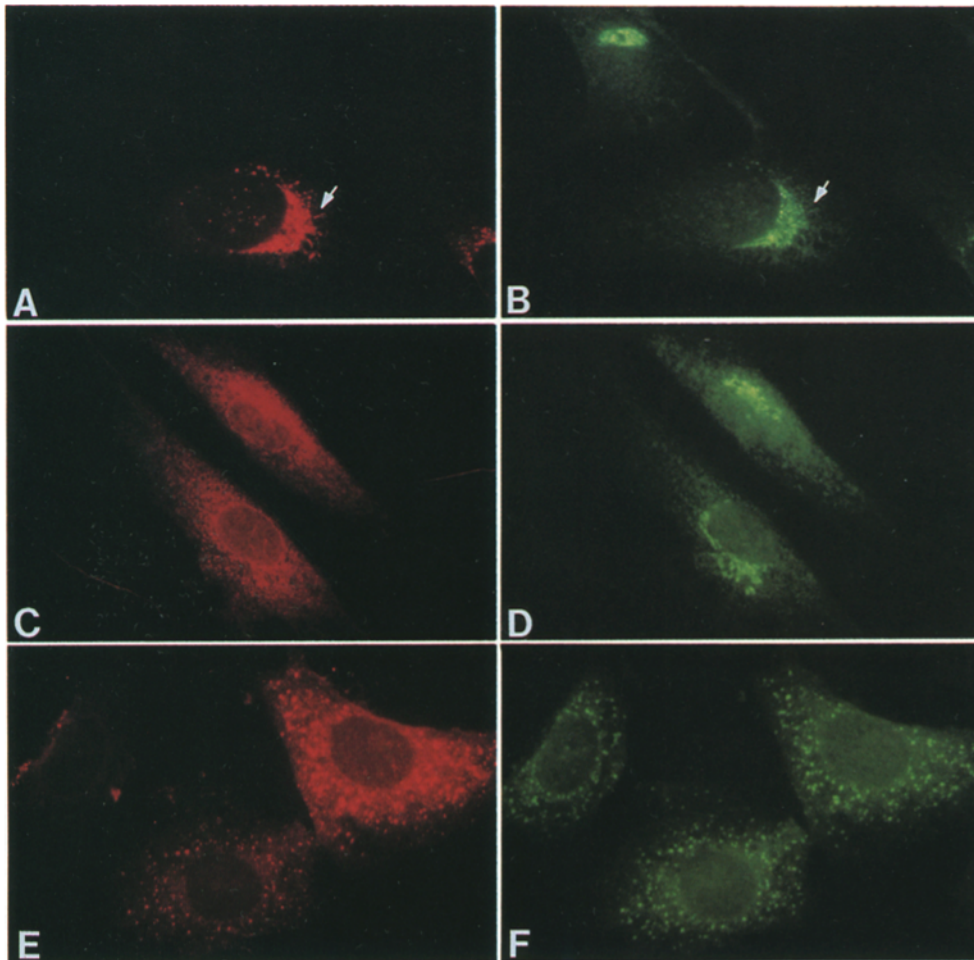
with a double-lysine or KDEL-motif have access to post ER compartment(s). However, in contrast to the KDEL receptors (Lewis and Pelham, 1992; Vaux et al., 1990), KDEL proteins have not been identified in these organelles.

To determine if proteins with the double-lysine motifs localize to post ER compartment(s), transfected 3T3 cells were costained for immunofluorescence microscopy utilizing antibodies against the tagged marker protein and p58 (Saraste et al., 1987). Immunolocalization has shown that p58 is a resident of the intermediate compartment (Schweizer et al., 1988; Chavrier et al., 1990), distributed among cisternae at the *cis* side of the Golgi apparatus and peripheral vesicular structures, that represent the intermediates in ER to Golgi transport (Saraste and Kuismenän, 1984; Saraste and Svensson, 1991). As the anti-p58 antiserum does not react with the human homolog, mouse 3T3 cells were used for these studies. Vesicular structures at the periphery of cells were identified which costained for CD8/E19 and p58, but not for the ER marker proteins, epoxide hydrolase (Galteau et al., 1985) or UDP glucuronosyltransferase (Jackson et al., 1987). However strong ER fluorescence made these results difficult to record. Clearer colocalization between p58 and an E19 tagged marker protein was obtained with CD4/E19 (CD4 protein with the entire cytoplasmic tail replaced by that from the adenoviral E19 protein, see Nilsson et al., 1989). Costaining vesicles were most apparent when the levels of protein expression were relatively low, see arrows in

Fig. 2, A and B. At higher expression levels strong ER fluorescence obscured details in the perinuclear region.

We have previously shown that the ER targeting motif in CD8/E19 can be substituted by replacing the last 10 amino acids of CD8/E19 with sequences derived from various ER resident proteins (See Fig. 2 E). This work allowed us to determine a common feature of these ER targeting motifs, the double lysine motif, and suggested that the various sequences were functionally similar (Jackson et al., 1990). Analysis of these various chimeras produced the surprising observation that only CD8/E19 and CD8/HMG CoA displayed costaining with the p58 vesicles. The other constructs, CD8/H25, CD8/HP1 and CD8/53kSER showed staining similar to the ER marker proteins with no obvious concentration in p58 staining vesicles. The common feature of the E19 and HMG CoA ER targeting motifs, which distinguishes them from the other three sequences, is that they have lysine residues at positions 3 and 4 residues from the carboxy terminus (see Fig. 2 E) with no additional basic residues among the last 9 amino acids of their tail sequence (Jackson et al., 1990).

Most interestingly, a similar phenomenon is observed if these exact same cytoplasmic tails are present on CD4. Whereas CD4/E19 and CD4/HMGCoA showed significant costaining with p58, CD4/HP1, CD4/H25 and CD4/53kSER only partially co-localized with p58 to an extent similar to CD8/E19 (data not shown). As morphological analyses show



**Figure 3.** Redistribution of the subcellular localization of CD4/E19 by temperature. 3T3 cells transfected with expression plasmids encoding CD4/E19 (A, B, E, and F) or CD8/H25 (C and D) were incubated at 16°C for 2 h followed by a warm-up to 37°C for either 5 min (A-D) or 30 min (E-F). Cells were fixed and permeabilized as in Fig. 2 and co-stained using anti-CD4 antibody (A and E) and anti-p58 antibody (B and F) or anti-CD8 antibody (C) and anti-p58 (D). FITC or Texas Red labeled secondary reagents were used to detect binding of anti-p58 and anti-CD antigens, respectively (see Fig. 2). An arrow in A and B indicates possible tubular structures that co-stain for p58 and CD4.

steady-state conditions, these data indicate that the extent to which the various chimeras access/dwell in the intermediate compartment may be dependent on the sequence context of the double lysine motif and whether the marker protein consists of a single chain or a homodimer.

