Dilysine signals confer localization of type I membrane proteins to the endoplasmic reticulum (ER). According to the prevailing model these signals target proteins to the ER by COP I-mediated retrieval from post-ER compartments, whereas the actual retention mechanism in the ER is unknown. We expressed chimeric membrane proteins with a C-terminal -Lys-Lys-Ala-Ala (KKAA) or -Lys-Lys-Phe-Phe (KKFF) dilysine signal in Lec-1 cells. Unlike KKFF constructs, which had access to post-ER compartments, the KKAA chimeras were localized to the ER by confocal microscopy and were neither processed by cis-Golgi-specific enzymes in vivo nor included into ER-derived transport vesicles in an in vitro budding assay, suggesting that KKAA-bearing proteins are permanently retained in the ER. The ER localization was nonsaturable and exclusively mediated by the dilysine signal because mutating the lysines to alanines led to cell surface expression of the chimeras. Although the KKAA signal avidly binds COP I in vitro, the ER retention by this signal does not depend on intact COP I in vitro because it was not affected in an e-COP-deficient cell line. We propose that dilysine ER targeting signals can mediate ER retention in addition to retrieval.

Many type I membrane proteins carry a dilysine signal in their cytosolic domain that confers localization to the ER (1). This signal has been defined as two lysine residues at positions -3 and -4 (KKXX) or -3 and -5 (KKXXX) from the C terminus exposed on the cytosolic side of the ER membrane (2). Chimeric proteins bearing a dilysine signal are localized to the ER but can acquire Golgi-specific carbohydrate modifications, suggesting that ER localization involves retrieval from the Golgi (3-5). Recent evidence implicates COP I (coat protein type I) in the recognition of the dilysine signal. COP I (coatomer) is a complex of seven proteins termed α-, β-, β′-, γ-, δ-, ε- and ζ-COP (6-8) mainly localized to the ER/Golgi intermediate compartment (ERGIC) and the Golgi (9). Functional dilysine signals bind to coatomer in vitro (10), and yeast mutants of COP subunits fail to localize reporter proteins to the ER (11-13). Some of these mutants are unable to bind to functional dilysine signals in vitro, supporting the notion that coatomer plays an essential role in retrograde Golgi-to-ER transport of dilysine-bearing proteins.

The ERGIC marker protein ERGIC-53 is a type I membrane protein carrying a dilysine signal with the sequence -Lys-Lys-Phe-Phe (KKFF) (14). ERGIC-53 is a mannos lectin continuously recycling in the early secretory pathway, suggesting that it may operate as a transport receptor for glycoproteins (15, 16). In support of this notion, antibodies to the cytoplasmic tail of ERGIC-53 inhibit transport of vesicular stomatitis virus G protein (17), mistargeting of ERGIC-53 to the ER impairs the secretion of cathepsin C (18), and mutations in ERGIC-53 can lead to combined factor V/VIII deficiency in humans presumably due to inefficient secretion of these coagulation factors (19). Targeting of ERGIC-53 is controlled by at least three signals. In addition to the dilysine signal the two C-terminal phenylalanines operate as an ER exit determinant (20). Moreover, the luminal and transmembrane domains of ERGIC-53 in combination contain information for intracellular retention that is abolished by exchanging the transmembrane domain of ERGIC-53 by that of the plasma membrane protein CD4 (20). If the two C-terminal phenylalanines are changed to alanines, resulting in the C-terminal sequence KKAA, ERGIC-53 is mislocalized to the ER (20, 21). Although the most straightforward interpretation of this finding is that the replacement of the two phenylalanines inactivates the ER exit signal, an alternative possibility is that the dilysine signal KKAA mediates direct retention in the ER. To test for retention, we have transferred the KKAA dilysine signal to a plasma membrane reporter protein, CD4, that is known not to carry intracellular retention information. Here we show that chimeric proteins possessing the C-terminal dilysine signal KKAA are permanently retained in the ER, in contrast to KKFF chimeras that can leave the ER. The results suggest that dilysine signals can operate in both ER retention and retrieval.

