A Different Intracellular Distribution of a Single Reporter Protein Is Determined at Steady State by KKXX or KDEL Retrieval Signals*

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To establish the specific contribution to protein topology of KKXX and KDEL retrieval motifs, we have determined by immunogold electron microscopy and cell fractionation the intracellular distribution at steady state of the transmembrane and anchorless versions of human CD8 protein, tagged with KKXX (CD8-E19) and KDEL (CD8-K), respectively, and stably expressed in epithelial rat cells (Martire, G., Mottola, G., Pascale, M. C., Malagolini, N., Turrini, I., Serafini-Cessi, F., Jackson, M. R., and Bonatti, S. (1996) J. Biol. Chem. 271, 3541–3547). The CD8-E19 protein is represented by a single form, initially O-glycosylated: only about half of it is located in the endoplasmic reticulum, whereas more than 50% of the total is present in the intermediate compartment and cis-Golgi complex. In the latter compartments, CD8-E19 colocalizes with β-coat protein (COP) (COP I component) and shows the higher density of labeling. Conversely, about 90% of the total CD8-KDEL protein is localized in clusters on the endoplasmic reticulum, where significant co-localization with Sec-23p (COP II component) is observed, and unglycosylated and initially O-glycosylated forms apparently constitute a single pool. Altogether, these results suggest that KKXX and KDEL retrieval motifs have different topological effects on their own at steady state: the first results in a specific enrichment in the intermediate compartment and cis-Golgi complex, and the latter dictates residency in the endoplasmic reticulum.

Many resident proteins of the ER bear short carboxyl-terminal sequences, KKXX in type I membrane proteins and KDEL in luminal proteins (1, 2). It has been clearly shown that both motifs are necessary and sufficient to dictate the recycling of reporter proteins from the Golgi complex to the ER (3, 4). Key events in this process are the binding of the KKXX signal, exposed in the cytosol, to members of the COP I coat protein (5, 6), and of the KDEL signal, exposed in the lumen, to a multispan membrane protein named KDELr (7, 8). Morphological evidence demonstrates that COPI coatomer and the KDELr are localized in the Golgi complex as well as in the IC (9, 10); thus, recycling to the ER could occur from both compartments. The precise pathways of KKXX and KDEL based recycles have not been unravelled yet, and it is not known whether they converge at some point.

The residency in the ER of KKXX and KDEL bearing proteins does not depend only on the recycling pathway. Evidence in favor of a direct retention, mediated by weak protein-protein interactions in the lumen of the ER, has been presented (11–14). Therefore, due to the likely involvement of retention mechanisms in addition to retrieval mechanisms, proteins naturally resident in the ER are not the ideal model system to study the contribution to protein topology of the KKXX- and KDEL-dependent recycling processes.

To overcome this problem, we decided to use a “neutral” reporter, the human CD8 glycoprotein (15). CD8 is a type I membrane protein, uniquely O-glycosylated, which is rapidly transported to the PM in heterologous cells after transfection (16). Anchorless versions of CD8, comprising only the luminal domain, are efficiently secreted (17). Thus, retention determinants, thought to reside in the luminal portion, should not be present in CD8. Two engineered forms of the reporter were used in our study: (a) the KKXX form, CD8-E19, formed by the ecto- and transmembrane domains of CD8 joined to the cytosolic tail of adenovirus E19 protein, which has a carboxy-terminal KKMP sequence (18), and (b) the KDEL form, CD8-K, formed by the ectodomain of CD8 elongated with the SEKDEL sequence (18). A striking difference was found in the fate of the two recombinant proteins stably expressed in transfected Fisher rat thyroid cells (19). Pulse-chase experiments and oligosaccharide analysis showed that CD8-E19 spends its entire life span within the ER and cis-Golgi complex (where the O-glycosylation process starts), whereas newly synthesized CD8-K spends long time routing within the ER and cis-Golgi complex but eventually escapes from this routing, becomes terminally glycosylated, and is rapidly secreted. Although these results implied that in FRT cells the KDEL-based retrieval system is more leaky than the KKXX-based one, immunofluorescence observations of the steady-state distribution of the two CD8 forms suggested that CD8-E19 might have a relatively higher concentration in the Golgi complex and a correspondingly lower concentration in the ER than its soluble counterpart CD8-K, with important implications for the retrieval mechanisms operating between ER and cis-Golgi complex.