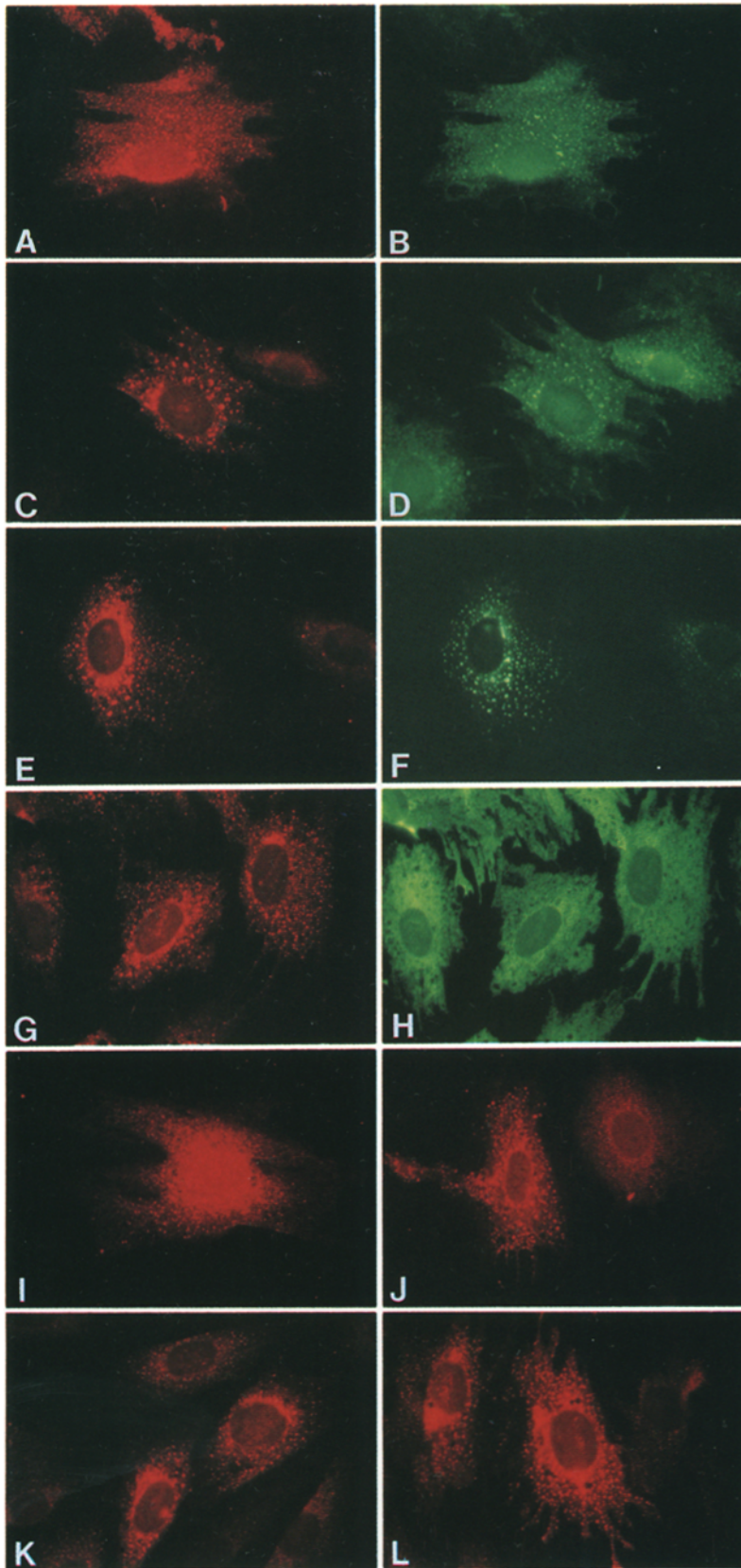
#### **Redistribution of CD4/E19 to the Golgi Region after Warm-up from a 16°C Incubation**

Incubation of cells at 16°C followed by brief warm-up to 37°C has been shown to result in a dramatic redistribution of both p58 and p53 (Schweizer et al., 1990; Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991). At 16°C, these molecules concentrate in pre-Golgi structures located throughout the cytoplasm. Upon warm-up to 37°C they move synchronously into the Golgi region before relocating back to the ER and returning to steady-state distribution intermediate between the ER and Golgi (Schweizer et al., 1990; Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991). These data have been interpreted as demonstrating that marker proteins of the intermediate compartment are recycling between the ER and the Golgi complex. We have carried out temperature shift experiments in order to determine if the subcellular distribution of our marker proteins tagged with the double-lysine motif were affected by this treatment (Fig. 3). CD4/E19 was found to colocalize almost entirely with p58 in these experiments, after a brief warm-up to 37°C both molecules were observed to concen-

trate in the Golgi region and in some cells in structures that appear more tubular than vesicular in nature (see arrow in Fig. 3, A and B) that emanate from this region. p53-positive tubular structures have previously been observed upon warm-up from a 16°C incubation, such structures may reflect the tubular nature of the retrograde transport (see Hauri and Schweizer, 1992; Lippincott-Schwartz et al., 1990). After a 30 min warm-up at 37°C, CD4/E19 and p58 colocalized to more peripheral vesicular structures located throughout the cytoplasm (Fig. 3, E and F). Similar results were observed for CD8/E19, however, the level of ER staining was always much greater compared to that observed for CD4/E19 (as described above). In contrast, temperature shift experiments had little effect on the subcellular distribution of CD8/H25 (Fig. 3, C and D) and there was no noticeable accumulation of these proteins in structures that costained for p58. The finding of these temperature shift experiments are fully consistent with a model whereby CD4/E19 and CD8/E19, like p53 and p58, recirculate between the ER and the Golgi.

#### **Disruption of Microtubules Results in Changes in the Subcellular Distribution CD8/E19 and CD4/E19**

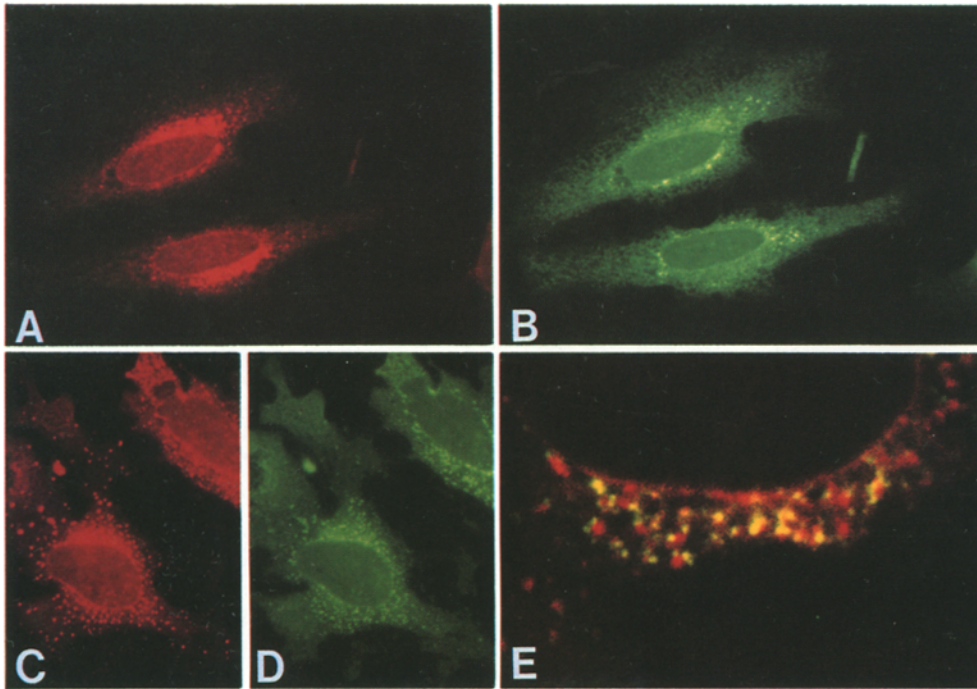
Morphological evidence has suggested that the return of p58 and p53 from the intermediate compartment/cis Golgi to the ER (Lippincott-Schwartz et al., 1990; Schweizer et al., 1990; Saraste and Svensson, 1991) may be directed by mi-



**Figure 4.** Accumulation of CD8/E19 and CD4/E19 in post ER compartment(s) in cells treated with nocodazole and/or brefeldin A. 3T3 cells transfected with expression plasmids encoding CD8/E19 (*A* and *B*) or CD4/E19 (*C–H*) were placed at 4°C for 20 min followed by a 2-h incubation at 37°C in the presence of nocodazole. Cells were fixed and permeabilized as described in Fig. 2 and co-stained for CD8/E19 and p58 (*A* and *B*), CD4/E19 and p58 (*C* and *D*), CD4/E19 and a resident Golgi protein, p135, (*E* and *F*) and CD4/E19 and a resident ER protein, epoxide hydrolase (*G* and *H*). The subcellular distribution of CD8/E19 (*I* and *K*) and CD4/E19 (*J* and *L*) was also determined in transfected 3T3 cells after a 1-h treatment with brefeldin A (*I* and *J*) or after this treatment followed by a further 1 1/2-h exposure to nocodazole and brefeldin A (*K* and *L*). In all cases the E19 chimeras were identified using Texas red reagents and the other proteins were identified using fluorescein secondary antibodies.

cro-tubules. Furthermore, biochemical and morphological data suggest that the retrograde transport of Golgi proteins to the ER in brefeldin A-treated cells is also facilitated by microtubules (Lippincott-Schwartz et al., 1990).

Treatment of cells with nocodazole, a microtubule-disrupting drug (De Brabander et al., 1976) resulted in pronounced colocalization of both CD8/E19 and CD4/E19 with p58 in vesicular structures (Fig. 4) that did not stain with the anti-



**Figure 5.** Comparison of the immunofluorescence localization of KDEL and E19 proteins. HeLa cells transfected with plasmids encoding CD4/E19 and CD8/KDEL (*A*, *B*, and *E*) or CD8/E19 and prealbumin/KDEL (*C* and *D*) were fixed and processed for immunofluorescence microscopy either without pretreatment (*A* and *B*), or after pretreatment with brefeldin A and nocodazole (*C–E*) as described in Fig. 4. In each case, E19 proteins were detected with Texas red reagents and KDEL proteins with fluorescein secondary antibodies. Fig. 4 *E* is a higher magnification image of the perinuclear region of a cell stained as in Fig. 4, *C* and *D* obtained by confocal microscopy.