**EXPERIMENTAL PROCEDURES**

Recombinant DNA—The cytoplasmic tails of the CD4 chimeras were generated by polymerase chain reaction and fused to the luminal and transmembrane domain of the CD4 molecule after codon 395 using a *Bsu36I* site as described (21). A construct encompassing the luminal and transmembrane domain of CD4 fused to a cytosolic tail with the sequence RRAAAASAKKFF was used as a template. This construct was generated according to Itin et al. (21). The CD4 chimeras were cloned into the pcDNA3 vector under the CMV promoter. The GM construct was as reported (21). The L5374KKAA mutant was constructed by polymerase chain reaction using the L5374C53 construct (21), as a template. All constructs were confirmed by DNA sequencing.

Antibodies—Antibodies used were: mAb G1/93 (IgG1) against human ERGIC-53 (22), mAb 9E10.2 (IgG1) against a c-Myc epitope (ATCC CRL 1729), mAbs HP2/6.1 (IgG2A; kindly provided by Francisco Sanchez-Madrid (23)) and RFT4 (IgG1; kindly provided by George Janossy through the MRC AIDS reagent project (24)) against CD4, mAb A1/59 (IgG1) against the ER protein p63 was produced by the hybridoma technique (22) using an ERGIC fraction of Vero cells (25) as an antigen.

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Cell Culture and Transfection—Chinese hamster ovary Lec-1 cells (ATCC CRL 1735; Ref. 27) were grown in α-minimum essential medium
supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml fungizone (Life Technologies, Inc.). ldlF cells (kindly provided by Monty Krieger) were grown in Ham’s F-12 medium supplemented with 5% fetal calf serum, antibiotics, and fungizone. Stable cell lines expressing the GM and L53T4KKAA constructs were generated as described in Kapperle et al. (20). For transient expression, Lec-1 cells were transfected by the calcium phosphate precipitation method and ldlF cells using Transfectam® (Promega, Madison, WI). For pulse-chase experiments 60-mm dishes were each transfected with 5–8 μg of DNA. For immunofluorescence microscopy cells were grown in eight-well multichamber Permanox slides (Nunc, Life Technologies, Inc.) and transfected with 0.6 μg of DNA/well. For cotransfections, 0.3 μg of DNA of each construct was used.

### Metabolic Labeling, Immunoprecipitation, and endo-D Treatment—
Cells of a 60-mm dish were metabolically labeled with 50–100 μCi of [35S]methionine in a total volume of 1 ml of medium (minimum essential medium, 10% dialyzed fetal calf serum, 1% nonessential amino acids). After the chase, the cells were lysed in 1 ml of lysis buffer (100 mM sodium phosphate, 1% Triton X-100, pH 8, and protease inhibitors). Lysates were cleared by centrifugation at 100,000 × g for 1 h. Each supernatant was added to 40 μl of protein A-Sepharose (Pharmacia) to which 1 μl of mAb HP2/6.1 had been prebound. After incubation overnight, samples were split in two, and proteins were eluted from the protein A beads by boiling for 3 min in 30 μl of endo-D buffer (50 mM NaF, 20 mM Na2HPO4, 6.5 (sodium citrate), 2% Triton X-100, 0.1% SDS, 5 mM EDTA, and protease inhibitors). Another 30 μl of endo-D buffer was added to each sample, and 3 milliunits of endo-D (Roche Molecular Biochemicals) was added to each sample and incubated at 37 °C for 20 h. Samples were then boiled in sample buffer and analyzed by SDS-PAGE followed by fluorography. Endoglycosidase H was used as described (20).

### Immunofluorescence Microscopy—
48 h posttransfection Lec-1 cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% saponin, and processed for indirect immunofluorescence as described (21). Cells were analyzed with a Reichert Polyvar immunofluorescence microscope or a Leica confocal laser scanning microscope (0.25-μm optical sections).