In the present study, we have undertaken a quantitative analysis of the intracellular distribution at steady state of the two tagged reporters by immunoelectron microscopy and cell fractionation. We indeed found that CD8-E19 protein is much more present in the IC and cis-Golgi complex respect to CD8-K,
which is largely accumulated in the ER. The possible relevance of these results for the understanding of the retrieval mechanisms responsible for the localization of endogenous protein within ER, IC, and cis-Golgi complex is discussed.

**Experimental Procedures**

**Antibodies**—The following antibodies were used: mouse mAb OKT8 (anti-CD8 protein), from Ortho (Raritan, NJ), mouse mAb N1 (anti-CD8 protein) (19), rabbit polyclonal anti-CD8 (kindly provided by Dr. M. Jackson, R.W. Johnson Pharmaceutical Research Institute, San Diego, CA), affinity purified rabbit polyclonal anti-Sec32p (kindly provided by Dr. J. P. Paccaud, University of Geneva, Switzerland), rabbit polyclonal anti-β-COP (anti-EAGE, kindly provided by Dr. T. Kreis, University of Geneva, Switzerland), mouse mAb anti-KDELr (kindly provided by Dr. B. Tang, National University of Singapore, Singapore), rabbit polyclonal anti-calnexin (kindly provided by Dr. A. Helenius, Swiss Federal Institute of Technology, Zurich, Switzerland), rabbit polyclonal anti-SSra (kindly provided by Dr. G. Migliaccio, Istituto di Ricerche di Biologia Molecolare *“P. Angeletti,”* Pomezia, Italy), and rabbit polyclonal anti-ERGIC-p58 (kindly provided by Dr. J. Saraste, University of Bergen, Norway).

**Cell Culture, Samples Preparation, and Analysis**—Cell culture, preparation of cell extracts, immunoprecipitation, SDS-PAGE, and indirect immunofluorescence were performed as detailed previously (20–22).

**Cell Fractionation**—A detailed description of the procedure will be presented elsewhere. All manipulations were done at 4 °C. Briefly, 7–10 × 10⁶ cells were homogenized by 10 passages through a cell cracker (8.020-mm chamber, 8.008-mm sphere) in 20 mM Hepes-KOH, pH 7.3, 120 mM sucrose. A postnuclear supernatant fraction was obtained by centrifugation at 2500 rpm for 5 min in an Eppendorf centrifuge and loaded on top of a discontinuous sucrose gradient made up in 35–10% sucrose, and was spun in a SW 50.1 rotor tube for 1 hour at 43,000 rpm in a Beckman ultracentrifuge. Sections were incubated with goat anti-biotin colloidal gold (10 nm) for 1 hour at room temperature. After thorough washing, thin sections were incubated at 4 °C overnight with biotinylated primary antibodies followed by 18 nm of protein-A gold. Control experiments, the sections were first incubated with anti-CD8 antibody followed by protein A-gold (18 nm; 24). In double labeling experiments, the sections were first incubated with anti-CD8 antibody followed by 10 nm of protein-A gold and then treated with the second set of antibodies followed by 18 nm of protein-A gold. Control experiments were performed by omission of the primary antibodies. For HPL labeling, thin sections were incubated at 4 °C overnight with biotinylated HPL at a concentration of 20 μg/ml. After extensive washing, thin sections were incubated with goat anti-biotin colloidal gold (10 nm) for 1 hour at room temperature. The specificity of the staining was assessed in control experiments adding 50 μM N-acetylgalactosamine to the first incubation. For double labeling experiments, thin sections incubated with biotinylated-HPL and anti-biotin colloidal gold were successively immunolabeled with anti-CD8 followed by protein A-colloidal gold (18 nm). All sections were finally stained with uranyl acetate and lead citrate before examination with EM. Quantitative evaluation of immunolabeling was performed as described previously (25).

