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A novel endocytosis signal related to the KKXX ER-retrieval signal

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Membrane proteins often contain a sorting signal in their cytoplasmic tail that promotes their clustering into coated vesicles at a specific cellular site. ERGIC-53 contains a cytoplasmic ER-retrieval signal, KKFF. However, overexpressed ERGIC-53 is transported to the cell surface and rapidly endocytosed. Here we report that ERGIC-53 carries a previously undescribed endocytosis signal. Surprisingly, the signal was KKFF and like the ER-retrieval signal required a C-terminal position. In fact, the minimal consensus sequence determined by substitutional mutagenesis (K-K/R-F/Y-F/Y) was related to the ER-retrieval consensus (K-K-X-X). Furthermore, we provide evidence that internalization of VIP36, a protein that cycles between plasma membrane and Golgi, is mediated by a signal at its C-terminus that matches the internalization consensus sequence. The relatedness of the two signals suggests that coatomer-mediated retrieval of proteins may be mechanistically more related to clathrin-dependent sorting than previously anticipated.

Key words: endocytosis/ERGIC-53/ER-retrieval/sorting signal

Introduction

The cellular localization of membrane proteins often depends on sorting signals present as short stretches of amino acids in their cytoplasmic domains (Pelham and Munro, 1993). Two such signals are the tyrosine-containing and the di-leucine-containing internalization signals (Letourneur and Klausner, 1992; Trowbridge *et al.*, 1993). Membrane proteins containing either of these signals are efficiently sorted into clathrin-coated pits at the plasma membrane (Anderson *et al.*, 1978; Dietrich *et al.*, 1994). This process involves at least three steps: binding of the clathrin adaptor complex to the membrane, recognition of the sorting signal and assembly of the adaptor complexes with clathrin to form coated pits (Schmid, 1992). Structural analysis of the tyrosine signals indicate that they form a tight β-turn fold (Bansal and Gierasch, 1991; Eberle *et al.*, 1991), while the conformation of the di-leucine signals has not been determined so far. Nevertheless, recognition of both signals is likely mediated by the plasma membrane clathrin adaptor complex, AP-2, suggesting that it has binding sites for at least two signals (Sorkin and Carpenter, 1993; Dietrich *et al.*, 1994).

The tyrosine- and di-leucine-containing signals also mediate sorting at the trans Golgi network (TGN). The presence of either of the two signals in lysosomal membrane proteins and mannose 6-phosphate receptor promotes their incorporation into TGN-derived vesicles destined for late endosomes (Williams and Fukuda, 1990; Johnson and Kornfeld, 1992; Ogata and Fukuda, 1994). In epithelial cells two tyrosine signals in LDL receptor and a di-leucine signal in the Fc receptor promote incorporation into vesicles routed from the TGN to the basolateral membrane (Matter *et al.*, 1992, 1993, 1994; Hunziker and Fumey, 1994). Similar to the mechanism at the plasma membrane, sorting at the TGN involves membrane binding of a Golgi–clathrin adaptor complex (AP-1), recognition of the signals and assembly into clathrin-coated vesicles (Stamnes and Rothman, 1993; Traub *et al.*, 1993). Although both Golgi and plasma membrane adaptors recognize the same signals, not all signals are recognized by the two adaptor complexes with equal efficiency. The tyrosine signals in transferrin receptor and lysosomal acid phosphatase promote endocytosis but not sorting at the TGN (Braun *et al.*, 1989; Trowbridge *et al.*, 1993). Conversely, one of the two tyrosine-containing basolateral targeting signals in LDL receptor is active at the TGN, but does not mediate clustering into coated pits at the plasma membrane (Matter *et al.*, 1992). These results suggest that subtle changes in the cytoplasmic signal can favor interaction with one of the two adaptor complexes and hence determine its site of function.

Thus at least two different types of signals promote internalization from the plasma membrane, and for each signal a closely related form also promotes sorting at the TGN. Here we identify a new internalization motif present in the protein ERGIC-53 (Schweizer *et al.*, 1988; Schindler *et al.*, 1993) that is also very similar to a sorting determinant used at a distinct intracellular site.