### In Vitro Budding Assay—
The in vitro budding assay was as described (30) with the following modifications. Microsomes were prepared from eight 15-cm dishes of confluent Lec-1 cells stably transfected with either the GM or the L53T4KKAA construct. The cells were homogenized by passing them 10 times through a ball-bearing homogenizer with a clearance of 20 μm. The final pellet was resuspended in 80 μl of transport buffer giving a protein concentration between 5–15 mg/ml and used directly in a single budding assay. Samples were analyzed by SDS-PAGE followed by Western blotting (ECL). Blots were detected at the cell surface of nonpermeabilized cells (Fig. 2, a and c) and at the cell surface (Fig. 2, c and d). The inability of the KKAA signal to mediate complete intracellular retention is likely due to saturation of the retention mechanism by overexpression. Such a leakage has already been observed for wild type ERGIC-53 (21, 33), and suggests that the dilysine-dependent retrieval mechanism is saturable. The CD4R2A10 construct showed cell surface staining as expected for a construct without retention signals. These results indicate that the targeting mechanisms mediated by KKAA and KKFF are different or that the two signals have different affinities for the same sorting machinery.

### Results

**A KKAA Chimera Is Localized to the ER**—We have previously shown that a construct of ERGIC-53 containing the dilysine signal KKAA is retained in the ER (20, 21). Because the combined presence of the luminal and transmembrane domains of ERGIC-53 already confer some retention, the contribution of the KKAA signal to ER retention could not be evaluated using this reporter protein.

To elucidate whether KKAA can mediate ER retention, it was fused to the transmembrane and luminal domain of CD4. CD4 is a cell surface protein known not to contain intracellular retention information. The CD4 luminal domain has two N-linked glycoylization sites, which are useful for determining the intracellular location of CD4 chimeras biochemically. The chimeras CD4KKK, CD4KKFF, and CD4R2A10, depicted in Fig. 1, were transiently expressed in Lec-1 cells, and their expression and intracellular localization was determined using immunochemical methods. CD4KKK exhibited typical ER staining and could not be detected at the cell surface of nonpermeabilized cells (Fig. 2, a and b). The CD4KKFF construct gave an ER pattern but was also detectable in the Golgi area (Fig. 2c) and at the cell surface (Fig. 2, c and d). The inability of the KKFF signal to mediate complete intracellular retention is likely due to saturation of the retention mechanism by overexpression. Such a leakage has already been observed for wild type ERGIC-53 (21, 33), and suggests that the dilysine-dependent retrieval mechanism is saturable. The CD4R2A10 construct showed cell surface staining as expected for a construct without retention signals. These results indicate that the targeting mechanisms mediated by KKAA and KKFF are different or that the two signals have different affinities for the same sorting machinery.

**The KKAA Signal Prevents Transport to cis-Golgi**—To investigate whether the CD4KKAA protein had access to the cis-Golgi and would be localized to the ER by retrieval from the Golgi, its carbohydrate modifications were studied in Lec-1 cells. Because these cells lack the cis-Golgi enzyme N-acetylgalcosaminyltransferase, glycoproteins passing through the cis-Golgi acquire permanent endo-D sensitivity (27). Lec-1 cells...
transiently transfected with either CD4KKAA, CD4KKFF, or CD4R2A10 were labeled with [35S]methionine for 30 min and chased for 6 h. Cell lysates were immunoprecipitated with a mAb against CD4, and the precipitates were incubated with or without endo-D prior to SDS-PAGE analysis. In the absence of endo-D (Fig. 3, upper panel) CD4KKFF (lane 4 versus lane 3) and CD4R2A10 (lane 6 versus lane 5) showed increased electrophoretic mobility after the 6-h chase, indicating posttranslational trimming. By contrast, the mobility of CD4KKAA changed only minimally (lane 2 versus lane 1), suggesting that this construct did not have access to the compartment(s) containing the trimming enzymes that processed CD4KKFF and CD4R2A10. Because the CD4 chimeras contain two N-linked glycosylation sites, the small shift of CD4KKAA can be explained by α-1,2-mannosidase-mediated trimming in the ER, whereas CD4KKFF and CD4R2A10 would be further trimmed by Golgi mannosidase I (28). Fig. 3 (lower panel) indeed shows that a fraction of CD4KKFF (lane 4) and CD4R2A10 (lane 6) was endo-D-sensitive after the 6-h chase, whereas CD4KKAA remained entirely endo-D-resistant (lane 2). The apparent incomplete endo-D sensitivity of CD4KKFF and CD4R2A10 after 6 h is likely due to incomplete digestion with endo-D, a well known phenomenon, and not to incomplete trimming to mannos 5. The slight shift of CD4KKAA seen both in the absence and presence of endo-D was prevented by the mannosidase inhibitor deoxymannojirimycin (not shown), which, together with the endo-D insensitivity, corroborates the notion of a mannose trimming event in the ER. These results suggest that CD4KKFF and CD4R2A10 had access to the cis-Golgi, whereas CD4KKAA did not.