**Results**

**The Bulk of Intracellular CD8-E19 and CD8-K Proteins Consists of Immature Forms**—Detailed studies on the biosynthesis and glycosylation in FRT cells of CD8, CD8-E19, and CD8-K have been previously reported (19, 22, 26, 27). As shown in Fig. 1, Western blotting analysis indicates that untagged CD8 accumulates mostly as the broad mature band of 32–34 kDa, in addition to the unglycosylated and initially glycosylated minor forms of 27 and 29 kDa, respectively (lane 1); CD8-K was represented by the unglycosylated 20 kDa form (70% of total) and the initially glycosylated 22–24 kDa forms (lane 2); and CD8-E19 accumulated mostly as the initially glycosylated 28 kDa form (lane 3), which has a half-life longer than 2 days (19). Therefore, at steady state, almost all CD8-E19 and CD8-K proteins present in the cells consist of immature forms, no more than initially glycosylated.

CD8-E19 co-localizes with ERGIC-p58 Protein at Light Microscopy Level.—To ascertain the relative distribution of the two tagged reporters among ER and IC/cis-Golgi complex, we first performed double indirect immunofluorescence microscopy using calnexin and ERGIC-p58 as marker proteins, respectively (28, 29). As shown in Fig. 2, both CD8-K and CD8-E19 appeared to co-localize significantly with calnexin (compare panels a and d to panels e and f), whereas only CD8-E19 showed a pronounced co-localization with ERGIC-p58 protein (compare panels c and d to panels g and h), thus suggesting an enrichment in the IC/cis-Golgi area. It is noteworthy that the immunofluorescence labeling pattern of calnexin, ERGIC-p58, and of several other markers for ER and Golgi complex was undistinguishable among FRT cells and the clones isolated after transfection (data not shown), thus excluding alteration of the ER/Golgi

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**Fig. 1. Intracellular marker and formers expressed by the different transfected FRT clones.** Aliquots of 80 μg of total protein of cell lysate were analyzed by SDS-PAGE, blotted on nitrocellulose filters, and stained by immuno-ECL. Lane 1, FRT-U10 (untagged CD8); lane 2, CD8-E19; lane 3, CD8-K; lanes 4 and 5, parental FRT cells. Lanes 1–3, anti-CD8 mAb N1; lane 4, anti-calnexin polyclonal antibody; lane 5, anti-ERGIC-p58 polyclonal antibody.

**Fig. 2. Partial co-localization of CD8-E19 and ERGIC-p58.** Double indirect immunofluorescence analysis of CD8-K and CD8-E19 cells. Panels a–d, CD8-K cells; panels e–h, CD8-E19 cells. Panels a, c, e, and g, anti-CD8 mAb; panels b and f, anti-calnexin polyclonal antibody; panels d and h, anti-ERGIC-p58 polyclonal antibody.
Fig. 3. Immunoelectron microscopic analysis of the intracellular localization of CD8-E19 and untagged CD8 proteins. Thin sections of LR White-embedded (a–d) or lowicryl-embedded (e) cells were immunogold-labeled with anti-CD8 polyclonal antibody and protein A-colloidal gold conjugates. Double labeling with anti-CD8 and HPL (c and d) was also performed. In cells expressing CD8-E19, gold particles were clearly present on the IC, identified as tubular vesicular structure (a and b). Labeling was also observed on ER, NE, and the cis-most cisternae of the Golgi complex, whereas very little labeling was seen on the trans-Golgi (a). Colocalization of CD8-E19 (large golds) and HPL (small golds) was observed on the IC (c) and cis-Golgi cisternae (d, arrows). In cells expressing untagged CD8 (e), the immunogold labeling was mostly associated with the PM and with the cisternae of the Golgi complex. ER and NE appeared virtually unlabeled (e). G, Golgi complex; M, mitochondrion; Nu, nucleus; Ly, lysosome. Bars: a, 0.5 μm; b–e, 0.1 μm.