The cytoplasmic tail of ERGIC-53, a protein associated with the ER–Golgi intermediate compartment (Hauri and Schweizer, 1992), has the sequence KKFF that matches the ER targeting consensus sequence KKXX (Jackson *et al.*, 1990; Schindler *et al.*, 1993). KKXX-containing peptides bind the coat complex coatomer (Cosson and Letourneur, 1994), and mutations in coatomer subunits lead to loss of retrieval, suggesting that ER targeting is mediated by coatomer-dependent clustering of transported proteins into vesicles destined for return to the ER (Letourneur *et al.*, 1994). In the case of ERGIC-53, the presence of two phenylalanines weakens the retrieval signal significantly (C.Itin, R.Schindler and H.-P.Hauri, in preparation) such that at steady state a substantial portion of ERGIC-53 is localized outside the ER (Schweizer *et al.*,

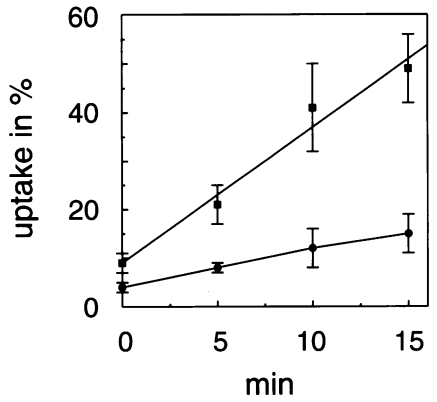

Fig. 1. Initial internalization of ERGIC-53 mutants. COS cells were transfected with GM (■) (a c-myc epitope-tagged and glycosylated variant of ERGIC-53) and a construct with the C-terminal tetrapeptide SSFF (●) in a GM background. Fourty four hours after transfection the cells were incubated at 4°C with [^{125}I]Fab fragments of mAb G1/93 against ERGIC-53 and rewarmed to 37°C for 0, 5, 10 and 15 min. Uptake was defined as the fraction of acid-resistant (pH3) counts. Values represent means ± standard deviations of three independent experiments.

1988), and overexpression results in plasma membrane appearance (Kappeler *et al.*, 1994). Surprisingly, once at the plasma membrane, ERGIC-53 is rapidly endocytosed and substitution of the two lysines to serines in the retrieval signal leads to a significantly reduced internalization rate (Kappeler *et al.*, 1994). We have now used chimeric proteins to demonstrate that the ERGIC-53 cytoplasmic tail contains an internalization signal, and we have generated further mutants to define precisely the sequence requirements of the signal. The derived consensus sequence for the signal is very similar to the ER-retrieval signal, and we provide evidence that this signal also mediates internalization of VIP36, a protein that cycles between the plasma membrane and the Golgi (Fiedler *et al.*, 1994). These results demonstrate the presence of a novel endocytosis signal, and also provide a unique example of related sorting signals that act at distinct cellular sites.