ER antibodies (Fig. 4, *G* and *H*). This treatment also caused a significant change in the morphology of the intermediate compartment (see Saraste and Svensson, 1991). The organelle became dispersed throughout the cytoplasm and numerous, much enlarged vesicles were stained by the anti-p58 antibody. This staining pattern is very similar to, and partially overlapping with that observed with an anti-Golgi antibody (Burke et al., 1982), making it difficult to determine to what degree the E19 tagged proteins are colocalizing with the intermediate compartment or with the Golgi membranes after this treatment (see Fig. 4, *E* and *F*) (see Saraste and Svensson, 1991). However, the staining pattern in untreated cells (Fig. 2) and the finding that some of the CD8/E19 and much of the CD4/E19 molecules were localized to the p58-staining compartment in brefeldin A-treated cells (Fig. 4 *J*) indicates that in the absence of nocodazole these proteins predominantly access the intermediate compartment (Lippincott-Schwartz et al., 1990). Treatment of cells with brefeldin A for 1 h followed by nocodazole for 1 1/2 h resulted in an apparent accumulation of CD4/E19 and CD8/E19 into vesicular structures scattered throughout the cell (Fig. 3, *L* and *K*), that contained with p58. However, none of the treatments promoted colocalization of the CD8/H25, CD8/HP1 or CD8/53kSER with the p58-staining vesicles.

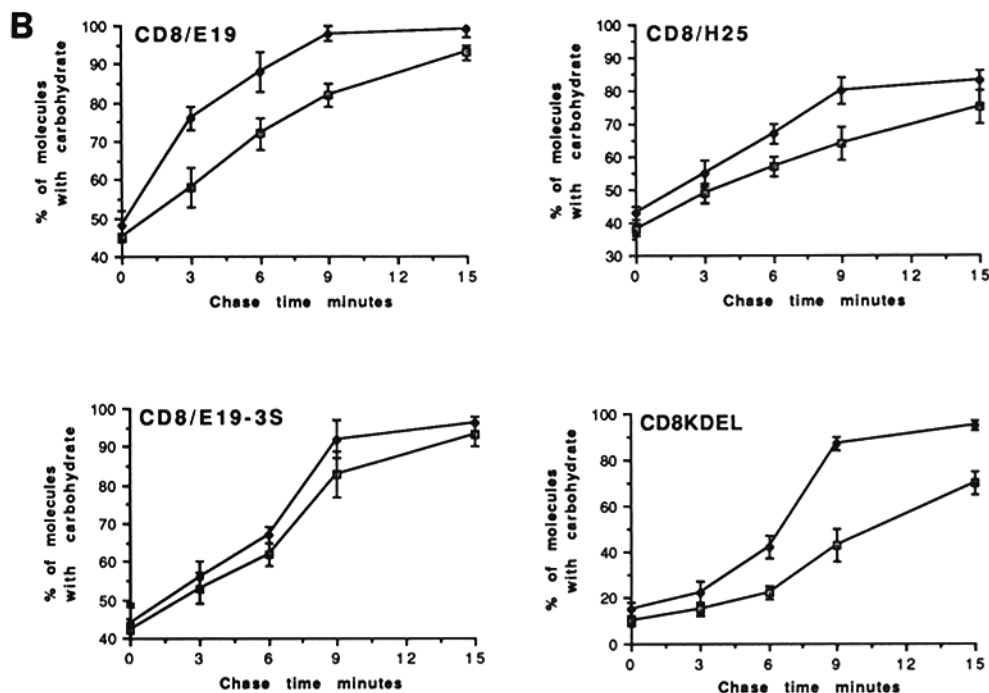
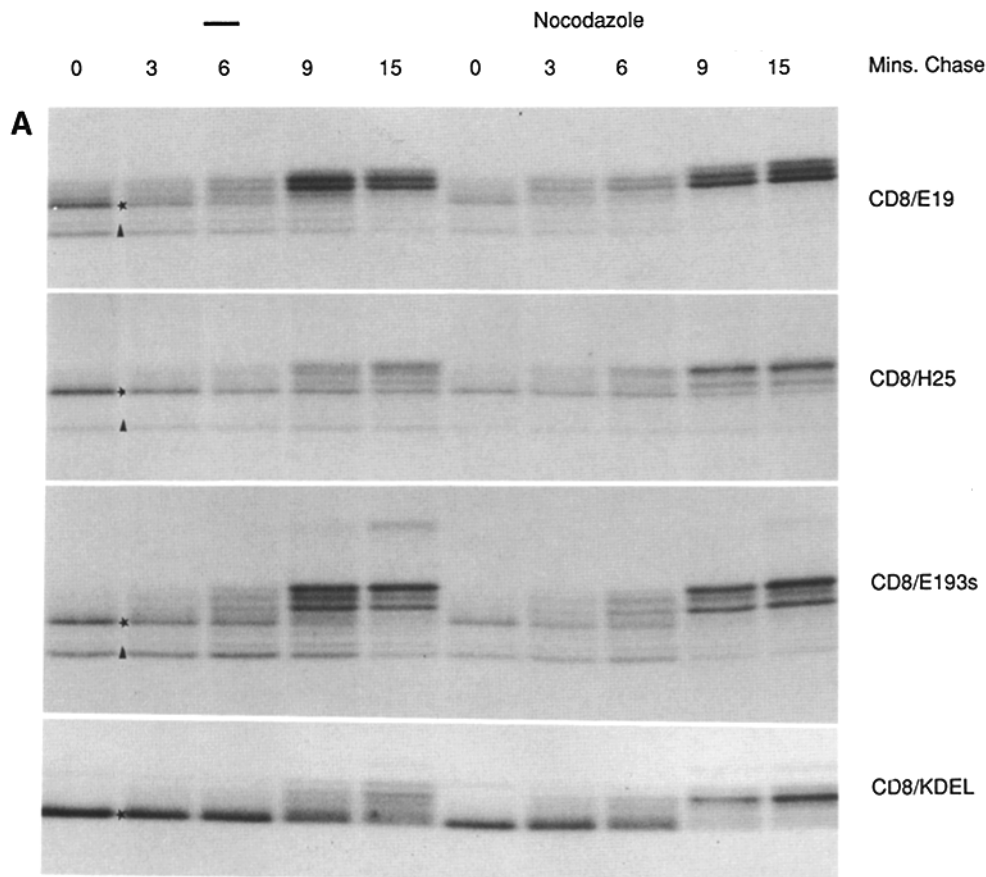
Three different marker proteins tagged with the KDEL motif, CD8/KDEL, HLA A2/KDEL and prealbumin/KDEL (see experimental procedures) were not localized to the intermediate compartment under any of the conditions described above. In untreated cells all three proteins colocalized with CD8/E19 in the ER but also occurred in small, brightly stained vesicles near the nucleus which did not stain for CD8/E19, CD4/E19, epoxide hydrolase or UDP-glucuronosyltransferase (Fig. 5, *A* and *B*). Nocodazole treatment resulted in a significant accumulation of the vesicles specifically stained by the KDEL tagged proteins. In many instances there was a spatial association of these vesicles and the inter-

mediate compartment vesicles. This association was more pronounced if cells are also treated with brefeldin A. Fig. 5, *C* and *D*, show that vesicles staining strongly for CD8/KDEL near the periphery of the cell were clustered around a vesicle that stained for CD4/E19, this association was also apparent in the peri-nuclear region when high magnification confocal microscopy was used (Fig. 5 *E*). The exact relationship between these two sets of vesicles is unclear. However, their spatial association raises the possibility that CD8/KDEL may be sorted and concentrated into specialized structures before retrieval.