Fig. 4. Immunoelectron microscopic analysis of the intracellular localization of CD8-K protein. Thin sections of LR White-embedded cells (a and c–f) were immunogold-labeled with anti-CD8 polyclonal antibody and protein A-colloidal gold conjugates. Double labeling with anti-CD8 and HPL (d and f) was also performed. Immunolabeling appeared dense and clustered over ER (a) and NE (a, arrows). Parallel morphological analysis on conventional thin sections of cells expressing CD8-K showed several protrusions extending from the NE and clusters of vesicles facing the NE (b, arrows). Immunolabeling with anti-CD8 was also present on IC (c) and on cis-Golgi complex (e). PM was virtually unlabeled (a). Colocalization of CD8-K (large golds) and HPL (small golds) was observed on IC (d, arrow) and over cis-Golgi cisternae (f, arrows). Symbols are as in Fig. 3. Bars: a and b, 0.5 μm; c–f, 0.1 μm.
area induced by the expression of the various CD8 forms.

**Immunoelectron Microscopical Analysis of the Intracellular Distribution of CD8-E19 and CD8-K Proteins**—Next, we analyzed at the ultrastructural level the intracellular localization of CD8-E19 and CD8-K. As shown in Fig. 3, a and b, CD8-E19 is clearly present on the IC, on one side of the Golgi complex, and, more dispersed, on the ER; very little labeling was detectable on the PM (not shown). Conversely, most wild type CD8 is on the PM and the entire Golgi complex (Fig. 3c), very little on the IC (not shown), and almost none on the ER (Fig. 3c). Double labeling with HPL demonstrated that CD8-E19 is present only in the cis-Golgi complex. HPL binds mostly to GalNAc-bearing glycoproteins, and the addition of GalNAc, the first step in the O-glycosylation process, takes place in the cis-Golgi (30–32). As shown in Fig. 3d, HPL labels only one side of the Golgi complex and overlaps very well with CD8-E19. Interestingly, some HPL labeling was detectable on the IC (Fig. 3c). Because the same occurred in CD8-K expressing cells (see Fig. 4) but never in parental FRT and CD8 expressing cells, this observation suggests that labeling in the IC was due to recycling tagged CD8 forms. Finally, double labeling experiments with KDELr, used as marker of the IC/cis-Golgi area, fully confirmed the distribution of CD8 and CD8-E19 described above (data not shown).

A strikingly different distribution was instead observed for CD8-K protein. It was present in high amounts on the ER and NE and appeared frequently clustered (Fig. 4a). Careful analysis of the immunolabeled regions of the ER and NE (see also Fig. 7, c, e–g) and parallel morphological observations of conventional thin sections (Figs. 4b and 7d) revealed the presence of several protrusions extending from the NE and of numerous labeled vesicles facing the NE. CD8-K was also clearly present in the cis-Golgi and IC (Fig. 4, c and e), as shown by the co-labeling with HPL (Fig. 4, d and f) and KDELr (not shown). Conversely, CD8-K was almost absent from the PM and barely detectable on the trans-side of the Golgi complex (Fig. 4, a and e). The untagged version of CD8-K, CD8-S, did not accumulate in the cell (19) and gave only low labeling of the Golgi complex (data not shown).

To better evaluate the distribution inside the cells of the different reporters, a quantitation of the immunogold labeling was performed. As shown in Table I, the absolute labeling density and its ratio respect to the ER within each cell clone, clearly indicates the enrichment of untagged CD8 in the Golgi complex and of CD8-E19 in the ER, whereas CD8-E19 is intermediate between the two reporters, with a relative higher density in the IC and cis-Golgi complex. In the absence of stereological measures of the organelles in FRT cells, we attempted to address the question of the quantitative distribution of the different reporters by a statistical approach, assuming that the compartments should be detectable proportionally to their abundance and size within the cell. Table II reports the result of this analysis: almost 75% of untagged CD8 is on the PM, with negligible amount on ER and IC; about 90% of CD8-K is in the ER and nuclear envelope; but only 50% of CD8-E19 is in the ER, because more than 30% is in the IC and cis-Golgi. Particularly striking appeared the amount of CD8-E19 located in the IC (about 17%), considering that this compartment is most likely the smallest of the three.