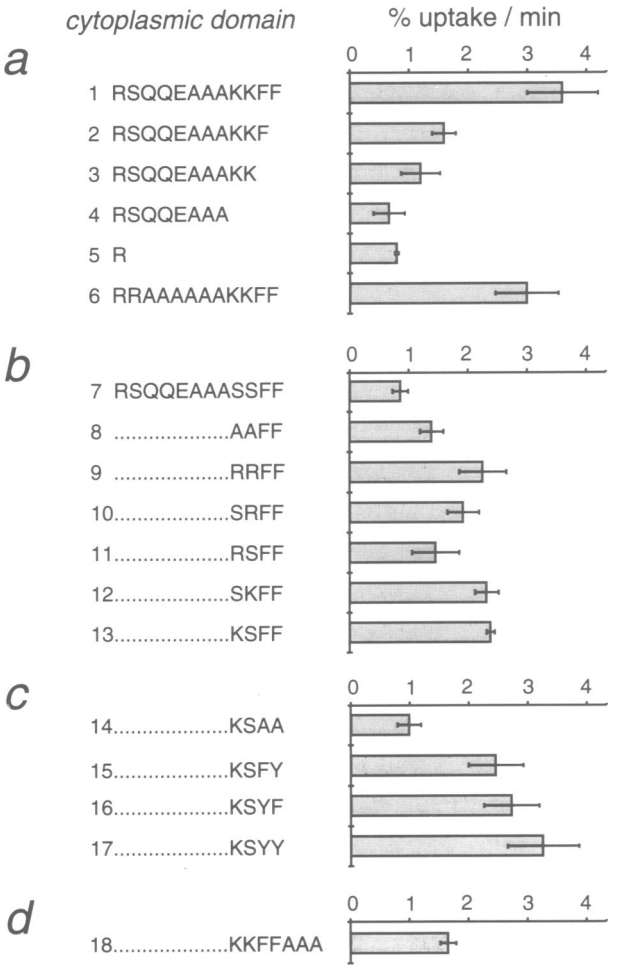

Fig. 2. Characterization of the endocytosis signal of ERGIC-53. Uptake was measured by means of [^{125}I]Fab fragments as outlined in Figure 1. All mutants were constructed in GM (corresponds to wild-type ERGIC-53; line 1). The values given represent means ± standard deviations (n = 3; except construct 1: n = 10). (**a**) Internalization of ERGIC-53 requires the C-terminal tetrapeptide KKFF. (**b**) Two arginines or a single lysine can partially replace the two lysines. (**c**) Phenylalanines or tyrosines are required for internalization. (**d**) The internalization signal is position dependent.

Results

The endocytosis rates of mutant ERGIC-53 expressed in COS cells was determined by quantifying the transfer of surface-bound iodinated monoclonal antibodies to an intracellular acid-resistant pool. Because we wished to compare endocytosis rates with previously determined pre-Golgi retention efficiencies, all mutations were made in the modified ERGIC-53 cDNA described elsewhere (C.Itin, R.Schindler and H.-P.Hauri, in preparation). This cDNA contained an engineered *N*-glycosylation site and a c-myc epitope tag (GM). Figure 1 presents an uptake time course for the corresponding protein containing either the wild-type C-terminus (KKFF) or a mutant terminating in SSFF. The values obtained were comparable with those of wild-type ERGIC-53 and the KK to SS mutant described in Kappeler *et al.* (1994), suggesting that the epitope tag and *N*-glycosylation on the exoplasmic domain of ERGIC-53 did not interfere with endocytosis. In the experiments reported below, the internalization efficiency of various mutants is given as percent uptake per minute calculated from the 15 min uptake time point. Time points at 30 and 60 min were also taken for all mutants and gave results consistent with those obtained at 15 min. Antibody uptake was also followed by immunofluorescence and confirmed the biochemical results.

The C-terminal tetrapeptide KKFF is required for internalization of ERGIC-53

One, two, four and eleven amino acids were truncated from the C-terminus to identify residues critical for internalization. Deletion of one or two residues already reduced the rate of endocytosis to about half the rate of wild-type (Figure 2a, lines 1, 2 and 3). A further truncation, deleting the four C-terminal residues, reduced the rate of endocytosis to the basal level observed for a mutant lacking 11 of the 12 cytoplasmic amino acids (Figure 2a, lines 4 and 5). Exchange of the residues adjacent to the membrane to alanines (RSQQEAAAKKFF to RRAA-AAAAKKFF) yielded a wild-type endocytosis rate (Figure 2a, line 6), suggesting that the residues adjacent to the membrane do not contribute to endocytosis. These results

establish that the C-terminal residues KKFF are required for endocytosis.

The two lysines are not absolutely required for endocytosis

As the two lysines are required for ER-retrieval and substitutions are not tolerated (Jackson *et al.*, 1990), we tested whether the two lysines were required for endocytosis. We first replaced both lysines by either serines, alanines or arginines. Replacement by serines gave basal rates of uptake (SSFF, Figure 2b, line 7). The rate of the alanine mutant was slightly increased compared with the serine mutant (AAFF, Figure 2b, line 8). However, the arginine mutant was endocytosed at two-thirds of wild-type (RRFF, Figure 2b, line 9). Substitution of either lysine with serine also gave a rate of internalization that was two-thirds of wild-type. Single arginines were less efficient than the single lysine constructs (Figure 2b, lines 10–13). We conclude that two arginines or a single lysine can partially replace the two lysines in KKFF, but these mutants did not reach the wild-type internalization rate.