#### ***Depolymerization of Microtubules Increases the Rate of GalNac Addition to CD8/E19 and CD8/KDEL***

If retrieval in normal cells is dependent on microtubules, then the rate of GalNac addition to CD8/E19 might be affected by nocodazole treatment. Cells transfected with CD8/E19, CD8/E19-3S, CD8/H25, CD8KDEL were pretreated with nocodazole or mock treated and labeled for 3 min followed by frequent chase time points. Fig. 6 *A* shows that pretreatment of cells with nocodazole increased the percentage of CD8/E19 that acquired GalNac at early chase times, with less of an effect on the additions to CD8/E19-3S (compare the 6-min chase time in the presence and absence of nocodazole for CD8/E19 and CD8/E19-3S). The effect on CD8/KDEL was more pronounced (compare the 9-min chase time with and without nocodazole). To allow for a more quantitative evaluation of this information we have used a laser densitometer to determine the relative amounts of protein with and without carbohydrate at each time point (the carbohydrate free protein species are marked with an asterisk and a triangle; see Fig. 1 for details). Fig. 6 *B* shows graphs of the rate at which each of the proteins acquire carbohydrate in the presence and absence of nocodazole. The mean values obtained from the data in Fig. 6 *A* and two simi-

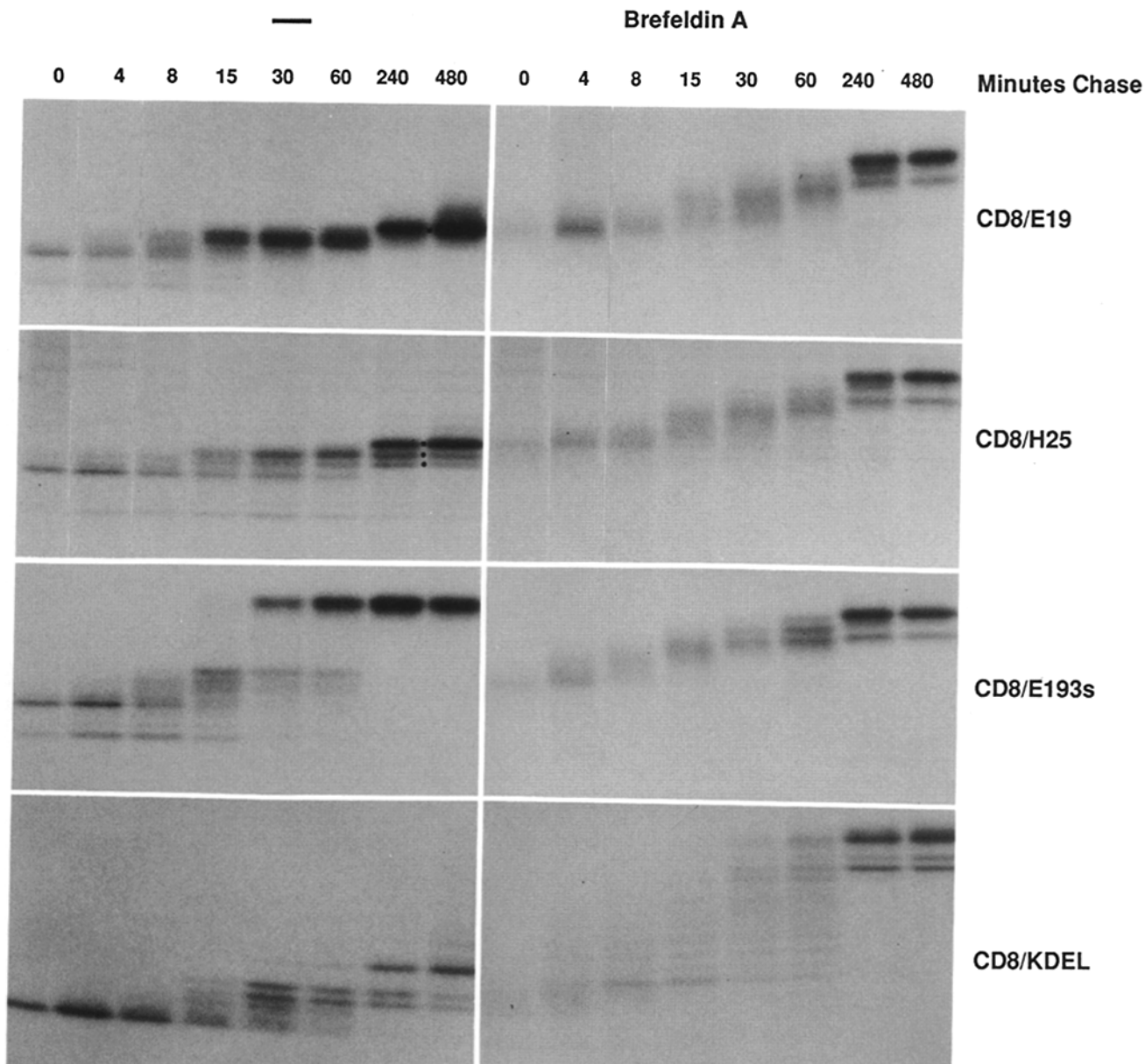




**Figure 6.** The rate of GalNAc addition to CD8/E19 and CD8/KDEL increases in cells treated with nocodazole. (A) HeLa cells transfected with the expression plasmids indicated in the figure were labeled for 3 min with [<sup>35</sup>S]-methionine and subsequently chased in the presence of medium containing unlabeled methionine for the times indicated. For each construct, two identical series of pulse/chase plates were set up. 2 1/2 h before labeling, both series were placed in ice cold medium for 20 min. Subsequently, one set of plates was returned to normal medium while the other was placed in medium containing 10 μg/ml nocodazole. Nocodazole was maintained in the prelabeling, labeling, and chase medium. Transport was stopped at the chase times indicated in the figure by addition of lysis buffer (1% Triton X-100/PBS). A shows analysis of the CD8 proteins immunoprecipitated from the lysates and analyzed by SDS PAGE as described in Fig. 1. The asterisk and triangle indicate species of CD8 devoid of carbohydrate (see Fig. 1). (B) The autoradiographs in A and two similar experiments were subjected to densitometry using an LKB (Pharmacia LKB Biotechnology, Inc.) ultrascan. The relative amounts of protein without (marked by the asterisk and triangle) and with carbohydrate at each time point were determined. These values allowed us to calculate the percent of protein with carbohydrate at each time point. B shows mean values (from three experiments) of these percentages, plotted against the corresponding chase time. (Note that these analyses also allowed us to determine that the levels of protein recovered over the chase period varied by <25%).

lar experiments show that there was a significant increase in the rate at which CD8/E19 and CD8/KDEL received carbohydrate in nocodazole treated compared to untreated cells, with a lesser effect on CD8/H25 and even less on CD8/E19-3S. Assuming that the various CD8 chimeras are equally good substrates for the transferase(s) (see below for details)

these data suggest that nocodazole treatment does not simply result in a general increase of GalNAc transferase activity in the ER. The observed effects of nocodazole are, however, consistent with the view that an intact microtubular network ordinarily limits the exposure of the ER-retrieved CD8 chimeras to GalNAc transferase.



**Figure 7.** Post-translational modification of CD8 chimeras in cells treated with brefeldin A. HeLa cells transfected with the expression plasmids indicated in the figure were labeled for 5 min with [<sup>35</sup>S]methionine and subsequently chased for the times indicated. Two series of pulse/chase plates were set up for each construct. These were treated identically except that brefeldin A (2 μg/ml) was added to one series 1 h before labeling and the drug was maintained in the labeling and chase media. The labeled CD8 proteins were collected and analyzed as described in Fig. 6. Note that due to its smaller size the various post-translationally modified forms of CD8/KDEL are better resolved on the SDS gels than the post-translationally modified forms of the transmembrane CD8 chimeras (see Fig. 1).