The KKAA Signal Prevents Transport to the ERGIC—The inability of CD4KKAA to reach the cis-Golgi may reflect efficient retrieval from the ERGIC. AlF₄⁻ was used to test this possibility. AlF₄⁻, an activator of trimeric G proteins, blocks anterograde and retrograde transport of proteins from the ERGIC (16, 20, 29), presumably by locking coatomer to membranes. If CD4KKAA would be transported from the ER to the ERGIC and rapidly retrieved from there, it should accumulate in the ERGIC in AlF₄⁻ treated cells. To this end, Lec-1 cells co-expressing CD4KKAA and GM were treated with AlF₄⁻ for 30 min, double stained for CD4 and ERGIC-53, and analyzed by confocal laser scanning microscopy. In the absence of AlF₄⁻, CD4KKAA was exclusively confined to the ER, whereas GM, in addition to the ER, was also localized to a juxtanuclear area in the Golgi region (Fig. 4, left panel). Upon AlF₄⁻ treatment, the staining pattern for CD4KKAA remained unchanged, very much in contrast to GM, which was depleted from the ER and concentrated in the juxtanuclear area (Fig. 4, right panel). We conclude that CD4KKAA is unable to recycle via the ERGIC.

A KKAA Chimera Is Excluded from ER Transport Vesicles—To investigate in a more direct way whether a KKAA signal prevents exit from the ER, we isolated ER transport vesicles using a recently described in vitro budding assay (26, 30). Microsomes were isolated from Lec-1 cells permanently expressing a fusion protein consisting of the luminal domain of ERGIC-53, the transmembrane domain of CD4 and a RSGQQEAAKKAA cytosolic tail (construct L53T4KKAA; Fig. 1). The reason for using this construct was that attempts to obtain a stable cell line expressing high amounts of CD4KKAA used above were unsuccessful. We have previously shown that the luminal and transmembrane domains of ERGIC-53 in combination contain pro-Golgi retention information (20). By exchanging the transmembrane domain of ERGIC-53 with that of CD4, this retention is abolished and any remaining retention is exclusively confined to the KKAA signal of the cytosolic tail (20, 21).

Transport vesicles were generated by incubating the microsomes with cytosol and an ATP-regenerating system at 32 °C for up to 40 min. As a positive control, microsomes were isolated from Lec-1 cells stably expressing GM. After incubation, the transport vesicles were separated from the microsomes by differential centrifugation. The medium speed pellet containing the microsomes and the high speed pellet (HSP) containing the vesicles were analyzed by SDS-PAGE followed by Western blotting. GM and L53T4KKAA were detected with anti-IgG2 antibodies. At no time point could the L53T4KKAA protein be detected in the HSP, indicating that the protein was not incorporated into transport vesicles (Fig. 5a, lower panel; Fig. 5b, quantification). In contrast, GM, which continuously recycles between the ER, ERGIC, and cis-Golgi, was detected in the HSP after a 20-min incubation (Fig. 5a, upper panel). The appearance of GM in HSP was not due to ER fragmentation because the ER resident protein p63 was absent from HSP (Fig. 5c). It was entirely inhibited in the absence of either an ATP regenerating system or cytosol in the budding reaction (Fig. 5d) or inhibited by >80% by a dominant negative mutant Sar1 protein, Sar1T39N (Fig. 5e), confirming that the appearance of GM in the HSP was due to vesicle budding.