Phenylalanines or tyrosines are required for endocytosis

The dramatic reduction in internalization rates after deletion of phenylalanines suggested that either they are required for internalization or that they function as a spacer that sets the distance of the lysines from the C-terminus. To distinguish between these possibilities the two phenylalanines were changed to alanines. Unfortunately this mutant was retained intracellularly and therefore not accessible to an endocytosis assay; this was not surprising as we have shown that the phenylalanines weaken the KKXX-retrieval signal and allow surface expression (C.Itin, R.Schindler and H.-P.Hauri, in preparation). To circumvent this problem we weakened the ER-retrieval signal by substitution of one of the lysines by a serine. This allowed surface appearance of a mutant lacking both phenylalanines. This protein, KSAA, had basal rates of endocytosis (Figure 2c, line 14) suggesting that the phenylalanines are required. Both phenylalanines appear to be required for full activity as removal of one led to a marked reduction (Figure 2a, line 2). Because phenylalanines and tyrosines are functionally interchangeable in many endocytosis signals (Davis *et al.*, 1987; McGraw and Maxfield, 1990; Canfield *et al.*, 1991; Fuhrer *et al.*, 1991) we changed the phenylalanines in KSFF to tyrosines generating KSFY, KSYF and KSYY (Figure 2c, lines 15–17). All three mutants were efficiently internalized suggesting that the signal requires aromatic residues in position –1, –2 from the C-terminus, in addition to charged residues (lysines or arginines) at –3 and –4.

The KKFF-tetrapeptide must be at the C-terminus for efficient endocytosis

Above, we have shown that the signal for endocytosis is located in the C-terminal tetrapeptide-KKFF that also mediates ER-retrieval. A key feature of the ER-retrieval signal is its strict dependence on its position at the C-terminus (Jackson *et al.*, 1990). Thus, the addition of three alanine residues to the C-terminus of ERGIC-53 abolishes retrieval (C.Itin, R.Schindler and H.-P.Hauri, in preparation). Similarly, the addition of three alanines to the

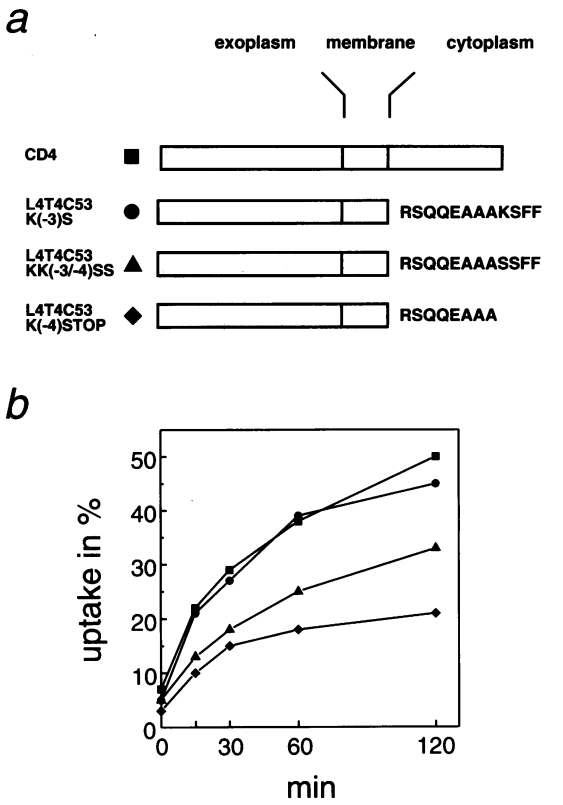

Fig. 3. The internalization signal is transplantable. (**a**) Schematic representation of the chimeric constructs. Abbreviations used are L for lumenal domain, T for transmembrane domain, C for cytoplasmic domain, 4 for CD4 and 53 for ERGIC-53. The cytoplasmic tails are given as amino acid sequences in the single letter code. (**b**) COS cells were transfected with cDNAs encoding CD4 (●) or chimerical proteins containing the exoplasmic and the transmembrane domain of CD4 combined with mutant cytoplasmic domains of ERGIC-53: L4T4C53 K(–3)S (■), L4T4C53 KK(–3/–4)SS (▲), and L4T4C53 K(–4)stop (◆). Internalization was determined as described in Figure 1 using [^{125}I]IgG purified from mAb HP2/6.1 against the exoplasmic domain of human CD4. Values given are the average of two independent experiments.