**Cell Fractionation Analysis of the Intracellular Distribution of CD8-E19 and CD8-K Proteins**—To evaluate with a different method the intracellular distribution of the two tagged reporters, we moved to an analytical cell fractionation approach. Postnuclear supernatant fractions from CD8-E19- and CD8-K-expressing cells were analyzed on a discontinuous sucrose gradient. Postnuclear supernatant fractions from CD8-E19- and CD8-K-expressing cells were analyzed on a discontinuous sucrose gradient, and the distribution of the recombinant forms of the reporter in the separate fractions was assessed by SDS-PAGE followed by Western blotting, ECL detection and densitometric quantitation. As shown in Fig. 5, CD8-E19 was present in fractions enriched for ER derived elements as well as for Golgi stacks membranes, but most striking was the coincidence with the sedimentation of IC elements, as indicated by the sharp peak of ERGIC-p58. Conversely, CD8-K was enriched in the ER zone and less present in the Golgi/IC region of the gradient. Therefore, the results obtained by cell fractionation are consistent with the evidence obtained with immunofluorescence and immunoelectron microscopy, i.e. that CD8-K is more present in the ER and CD8-E19 significantly enriched in the IC.

To investigate whether the CD8-K protein molecules residing in the ER contain Golgi modifications, we compared the distribution of the gradient of the ubiqcytosolated and initially glycosylated forms. A version of this experiment in which the CD8-K forms were immunoprecipitated and evidenced by Coomassie Blue staining of the SDS-PAGE gel is shown in Fig. 6. Remarkably, there was no significant difference in the dis-

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**Table I**

| CD8          | CD8-E19 | CD8-K          |
|--------------|---------|----------------|
| ER           | 5.6 ± 2.4 | 41.6 ± 4.0     | 435.0 ± 49.3 |
| IC           | 20.5 ± 2.9 | 12.6 ± 16.6   | 480.0 ± 8.3  |
| Cis-Golgi    | 42.0 ± 14.0 | 57.8 ± 6.0    | 175.0 ± 16.0 |
| Trans-Golgi  | 42.0 ± 14.0 | 11.0 ± 2.5    | 56.0 ± 8.4   |
| PM           | 14.9 ± 1.5 | 0.29 ± 0.02   | 0.28 ± 0.05  |

| Fold         |          |                |              |
|--------------|---------|----------------|
| ER           | 1.0     | 1.0            | 1.0          |
| IC           | 3.6     | 3.0            | 0.3          |
| Cis-Golgi    | 2.5     | 1.4            | 0.4          |
| Trans-Golgi  | 2.5     | 0.26           | 0.1          |
| PM           | 1.5     | 0.05           |              |

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**Table II**

|            | CD8 + NE | CD8-E19 | CD8-K |
|------------|----------|---------|-------|
| % of gold particles | 2.5      | 49.5    | 89.3  |
| IC         | 10.4     | 16.8    | 2.9   |
| Cis-Golgi  | 7.3      | 14.4    | 4.1   |
| Trans-Golgi| 7.3      | 5.1     | 1.7   |
| Endosomes  | 7.7      | ND      | ND    |
| PM         | 73.7     | 14.1    | 1.9   |

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*a* Labeling density represents the number of gold particles divided by the surface area, except for the plasma membrane, where the linear length of the membrane has been considered. The average value of labeling density is reported.

*b* Fold indicates, for each clone, the labeling density of the compartments divided by the labeling density of the ER. At least 15 images of each compartment for each clone have been used. For quantitation of the immunolabeling on cis- and trans-Golgi cisternae, Golgi complexes were separated into two halves by an arbitrary line drawn through the medial part of the complex. Total amounts of surface area measured for each clone (in μm): ER, 2–2.5 (NE was not considered due to the difficulty of estimating the surface area); IC, 1.5–1.8; cis-Golgi, 2–2.3; trans-Golgi, 2.5–2.9. Total amount of PM measured for each clone (in μm), 125–130. Total numbers of gold particles counted: CD8, 2966; CD8-E19, 458; CD8-K, 1666.
distribution of the unglycosylated form of CD8-K respect to its initially glycosylated forms, as expected for a protein quickly recycling from the Golgi complex.