***Different, ER-maintained CD8 Chimeras Receive GalNAc at Approximately the Same Rate in the Presence of Brefeldin A***

The data shown in Fig. 6 indicated that CD8/H25 acquired GalNAc more slowly than CD8/E19. After a chase period of 8 h (Fig. 7) some of the CD8/H25 (species marked by dots in Figs. 7 and 8 A) had still not acquired a full complement of GalNAc in contrast to CD8/E19. A similar analysis of CD8/HP1, CD8/53kSER and CD8/HMG CoA (not shown) revealed that CD8/HP1 and CD8/53kSER received GalNAc very slowly, like CD8/H25, while CD8/HMGCoA behaved like CD8/E19. One interpretation of these results is that

CD8/E19 and CD8/HMGCoA receive a greater exposure to post-ER localized transferase(s), a suggestion that is particularly attractive as it is the predicted consequence of the finding that only these two CD8 chimeras localized to the intermediate compartment (Fig. 2). The alternative explanation, that the different chimeras are not equally good substrates for the transferase, reflecting differences in either the rates of folding of the proteins or differences in the final conformation of the folded proteins, seemed less likely for a number of reasons. First, the different CD8 chimeras differ only in the sequence of the last 10 amino acids of their 15 residue long cytoplasmic tails. The sequences of their luminal and transmembrane domains are identical (see Fig. 2

**Table I. Morphological and Biochemical Characteristics of CD8 and CD4 Chimeras**

Chimeric protein	Intermediate compartment staining (Figs. 3 and 4)	Neuraminadase/Endo H resistant carbohydrate (Fig. 6, A and B)	Time* GalNAc + palmitate (Figs. 6 A and 7)	PNA† staining ER (Fig. 8)
CD8/E19	X	X	~4 h	X
CD8/HMG CoA	X	X	~4 h	X
CD8/H25	—	—	>8 h	—
CD8/HP1	—	—	>8 h	—
CD8/53kSER	—	—	>8 h	—
CD4/E19	XXX	X		
CD4/HMG CoA	XXX	X		
CD4/H25	X	—		
CD4/HP1	X	—		
CD4/53kSER	X	—		

\* These values were obtained from pulse chase analyses (eg., Figs. 6 and 7); the values shown are an approximation of the chase time required for >95% of a given chimera to migrate with a mobility adjacent to the band marked with a square (see Figs. 1, 6, and 7), the form we suggest has a full complement of GalNAc and palmitate.

† The data shown are from experiments described in Fig. 8 and similar analyses of CD8/HMG CoA, CD8/HP1 and CD8/53kSER.

E). It would be surprising if these differences were transmitted through the transmembrane portion to alter the folding of the luminal domain of CD8, the portion the transferase acts upon (see Nilsson et al., 1989). Secondly, three different conformation-sensitive anti-CD8 monoclonal antibodies reacted equally well with each of the CD8 chimeras and detected no differences in the time required for each chimera to fold into a conformation recognized by the antibodies.

To examine if each of the CD8 chimeras could be modified at approximately the same rate, if they were equally exposed to GalNAc transferase we treated cells with brefeldin A for 1 h before biosynthetic pulse chase experiments. This treatment has previously been shown to result in the collapse of post ER compartments into the ER (Lippincott-Schwartz et al., 1989). The most striking affect of this treatment was that all the CD8 chimeras now acquired GalNAc extremely rapidly (Fig. 7). Similar results were found for CD8/HP1, CD8/53kSER and CD8/HMGCoA (not shown). At the end of the labeling period little unglycosylated protein was seen in contrast to the results of untreated cells. With time, all the CD8 chimeras in the treated cells acquired post-translational modifications at approximately the same rate, indicating that the different proteins were about equally good substrates for the transferases. These data suggest that the majority of the GalNAc transferase activity in untreated cells is located in a post ER compartment(s) and that the differences in the rates at which the different CD8 chimeras acquired GalNAc might be due to their differential exposure to these post ER enzymes.

#### **Marker Proteins with E19 or KDEL Motifs Receive Golgi Modifications**

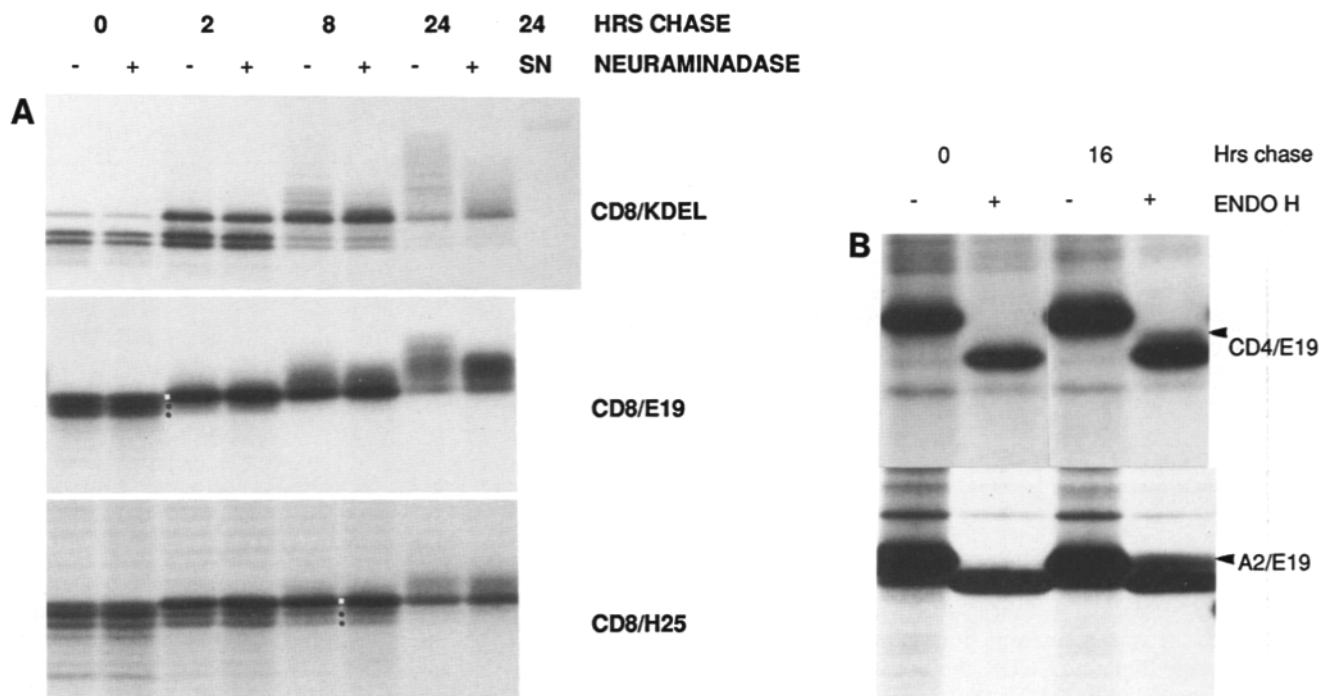
After 8 h of chase CD8/E19 had received modifications in addition to GalNAc (Fig. 7), suggesting that the O-linked sugars had been further modified, perhaps by Golgi enzymes. Golgi additions to wild type CD8 have been determined to be galactose, GlucNAc and sialic acid (Pascale et al., 1992). Neuraminadase treatment of CD8/E19 showed that after 24 h, a significant proportion of the molecules had indeed received sialic acid (Fig. 8 A). In contrast, little if any CD8/H25 had received sialic acid. Similar results were found for CD8/HP1 and CD8/53kSER. A parallel experi-

ment with CD8/KDEL showed that at the end of the chase period, ~50% of the protein had acquired sialic acid (Fig. 8 A). Moreover, a small amount of the CD8/KDEL had been secreted into the culture medium. The slow stepwise addition of carbohydrate to CD8/E19 and CD8/KDEL by enzymes considered to be of Golgi origin (Farquhar, 1985; Piller et al., 1989) is difficult to explain by leakage past a single pre-Golgi point of retention/retrieval. Transport past such a point would be expected to result in the rapid addition of sialic acid to all carbohydrates followed by expression on the cell surface or secretion as indeed we observed for CD8/E19-3S and CD8sol, respectively (Fig. 1, A and C). Furthermore, leakage of CD8/E19 is unlikely as we have been unable to detect CD8/E19 on the cell surface (Jackson et al., 1990).

The above data raised the possibility that the N-linked carbohydrates on CD4/E19 (Nilsson et al., 1989) and HLA A2.2/E19 (see Materials and Methods for details) might also be modified by Golgi enzymes. Endoglycosidase H (Endo H) was used to identify Golgi modification of the N-linked sugars, as resistance to this enzyme occurs if the carbohydrates have been modified by medial Golgi enzymes (Kornfeld and Kornfeld, 1985). Fig. 8 B shows that after a 16-h chase period a small portion of the N-linked sugars on CD4/E19 and ~20% of the N-linked carbohydrate on A2.2/E19 were found to be resistant to Endo H (indicated by an arrow in Fig. 8 B). Interestingly, the N-linked sugars on CD4/H25 or CD4/HP1 did not become endo H resistant even after a chase period of 24 h, results entirely analogous to those obtained with the CD8 chimeras (see Table I). Thus, using two different marker proteins and unrelated Golgi modifications we show that the degree of exposure to Golgi proteins of a given chimera is dictated by the sequence context of the double lysine motif. We conclude from these data that marker proteins maintained in the ER by either a double lysine or KDEL motif are slowly exposed to enzymes recognized as Golgi residents.