C-terminus resulted in a significant reduction in the endocytosis rate (KKFFAAA, Figure 2d, line 18). Formally, the addition of three alanines could disturb the signal unrelated to its positioning. Since alanine is widely used in mutational analysis and has not been shown to interfere with results due to its inherent properties, we suggest that the signal for endocytosis is indeed position dependent.

The KSFF-tetrapeptide confers internalization to a reporter protein

To test whether the endocytosis signal of ERGIC-53 promotes the internalization of a reporter protein, we generated chimeric molecules containing the lumenal and transmembrane part of human CD4 together with the ERGIC-53 cytoplasmic tail mutants (Figure 3a). Full-length CD4 is rapidly internalized following expression in fibroblasts (Pelchen-Matthews, 1989, 1991), but its internalization is dependent on a signal within its cytoplasmic domain (Aiken *et al.*, 1994). Elsewhere, we have demonstrated that a CD4 chimera containing the wild-type ERGIC-53 tail does not reach the surface. Surface appearance required the presence of the ERGIC-53 lumenal domain (C.Itin, R.Schindler and H.-P.Hauri, in

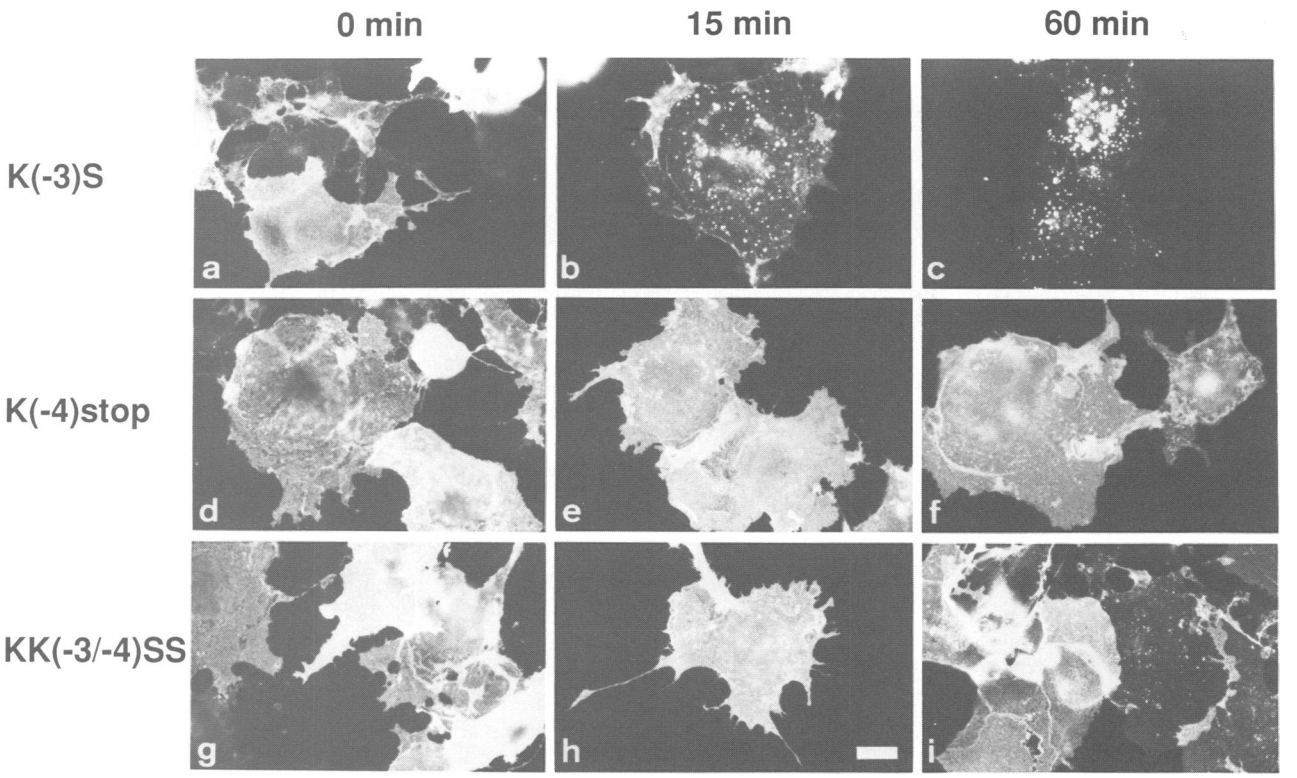

Fig. 4. Internalization of chimerical proteins visualized by indirect immunofluorescence microscopy. Transfected COS cells were incubated with mAb HP2/6.1 against human CD4 at 4°C and either directly fixed (0) or rewarmed to 37°C for 15 or 60 min before being fixed with paraformaldehyde. The cells were then saponine-permeabilized and stained with FITC-goat anti-mouse. Time points of uptake are given at the top. The mutations are indicated at the left margin: L4T4C53 K(–3)S (**a**, **b** and **c**), L4T4C53 K(–4)stop (**d**, **e** and **f**), L4T4C53 KK(–3/–4)SS (**g**, **h** and **i**). Bar corresponds to 10 μm.

preparation). Therefore, the ERGIC-53 tail mutated to the internalization sequence KSFF was used to test the activity of the signal. The CD4 chimera containing this sequence was internalized at a rate equal to wild-type CD4 (Figure 3b), indicating that the ERGIC-53 internalization signal could functionally replace the CD4 internalization signal. As a control, a chimera with substituted lysines (SSFF), and a chimera completely lacking the tetrapeptide internalization sequence, showed significantly reduced internalization (Figure 3b). Figure 