**Intracellular Distribution of COPI and COPII Coatomers**—Strong evidence suggest that COPI and COPII coated vesicles fulfill different functions inside the cell. COPI is involved in the retrograde traffic from the Golgi to the ER, and perhaps also in anterograde intra-Golgi traffic (5, 6, 33, 34). Although previous data had indicated a role for COPI in the anterograde traffic from the ER to the Golgi complex (35, 36), it now appears that only COPII is involved in the anterograde export of vesicles from the ER toward the IC and the Golgi complex (37–39). As shown in Fig. 7, significant co-labeling with β-COP (chosen as marker for COPII structure) was observed in immunoelectron

**Fig. 5. Cell fractionation analysis of the intracellular distribution of CD8-K and CD8-E19 proteins.** Postnuclear supernatant fractions were resolved on a discontinuous sucrose gradient (see under “Experimental Procedures” for details), and the collected fractions were analyzed by SDS-PAGE followed by Western blotting developed with antibodies against the indicated proteins. The amount of each protein per fraction is reported as a percentage of the total on the gradient. CLNX, calnexin protein.
microscopy for both CD8-E19 and CD8-K on the cis-Golgi and the IC (Fig. 7, a and b); conversely, no labeling for β-COP was observed on the ER (as well as on the PM). On the other hand, Sec-23p (representative of COPII structure) was detected only on the ER of both CD8-E19 and CD8-K expressing cells in single-labeling experiments; in several cases, Sec-23p was found on protrusions extending from the ER (Fig. 7g). Given that CD8-E19 shows a low labeling density on the ER (Table I), we investigated only the possible co-localization of CD8-K with Sec-23p. Convincing co-labeling of the two proteins was observed in the NE and the ER (Fig. 7c). Quantitative analysis of the electron micrographs showed that Sec-23p was almost 6 times enriched in the ER regions containing clusters of CD8-K labeling compared with the regions devoid of CD8-K clusters (not shown). Moreover, several vesicles facing the NE (Fig. 7c, arrow), and protrusions extending from either the NE or the ER (Fig. 7c, e, and f), were clearly co-labeled for Sec-23p and CD8-K, thus suggesting active export of CD8-K from the ER and a possible role of COPII in this export. Conventional electron microscopy clearly indicated the presence in these cells of a non-clathrin coat on many protrusions extending from the ER and NE (Fig. 7d, arrow) and on adjacent small vesicles (d, arrowheads) (40).

**DISCUSSION**

In this study, we examined the role of KKXX and KDEL retrieval motifs in the intracellular distribution of a reporter protein at steady state. Previous work, based on immunofluorescence and biochemical analysis after transient transfections (18), could not address this question. Human CD8 glycoprotein, expressed in FRT cells, has been chosen as reporter because (a) it does not bear any known retention/retrieval signal, (b) it readily travels through the secretory pathway, and (c) much information is available on its posttranslational maturation, as well as on the glycosylating apparatus of the host cell line. To reach quantitative conclusion, immunogold electron microscopy was used to extend the immunofluorescence analysis, and the results were confirmed by cell fractionation. The evidence obtained demonstrates that the KKXX and KDEL motifs determine a remarkably different intracellular localization of the two reporter forms at steady state: CD8-E19 is significantly enriched in the IC and cis-Golgi, whereas CD8-K is almost entirely located in the ER.

**Retrieval Motifs and the Early Region of the Secretory Pathway**—The data indicate that the reporter tagged with the KKXX motif is located along the entire early segment of the secretory pathway, i.e. ER, IC, and cis-Golgi complex, more than confined in the ER. This finding suggests that the steady state localization of a KKXX-bearing protein within the segment depends on other factors, such as the presence in the same protein of export/retention motifs operating in any one of the three compartments. Evidence in favor of this hypothesis is available, although much of it is only indirect, not based on quantitative measure of intracellular distribution. Indeed, Emp47, a yeast protein that recycles to the ER, has been found located in the Golgi complex at steady state, and the deletion mutant lacking the double lysine motif is transported downstream to the vacuole (41). ERGIC-p53/p58 protein is located in the ER and IC/cis-Golgi region (29, 42–44); recent work has evidenced the crucial role of two phenylalanine residues located at the carboxyl terminus, essential for exit from the ER, and the presence of an ER retention signal in the luminal and transmembrane domain of the protein (45). A similar situation was found in the emp24 protein family members, present among cis-Golgi and ER, which bear in the cytosolic tail a double phenylalanine motif and a sequence homologous to an endocytosis signal (46–48). Moreover, UDP-glucuronosyltransferase maintains its ER localization when deleted of the double lysine motif, thus suggesting the presence of a strong ER retention signal (4).