KKAA-mediated ER Retention Does Not Depend on Intact Coatomer—Previous studies have shown that peptides terminating in KKAA avidly bind coatomer (17, 20). To test whether KKAA-mediated ER retention requires coatomer, we used a mutant CHO cell line (ldlF) that expresses a temperature-sensitive point mutation in the coatomer subunit ε-COP (31). At the nonpermissive temperature (39.5 °C) ldlF cells have a defect in transport of newly synthesized glycoproteins through the Golgi as indicated by delayed or incomplete oligosaccharide
processing. If COP I was to play a role in KKAA-mediated ER retention, CD4KKAA can be expected to leave the ER and undergo cis-Golgi-dependent oligosaccharide processing in ε-COP-defective cells. IdIF cells grown at 34 °C (permissive temperature) were transfected with CD4KKAA or CD4R2A10 cDNA. 38 h posttransfection, the cells were transferred to a 39.5 °C incubator for 10 h. Then the cells were pulse-labeled with [35S]methionine for 30 min, chased for 3 h with methionine in excess, and subjected to immunoprecipitation with antibodies against CD4. The precipitates were analyzed by SDS-PAGE followed by fluorography (Fig. 6). CD4R2A10 remained entirely endoglycosidase H-sensitive after the 3-h chase (not shown), indicating that transport through the Golgi was impaired at 39.5 °C as expected (31). However, during the chase CD4R2A10 underwent an apparent size reduction characteristic for the cis-Golgi form (Fig. 6, lane 4; see also Fig. 3). By contrast, CD4KKAA did not show the Golgi-specific Mᵣ transi-

FIG. 5. A KKAA construct does not enter ER-derived vesicles. Microsomes of Lec1 cells stably transfected with GM or L53T4KKAA were incubated in an in vitro budding assay mixture at 32 °C for 0, 20, and 40 min. ER transport vesicles were separated from the microsomes by differential centrifugation and analyzed by SDS-PAGE followed by Western blotting. 20% of the medium speed pellet and 100% of the high speed pellet were applied per lane. a, budding assay with microsomes from GM cells (upper panel) and L53T4KKAA cells (lower panel). b, quantification of the amount of GM and L53T4KKAA appearing in the high speed pellet after a 20-min incubation at 32 °C. Shown are means of four independent experiments ± S.D. c, distribution of the p63 ER protein in a budding assay performed with GM microsomes for 20 min. First the GM protein was detected (left two lanes), then the blot was stripped and probed for p63 (right two lanes). d, cytosol and ATP dependence of the budding assay with GM microsomes. The reaction mixture was incubated for 20 min in the presence (normal) or absence of either ATP (-ATP) or cytosol (-cytosol). The constructs were visualized with anti-Myc (mAb 9E.10.2). e, Sar1 dependence of the budding assay. A dominant-negative mutant of Sar1, Sar1T39N (final concentration, 3 μM), inhibits vesicle formation from GM microsomes. Sar1T39N was added to the complete budding mixture for 20 min. M, medium speed pellet containing the microsomes; H, high speed pellet containing the transport vesicles.

FIG. 6. R2A10 but not KKAA allows transport to the Golgi in ε-COP-deficient IdIF cells. IdIF cells were transiently transfected with either the CD4KKAA or CD4R2A10 construct. 38 h posttransfection the cells were transferred to 39.5 °C and incubated for 10 h prior to labeling. Cells were labeled with [35S]methionine for 30 min, chased for 3 h, lysed, and immunoprecipitated with mAb HP2/6.1 against CD4. Precipitates were analyzed by SDS-PAGE followed by fluorography. The constructs are denoted KKAA and R2A10 for CD4KKAA and CD4R2A10, respectively.