4 shows the pattern of surface-bound anti-CD4 antibodies after internalization for the indicated time periods. As expected, only the chimera with the active endocytosis signal showed a change from the initially uniform surface pattern to a punctate endosomal pattern (Figure 4, a–c). The chimeras containing inactive signals remained mostly at the surface as indicated by their uniform pattern at each time point (Figure 4, d–i). These experiments demonstrate that the tetrapeptide sequence at the C-terminus of ERGIC-53 is a true internalization signal because its function is transferable.

The VIP36 KRFY C-terminal tetrapeptide promotes endocytosis of ERGIC-53

To test whether the ERGIC-53 internalization signal mediates the endocytosis of a protein that is internalized under normal conditions, we took advantage of the fact that VIP36, a protein that localizes to the plasma membrane and the Golgi (Fiedler *et al.*, 1994), contains a C-terminal tetrapeptide (KRFY) related to the KKFF of ERGIC-53

Table I. Amino acid sequence comparison of the cytoplasmic domains of ERGIC-53 with VIP36

Protein	Sequence	Ref
ERIGC-53	R S Q **Q E** A A A **K K F F**	Schindler *et al.*, 1993
VIP36	Q K R **Q E** R N . **K R F Y**	Fiedler *et al.*, 1994

The amino acid sequences are given in the one letter code. Note that the cytoplasmic domains differ only by one amino acid in length. Conserved and related residues are in bold letters.

(Schindler *et al.*, 1993; compare Table I). The internalization behaviour of the mutants described above suggested that this sequence should promote endocytosis. This was confirmed by determining the endocytosis capacity of two mutant proteins. First, using an immunofluorescent assay, we found that a chimera containing the VIP36 cytoplasmic tail fused to the CD4 lumenal and transmembrane domains was efficiently endocytosed (not shown). This result indicated that the VIP36 cytoplasmic domain contains an endocytosis signal. In the second mutant, the ERGIC-53 C-terminal tetrapeptide KKFF was changed to KRFY corresponding to that of VIP36. The internalization rate of this mutant was indistinguishable from wild-type ERGIC-53 (Figure 5b, lines 1 and 3), demonstrating that the VIP36 C-terminal tetrapeptide can promote endocytosis. These results strongly suggest that we have defined a physiologically relevant internalization signal.