#### **Retrieval of Golgi Modified CD8/E19 and CD8/KDEL to the ER**

Taken together the results described above and summarized in Table I, provide good evidence that proteins accumulated

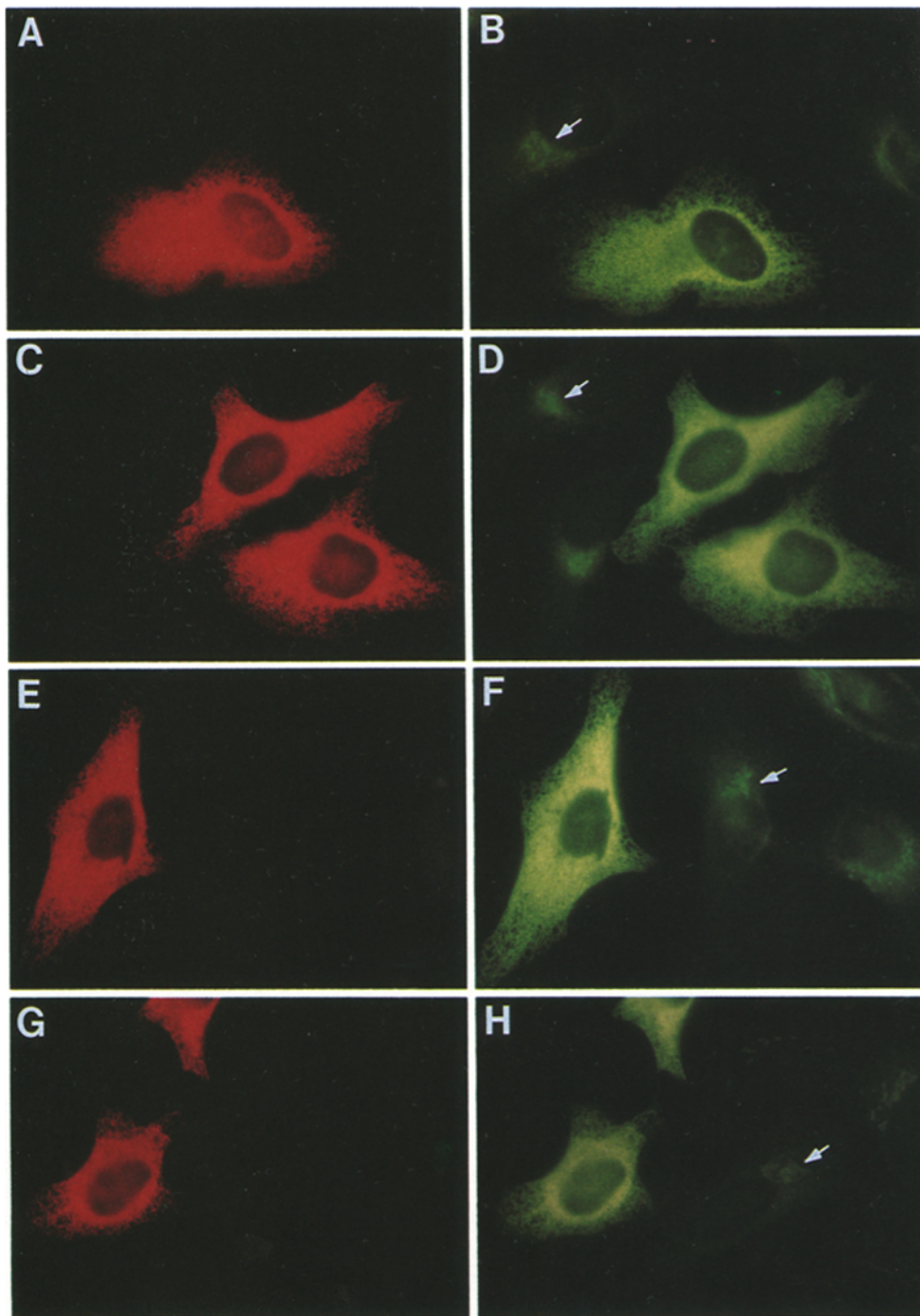


**Figure 8.** O- and N-linked sugars on -E19 and -KDEL tagged proteins receive Golgi modifications. HeLa cells transfected with an expression plasmid encoding the protein indicated in the figure were labeled for 1 h with [<sup>35</sup>S]methionine and then chased in the presence of medium containing unlabeled methionine for the times indicated before lysis of the cells with Triton X-100. (A) CD8 protein was immunoprecipitated from the cell lysates and also from the 24-h chase medium of CD8/KDEL. Before analysis by SDS PAGE, half of each sample was treated with neuraminidase. (B) CD4/E19 and A2.2/E19 were immunoprecipitated from the cell lysates using mAbs 19Thy/5D7 and BB7.2, respectively. Before SDS PAGE analysis, half of each sample was treated overnight with endoglycosidase H. Overexposure of the gel was required to see the endo H resistant forms of the protein which are indicated with an arrow in the figure.

in the ER as a result of a double lysine or a KDEL motif have access to post-ER compartments. However they do not prove that these escaped proteins modified by post-ER enzymes are subsequently retrieved to the ER. To investigate this, we analyzed whether the ER of cells expressing high levels of these CD8 chimeras could be stained with lectins (see Roth, 1984; Piller et al., 1989; 1990) which recognize specific terminal glycans. *Helix pomatia* (HPA) and *Arachis hypogaea* (PNA) lectins were used to stain for terminal GalNAc and terminal Gal(1-3)GalNAc, respectively (Hammarstrom and Kabat, 1969). It is important to note that binding of the lectin requires the glycan to occupy a terminal position. Thus, if galactose is added to GalNAc it is not recognized well by HPA. Similarly if sialic acid is added to Gal-GalNAc, reactivity to PNA is virtually eliminated (Piller et al., 1989). HeLa cells transiently transfected with plasmid DNA encoding various CD8 chimeras (see Table I) were stained with HPA and PNA lectins. Fig. 9 shows that in cells expressing high levels of each of these CD8 chimeras (identified by staining with anti-CD8 antibodies) the cells stain strongly with HPA (Fig. 9, B and D, see Table I). The staining pattern by HPA in transfected cells colocalized with that for the CD8 chimeras and also with the ER marker protein epoxide hydrolase indicating that the lectin is staining the ER. Furthermore staining by HPA of the characteristic membrane network of the ER was clearly visible at the periphery of cells, see Fig. 9, B and D. In the adjacent untransfected cells (not stained by anti-CD8 antibodies, Fig. 9, A and C), HPA lectin primarily stained the Golgi region of cells, see arrow in Fig. 9, B and D, the expected location for endogenous proteins with only partially processed carbohydrate, whereas the ER

stained very weakly (see Discussion). Similar results were found for CD8/E19, CD8/HMGCoA and CD8/KDEL when cells were stained with PNA lectin (Fig. 9, E-H), in untransfected cells only the Golgi stained (arrows, Fig. 9, F and H) whereas in transfected cells the ER stained strongly with the PNA. However when we analyzed the other CD8/chimeras (see Table I), we found that even in cells expressing high levels of CD8/H25, CD8/HP1 or CD8/53kSER the ER did not stain with PNA. These observations complement the pulse chase data which showed significant Golgi additions to CD8/E19, CD8/HMG CoA and CD8/KDEL, but not to the other ER maintained CD8 chimeras after 24 h of chase (see Table I).