DISCUSSION

It is currently assumed that dilysine targeting signals operate by retrieval rather than retention (1). This notion is based on the findings that chimeras bearing some dilysine signals carry Golgi-specific carbohydrate modifications despite their localization to the ER both in mammalian and yeast cells (2–4).

Surprisingly, we were unable to find evidence for recycling of chimeric proteins with a C-terminal KKAA dilysine signal. These chimeras localized to the ER, did not acquire Golgi-dependent endo-D sensitivity in Lec1 cells, were not concentrated in the ERGIC by AlF₄⁻, and were excluded from ER-derived transport vesicles. These data strongly support the notion that KKAA is a true ER retention signal. ER retention is not simply due to the lack of an ER-export signal because chimeras with a polyanaline tail lacking transport information were not retained. Moreover, the inactivation of an ER exit signal does not entirely block ER exit (20, 32). It has previously been noted that the rate at which various dilysine signal-tagged reporter proteins acquired Golgi modifications greatly varied, as did the degree to which they co-localized to post-ER compartments (4). It was therefore proposed that the sequence context of a dilysine motif may affect the efficiency of retrieval (1). Our data with KKAA suggest retention as an additional function of dilysine signals, whereby the sequence context of dilysine signals determines the efficiency of ER retention. Although it is likely that the sequence context also affects the efficiency of protein retrieval, this issue remains to be reinvestigated in light of our present findings.

What is the mechanism by which KKAA mediates ER retention? Previous transfection experiments in COS cells have shown that the intracellular retention of ERGIC-53 and CD4 chimeras carrying the wild type KKFF signal is saturable by overexpression very much in contrast to that of ERGIC-53 terminating in KKAA (21, 33). It was of interest therefore to determine whether COP I known to specifically recognize dilysine signals plays a role in the ER retention mediated by the KKAA signal. Although the KKAA signal avidly binds COP I has been localized to some areas of the ER (20) and COP I has be localized to some areas of the ER (34), our results with the ε-COP-deficient IdIF cells suggest that intact coatomer is not required for ER retention by the KKAA signal. 

2 M. Gomez, personal communication.
with the notion that COP I is primarily involved in retrograde transport (11), although other traffic routes may also depend on coatomer. Another possibility for retention would be tight interaction of the KKAA signal with microtubules. Studies with a synthetic peptide containing the dilysine signal of the adenovirus E19 protein indicate that this peptide can bind β-tubulin and promote tubulin polymerization in vitro (35). However, if microtubules were destroyed by nocodazole, KKAA-mediated ER retention was not affected, rendering such a mechanism less likely. Yet another notion for protein retention in a membrane organelle is the formation of oligomers too large to be included into transport vesicles (36). Such a mechanism appears unlikely because CD4 is a monomer and KKAA-bearing ERGIC-53 oligomerizes with normal kinetics and no such large aggregates were observed (Ref. 21 and this study). Interaction with an ER resident protein appears a likely possibility for explaining the ER retention, although our attempts to identify such a partner protein were unsuccessful. Interaction with ER-specific lipids is another possibility that remains to be tested.

In conclusion, we have shown that the dilysine signal KKAA mediates permanent retention of type I membrane proteins in the ER. We propose that dilysine ER targeting signals can mediate retention in addition to retrieval. Thus protein targeting to the ER by dilysine signals can be mediated by at least two mechanisms that are not mutually exclusive: signal-mediated retention and signal-mediated retrieval.

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REFERENCES

3 H. Andersson and H.-P. Hauri, unpublished observations.
Protein Targeting to Endoplasmic Reticulum by Dilysine Signals Involves Direct Retention in Addition to Retrieval
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