We also measured the ER to Golgi transport rate of

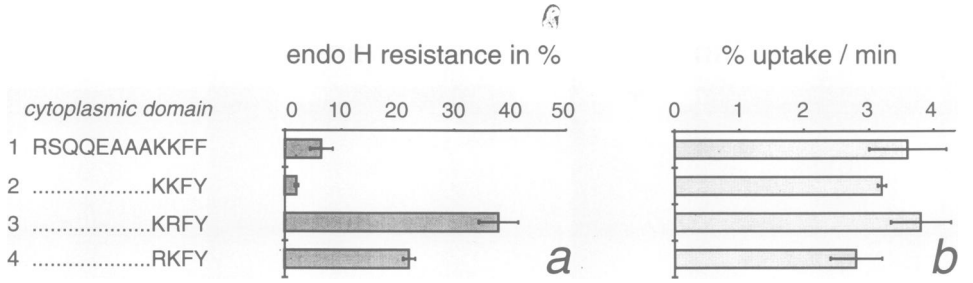

Fig. 5. The KRFY tetrapeptide of VIP36 promotes internalization but not pre-Golgi retention of ERGIC-53. (**a**) ER to Golgi transport rates of newly synthesized protein after a 1 h chase. COS cells were transfected with GM (construct 1) or mutants in a GM background (constructs 2–4) pulsed for 5 min with [^{35}S]methionine and chased for 1 h with unlabeled methionine in excess. The proteins were immunoprecipitated with mAb 9E10 against the c-myc epitope and digested with endoglycosidase H (endo H). The values represent the fraction of endo H-resistant protein given as means ± standard deviations of triplicate cultures treated in parallel. Note that mutant 3 corresponds to the tetrapeptide found in VIP36. (**b**) Internalization of [^{125}I]Fabs against ERGIC-53 was determined as described in Figure 1. The values given represent means ± standard deviations (n = 3).

the ERGIC-53 mutant containing the VIP36 C-terminal tetrapeptide. Transport to the Golgi was determined from the percent of the mutant protein that became endoglycosidase H (endo H)-resistant following a 1 h chase. The mutant bearing the VIP36 sequence was transported at four times the rate of wild-type ERGIC-53 (Figure 5a, lines 1 and 3). Thus in contrast to the ERGIC-53 sequence which promotes both ER-retrieval and endocytosis, the VIP36 C-terminal tetrapeptide only functions to promote internalization. Clearly, these signals for ER-retrieval and endocytosis are related but not identical.

Discussion

A new type of endocytosis signal

We have localized the endocytosis signal of ERGIC-53 to the C-terminal tetrapeptide KKFF. By a mutational analysis we have characterized the signal, and in order to simplify the comparison with other endocytosis signals we defined a minimal consensus sequence for internalization. Arbitrarily we decided to include only those mutants with equal rates of uptake as wild-type. This criterion leads to the minimal C-terminal consensus K-K/R-F/Y-F/Y, which allowed the identification of one other protein containing such an internalization signal, VIP36 (Fiedler *et al.*, 1994). The endocytosis signal K-K/R-F/Y-F/Y contains three features: position dependence from the C-terminus, aromatic residues in positions –1 and –2 and lysines or arginines in positions –3 and –4. These features distinguish the ERGIC-53 endocytosis signal from the previously defined mammalian endocytosis signals. Both the tyrosine and the di-leucine signal function at various distances from the C-terminus (Letourneur and Klausner, 1992; Trowbridge *et al.*, 1993). Moreover, the tyrosine signal of LDL-R is not functional when a stop codon is introduced after the NPXY endocytosis motif (Davis *et al.*, 1987). The dependence on lysines and arginines for activity is different from the di-leucine and part of the tyrosine signals (Chen *et al.*, 1990; Letourneur and Klausner, 1992; Ogata and Fukuda, 1994). However, some tyrosine signals contain single arginines or lysines in their motif that, when mutated to uncharged amino acids, reduce the rate of internalization to ~50% of the corresponding wild-type rate (Jing *et al.*, 1990; Canfield *et al.*, 1991). Nevertheless, based on the position dependence of the ERGIC-53 endocytosis signal at the C-terminus and the requirement of two aromatic and two positively charged residues, lysines and arginines, we suggest that the endocytosis signal of ERGIC-53 is different from mammalian tyrosine- or di-leucine-related endocytosis signals.