Further evidence that Golgi modified CD8/E19 were located in the ER was obtained by subcellular fractionation on sucrose density gradients (Bole et al., 1986; Hsu et al., 1991). CD8/E19 modified by the addition of sialic acid (identified by neuraminidase treatment, see Fig. 8 A) was found to be primarily localized to the more dense membrane fractions, previously defined as the rough and smooth ER (Bole et al., 1986), with only low levels in the lighter membrane fractions marked by the Golgi enzyme, galactosyltransferase (Fig. 10). Sialylated CD8/E19 and a smooth ER marker protein, epoxide hydrolase were found to be distributed in a quantitatively similar manner over the gradient, (compare Fig. 10, B and C). Furthermore, sialylated CD8/E19 had an almost identical distribution over the gradient as CD8/E19 modified by addition of only GalNAc (compare Fig. 10, C and D). Taken together, the results from these lectin binding and subcellular fractionation experiments provides strong evidence that proteins maintained in



**Figure 9.** Immunofluorescence localization of Golgi modified CD8 proteins to the ER by lectin staining. HeLa cells transiently transfected with expression plasmids encoding CD8/H25 (A and B), CD8/KDEL (C, D, G and H) or CD8/E19 (E-F) were fixed and permeabilized as in Fig. 2 followed by incubation with *Helix pomatia* (HPA) lectin (A-D) or *Arachis hypogaea* (PNA) lectin (E-H). Rabbit antilectin antisera and mouse anti-CD8 antibody, OKT8, were used in conjunction with fluorescein anti-rabbit and Texas red anti-mouse secondary reagents to detect, lectin binding and CD8, respectively. To reduce binding of PNA to the cell surface cells (E-H) were incubated with *Bauhinia purpurea* agglutinin (BPA) for 20 min before fixation, permeabilization and PNA staining. Note that as transiently transfected cells were used for these experiments both transfected (stained by anti-CD8 antibodies) and nontransfected cells are shown in each panel. Golgi staining of untransfected cells by the lectins is indicated by arrows in the figure.

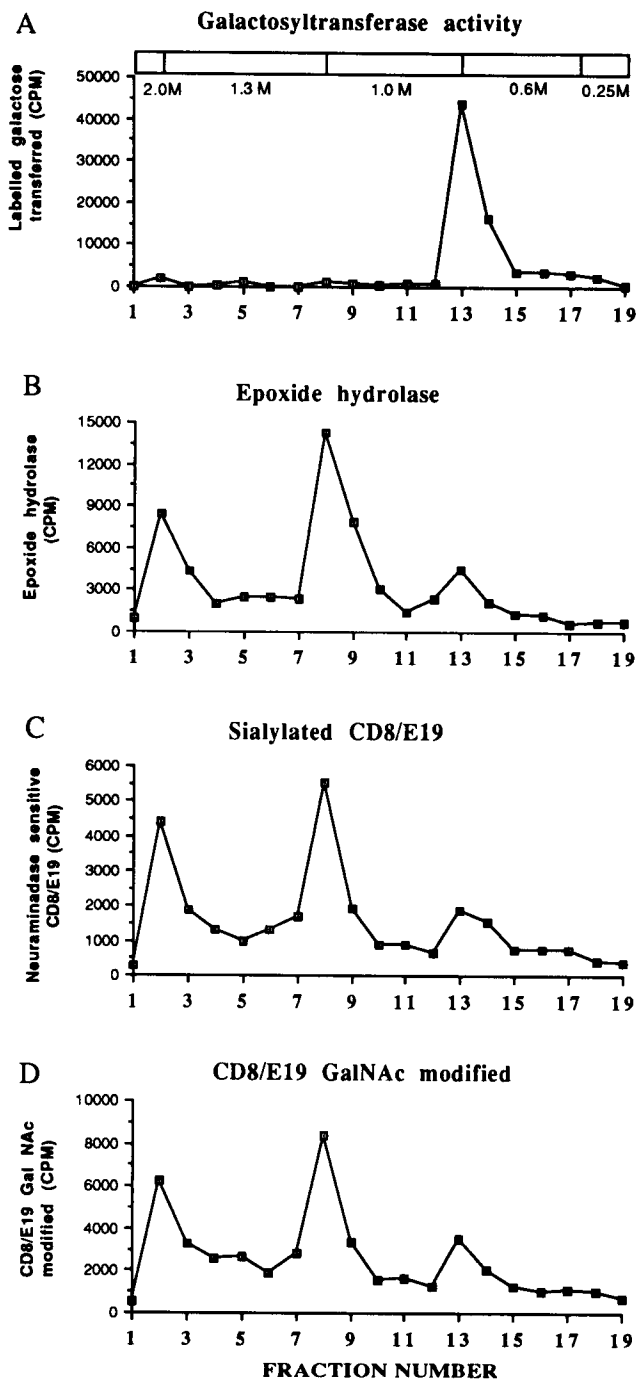
the ER by an E19 or KDEL motif are actually retrieved to the ER from post ER compartments. A conclusion strongly supported by our observation that marker protein tagged with the E19 motif redistribute initially to the Golgi region and subsequently back to the ER/intermediate compartment upon warming of cells to 37°C after a 2-h incubation at 16°C (Fig. 3).

## Discussion

### *Double-Lysine Tagged Proteins are Modified in Post-ER Compartment(s)*

To determine whether the double-lysine motif serves as a re-

trieval rather than as a retention signal, we analyzed the post-translational modifications of CD8/E19. Conclusive evidence is provided that this protein rapidly receives GalNAc and palmitate, modifications reported to occur in post ER location(s). However these reports (Bonatti et al., 1989; Tooze et al., 1988) could not rule out low levels of activity in the ER. Indeed herein lies one of the fundamental problems with using post-translational modifications to map the intracellular trafficking of proteins. This problem is particularly acute in the secretory pathway as the various modifying enzymes and the protein in question are first synthesized into the ER. Furthermore, although recent data suggest Golgi proteins are most likely targeted by aggregation (Nilsson et al., 1991) we cannot rule out that their location may be determined by



**Figure 10.** Sialylated CD8/E19 co-localizes with ER marker proteins upon subcellular fractionation. HeLa cells expressing CD8/E19 were metabolically labeled with [<sup>35</sup>S]methionine for 4 h followed by a 24-h chase period in the presence of unlabeled methionine. Subcellular fractionation of homogenates of these labeled cells was carried out on discontinuous sucrose gradients as described by Bole et al., 1986 (see Hsu et al., 1991). *A* shows the distribution of the Golgi marker, galactosyltransferase over the gradient, as measured by the CPM of radiolabeled galactose transferred to ovalbumin (Bole et al., 1986). The distribution of epoxide hydrolase (*B*) and CD8/E19 (*C* and *D*) over the gradient was determined by immunoprecipitation followed by SDS PAGE and quantitation of the amount of radioactivity (CPM) in specific bands using an Ambis radioanalytic imager. *C* and *D* show the distribution over the gradient of sialylated CD8/E19 (i.e., the neuraminidase sensitive forms, Fig. 8 *A*) and GalNAc modified CD8/E19, (Fig. 8 *A*, *square*), respectively.

the balance of retrograde targeting and anterograde bulk flow.

To control these problems, we have analyzed the rates of post-translational modification of a series of CD8 chimeras, all maintained in the ER, but by double-lysine motifs from different ER resident proteins. We show that these chimeras receive O-linked carbohydrate at quite different rates, a finding that is difficult to explain by low levels of ER located transferase. We suggest that these rate differences reflect differences in the strength of the various retrieval signals, whereby the strength of the motif dictates the dwell time in, and thus, exposure to, post ER locations. This suggestion is supported by the subcellular distribution of the different CD8 and CD4 chimeras (see Table I) and is an obvious extension of our previous findings, that the transport rates of CD8 chimeras is directly related to the sequence context and position of the double-lysine motif (Nilsson et al., 1989; Jackson et al., 1990). We have not found evidence to support the alternative interpretation, i.e., that the different chimeras are not equally good substrates for ER located transferases. Conformational-specific anti-CD8 antibodies did not detect any difference in either the rate at which the various chimeras fold or their final folded state. Further, we showed that the various chimeras received GalNAc with very similar rates in brefeldin A treated cells, indicating that they are more or less equally good as substrates for the transferases. Perhaps the most convincing argument that the sequence of cytoplasmic tail effect the retrieval process, rather than a conformation of the marker protein, is that transferring the same set of cytoplasmic tails from CD8 to a different marker protein, CD4, also transfers the rank order in both the rates at which the carbohydrates on this marker protein are modified by post-ER enzymes and the degree to which each chimera colocalizes with the p58 marker protein (Table I).

Thus, the most plausible explanation for the types and rates of post-translational modifications of CD8/E19 observed is that these are received primarily outside the ER. This suggestion is supported by the observation that CD8/E19 and CD8/KDEL (a protein maintained in the ER by retrieval from post ER compartments [Pelham, 1988, 1989]) receive these modifications at about the same rate (Figs. 6 and 8).