Conversely, for KDEL-bearing proteins, a different situation may exist, although also in this case much of the evidence is
indirect, not based on quantitative measure of intracellular localization. On one hand, the endogenous proteins bearing this signal have been all located in the lumen of the ER, with the exception of exocrine pancreas, where some secretion has been detected (49). On the other hand, deletion of the signal resulted in slow secretion, again suggesting the involvement of retention in the natural localization (11, 12, 50).

**Differences between the KDEL and KKXX Determined Retrieval**—Our results and the previous evidence discussed above strongly suggest that luminal proteins carrying a KDEL motif are more efficiently located in the ER respect to membrane proteins equipped with a KKXX signal. This behavior could be due to differences either in the retrieval mechanisms or in the trafficking of luminal respect to transmembrane proteins. In the first case, the KDEL based retrieval could be more effective because operating more efficiently from the IC. That the IC may represent the first site of sorting/retrieval after the ER has been claimed (1, 51), but the absence of specific enzymatic modifications that could mark protein transiting through this compartment has impaired to reach a firm conclusion. However, indirect biochemical data may support this hypothesis. The half-time of glycosylation of CD8-K is about 12 h (19), and 70% of the intracellular CD8-K is represented by the unglycosylated form; thus, it is conceivable that the protein exits from and returns to the ER many times, without reaching the cis-Golgi complex. Conversely, the findings that CD8-E19 is (a) localized among ER, IC, and cis-Golgi, but more evenly distributed among the three compartments, (b) represented by almost a single, initially glycosylated form, and (c) a long lasting protein (19) strongly suggest in this case a recycling mechanism that relies mostly on the Golgi complex. On the other hand, this view does not deny that CD8-K may be quickly recycled from the cis-Golgi, as indicated by the finding that at steady state the initially glycosylated forms of CD8-K do not constitute a separate pool respect the total (and reinforced by the observation that cell fractionation analysis after glucosamine pulse-labeling of CD8-K expressing cells shows the same result; data not shown). In conclusion, a retrieval mechanism from the IC to the ER that would operate better (or exclusively) for the KDEL bearing proteins than for the KKXX ones could well explain the greater accumulation of CD8-K in the ER and of CD8-E19 in the IC and cis-Golgi complex.

The second hypothesis, that no significant differences exist in the two retrieval mechanisms because the critical factor is being a soluble or a transmembrane protein, has to be considered (52). Indeed, soluble proteins may be at a disadvantage for geometric reasons in a transport process based on vesicular trafficking. This could in principle explain the accumulation of CD8-K in the ER, given that the interaction with the membrane-bound KDELr in the IC and cis-Golgi complex could make the packaging of the protein in vesicles a different process in the ER respect to the other two compartments. We consider this hypothesis unlikely for several reasons. Strong evidence indicates that ER export does not occur by default (53, 54), but instead it involves sorting and concentration of the cargo, processes that must depend upon the interaction of the cargo with receptor/adaptor proteins. The clustering of CD8-K in the ER and its co-labeling with COPII suggest active export of the protein. Along these lines, preliminary cell fractionation results of pulse-chase experiments show a rapid movement of
newly synthesized CD8-K from the ER to lighter membrane fractions.3

COPs and ER/IC/cis-Golgi Complex Traffic—Finally, we looked at the distribution of COPI and COPII in the cells expressing the engineered reporters. A complementary situation was found: the first was detected exclusively in the IC and cis-Golgi complex, the second only in the ER, often in protrusions and small vesicles very close to them. Significant co-labeling was observed for both COPs and the two CD8 forms. Altogether, this analysis fully supports the current view that COPI is primarily involved in the retrograde traffic to the ER, and possibly in the transport from the IC to the Golgi and within the Golgi complex, whereas COPII plays an important role only in the anterograde transport from the ER to the IC.

Conclusion—The heterologous system we are using to study in vivo the retrieval processes operating among ER, IC, and cis-Golgi complex is giving significant information. Our current focus is on the IC, to address the question of the role of this compartment in the retrieval process.

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