### Retrieval Versus Retention

The best evidence that the double-lysine motif directs the retrieval of these post-translationally modified proteins back to the ER is provided by the lectin staining. We show that the ER of cells expressing high levels of CD8/E19 stained with PNA (Fig. 9 *F*), a lectin that specifically recognizes the characteristic Golgi addition of galactose. Most interesting was the relative lack of ER staining by both HPA and PNA in untransfected cells (Fig. 9, *A*, *C*, and *E*), indicating that few ER resident proteins are modified by addition of GalNAc or galactose. This finding indicates that either the majority of ER resident proteins are not substrates for O-glycosylation or, that in contrast to our tagged marker proteins, the majority of ER resident proteins are not continuously recirculating between the ER and post-ER compartments. The latter suggestion is consistent with reports that in contrast to CD4/E19, endogenous ER resident proteins lack Endo H-resistant carbohydrate moieties (Kornfeld and Kornfeld, 1985). Moreover, removal of the ER targeting motif from ER proteins such as UDPGT HP1 (M. Jackson, unpublished

data), BiP (Munroe and Pelham, 1987) and ERp59/PDI (Mazzarella et al., 1990) results in slow if any transport of the truncated molecules. These and other observations (see Hurtley and Helenius, 1989; Rose and Doms, 1988) indicate that many ER proteins never leave this organelle. How such proteins are retained in the ER remains enigmatic, but various models have been proposed (Hortsch and Meyer, 1985; Booth and Koch, 1989; Suzuki et al., 1991). In terms of efficiency, it would make much sense if the majority of the ER proteins were prevented from leaving the ER. A combination of retention and retrieval would ensure minimal leakage, and would explain why marker proteins with a KDEL motif, eg., CD8/KDEL (see Zagouras and Rose, 1989), which rely on retrieval only, are very slowly secreted while endogenous ER soluble KDEL proteins (Booth and Koch, 1989) are fully retained in the cell. This would imply that the double-lysine motif and the KDEL sequence are salvage sequences (Warren, 1987) and they should accordingly be referred to as retrieval motifs.

### ***The Subcellular Distribution of Marker Proteins Depends on the Sequence Context of the Retrieval Motif and Intact Microtubules***

The immunofluorescence data colocalizing CD4/E19 and CD8/E19 with the p58 antigen is the first demonstration that protein tagged with a retrieval motif is present in the intermediate compartment. The finding that these two proteins, like the marker proteins of the intermediate compartment p58 and p53, accumulate initially in the Golgi region and subsequently redistribute to more peripheral ER locations in the cell upon warm-up from 16°C (Fig. 3) supports a model (Hauri and Schweizer, 1992) in which these proteins are continually recirculating between the ER and the Golgi. These experiments also confirm that the E19 tagged proteins colocalizing with p58 are localized to post-ER structures rather than to specialized exit sites from the ER as although significant overlap occurs in the staining pattern for p53 and p63 (p63 is recognized as a marker protein for these exit sites), the distribution of p63 is unaffected by such temperature shift experiments (Hauri and Schweizer, 1992).

Not all of the CD8 chimeras could be clearly visualized in the intermediate compartment, for example CD8/H25, CD8/HP1, CD8/53kSER and CD8/KDEL, like the endogenous ER resident proteins BiP, PDI (Schweizer et al., 1990, 1991) and UDPGTs (M. Jackson, unpublished data) were not noticeably accumulated in p58 staining vesicular structures. Because morphological analyses reflect steady-state distributions of proteins, it is possible that our inability to detect these proteins in post-ER structures reflects the fact that they are very rapidly retrieved and thus never accumulate in post-ER locations to a level sufficiently high to be detected. This assumption predicts that one and two copies, of a retrieval motif on a molecule would confer different subcellular distributions. The observation that CD4/E19 and HLA A2.2/E19 (data not shown), which are monomers, strongly colocalized with the p58 antigen while CD8/E19, a homodimer, with an otherwise identical cytoplasmic tail sequence, weakly localizes to the p58 antigen compartment is consistent with this notion. A retrieval process whose efficiency is dependent on the context of the double-lysine motif would also explain our previous observation that the exit rate of CD8/E19 mutants was found to be more or less directly proportional to the

length of the truncation/addition to the carboxyterminus (Nilsson et al., 1989; Jackson et al., 1990).

The recent finding that the carboxy-terminal sequence of the intermediate compartment marker protein p53 contains lysine residues located 3 and 4 residues from the carboxy terminus of the protein (Hauri and Schweizer, 1992) lends further support to our contention that the double lysine motif is a retrieval rather than a retention signal. It also suggests that the localization of a protein to the intermediate compartment may simply reflect the steady-state distribution of a protein continually recycling between the ER and Golgi complex. If the balance of anterograde and retrograde transport were to be altered then one would predict a change in the steady-state distribution of the recirculating proteins (see Hauri and Schweizer, 1992). At 16°C, the delivery of proteins to the Golgi complex is recognized as being blocked however exit out of the ER is not. Reduced temperature has thereby upset the normal balance of anterograde and retrograde transport. The synchronized redistribution of molecules upon warm-up has been interpreted as the result of a wave of molecules being delivered to the Golgi with subsequent retrieval of p58 and E19 tagged proteins to the ER and with time relocation of these proteins back to their original steady-state distribution before perturbation. Similarly, the apparent increase in the concentration of CD8/E19 and CD4/E19 in the intermediate compartment in nocodazole-treated cells may be the result of this drug reducing the retrograde relative to the anterograde transport. A suggestion supported by the observed alterations in the kinetics of post-translational modification of CD8/E19 in the presence of nocodazole (Fig. 6) and one that is consistent with the previous data that depolymerization of microtubules affects retrograde delivery of Golgi proteins to the ER in the presence of brefeldin A (Lippincott-Schwartz et al., 1990). However, nocodazole treatment did not result in an obvious accumulation of CD8/H25 in intermediate compartment (Fig. 3, C and D) nor did it affect the kinetics of GalNAc addition to CD8/H25 to the same extent as to CD8/E19 (Fig. 6 B). We suggest that if microtubules are utilized in retrograde transport their role is to facilitate more long distance retrieval operations. Thus, it is possible that the more efficiently a protein is retrieved, the less dependent this process is on microtubules. The anterograde transport also seemed to be affected by microtubules because CD8/E19-3S acquired sialic acid more slowly in nocodazole-treated cells (Fig. 6 A). Thus, the disorganization of the Golgi and the intermediate compartment in nocodazole-treated cells may result in reduced rates both of antero- and retrograde transport, but it is obvious that neither process is obligatorily dependent on an intact microtubule network.

### ***ER Retrieval Does Not Occur from a Distinct Compartment***

The occurrence of O-linked sialic acid on CD8/E19 and CD8/KDEL, and the existence of endo H-resistant forms of CD4/E19 and HLA A2.2/E19 suggest that these proteins had access to the *cis* and probably also medial Golgi compartments (Kornfeld and Kornfeld, 1985; Farquhar, 1985). However, simply altering the sequence context of the ER targeting motif on CD8/E19 or CD4/E19, resulted in proteins that were still accumulated in the ER but which received little or no modifications by Golgi enzymes (Fig. 8). These data in

conjunction with the different rates of GalNAc addition to the CD8 chimeras (see Table I) raise the possibility that the various chimeras are retrieved to the ER with different kinetics. Assuming that the forward rates of transport of these proteins are identical, (which is quite possible given that the chimeras differ only in their tail sequences) then one might expect on average, that retrieval of the different chimeras would occur from different sites in the exocytotic pathway; a view supported by differences in subcellular distribution of the various CD8 and CD4 chimeras (Table I). We envisage (see below) that retrieval of proteins to the ER as a continuous process, sorting occurring as soon as the proteins exit the ER and continuing from through out the secretory pathway. By altering the strength (context) or number of retrieval signals the steady state distribution of a protein may be altered from almost exclusively ER, eg., CD8/H25 to almost entirely post-ER eg. p53.

Small but significant amounts of CD8/KDEL were secreted from the cells. A likely explanation for this observation is that CD8/KDEL is recognized by at least one receptor, which resides in the Golgi (Lewis and Pelham, 1992). CD8/KDEL molecules that succeed in penetrating further along the exocytotic pathway than the receptor distribution would thus be secreted. In contrast, proteins containing the double-lysine motif have never been detected on the cell surface (Jackson et al., 1990). This would be fully consistent with a stochastic retrieval process where the double-lysine motif is recognized by cytosolic factors, which are not compartmentalized.

In conclusion, this study demonstrates that type I transmembrane proteins with the double-lysine motif may exit the ER to be retrieved from subsequent compartments in the secretory pathway. As predicted by Rothman (1981) more than 10 years ago, retrieval of proteins might occur from multiple compartments in the exocytotic pathway to increase the efficiency of the sorting process. Indeed, the present data fully supports that model.

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