In yeast, a lysine-dependent internalization signal, DAKSS, has been identified in the cytoplasmic domain of the G-protein-coupled α-pheromone receptor Ste2p (Rohrer *et al.*, 1993). However, internalization of this receptor is pheromone-induced and no aromatic residues are found in the vicinity of the DAKSS internalization signal. A potential relationship to endocytosis signals in higher eukaryotes has not been investigated so far. As ERGIC-53 internalization is constitutive, not strictly lysine-dependent and requires aromatic residues, it is unlikely to be related to the signal of Ste2p.

The above considerations suggest that the endocytosis signal of ERGIC-53 is substantially different from previously described endocytosis signals. Because we have identified this signal in another protein, VIP36, it seems likely that the ERGIC-53 internalization signal belongs to a new class of internalization signals.

Endocytosis and ER-retrieval signals of ERGIC-53 are related but not identical

Our finding that the ERGIC-53 internalization signal comprises the same tetrapeptide, KKFF, as the ER-retrieval signal, indicated an unexpected correspondence. A comparison of the minimal consensus for internalization, K-K/R-F/Y-F/Y, with the consensus for ER-retrieval, K-K-X-X (Jackson *et al.*, 1990), shows that the signals are not identical but share two similarities. Both signals are C-terminal positioned and require positively charged residues in positions –3, –4. However, the signals differ in two aspects: the requirement of two lysines for ER-retrieval while arginines are tolerated for endocytosis, and the dependence on aromatic residues for endocytosis but not for ER-retrieval. Nevertheless, the identification of two specific signals, KKFF and KKFY, efficient in ER-retrieval and endocytosis, underscores the relatedness of the two signals (Figure 5). The similarity of the ER-retrieval and the endocytosis signal implies that the underlying sorting machineries contain structurally similar binding sites.

Relationship of coatomer-dependent sorting to clathrin-mediated sorting

Sorting of di-lysine signal-containing proteins for return to the ER is likely mediated by coatomer (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994). However, at the plasma membrane ERGIC-53 and VIP36 are at least

in part localized to coated pits (Fiedler *et al.*, 1994; C.Hopkins, C.Itin and H.-P.Hauri, unpublished) suggesting that endocytotic sorting is mediated by AP-2. Thus it is likely that the signals for ER-retrieval and endocytosis are recognized by two different coat structures, coatomer and AP-2.

Both AP-1 and AP-2 bind related tyrosine- and di-leucine signals on the cytoplasmic domains of membrane proteins (Glickman *et al.*, 1989; Sosa *et al.*; 1993) suggesting that they contain related binding sites. Sequence homologies between coatomer and clathrin adaptor complex subunits, β-COP with β-adaptins and ζ-COP with AP17 and AP19, suggest a structural and evolutionary relatedness of these coat structures (Duden *et al.*, 1991; Kuge *et al.*, 1993). In addition, coatomer and AP-1 depend on GTP-bound ARF-1 as a prerequisite for membrane binding of the coat complexes (Orci *et al.*, 1993; Stamnes and Rothman, 1993; Traub *et al.*, 1993). However, there is no evidence for a small GTP-binding protein involved in plasma membrane recruitment of AP-2 (Carter *et al.*, 1993).

That the ERGIC-53 endocytosis signal is recognized by coatomer and likely by clathrin adaptor complex suggests that the two coats contain similar signal binding sites, adding a new feature to the list of similarities of coatomer- and clathrin-mediated protein sorting.

Materials and methods

Recombinant DNAs

All ERGIC-53 mutants were generated as described (C.Itin, R.Schindler and H.-P.Hauri, in preparation). Briefly, PCR-based splicing and mutagenesis (Ho *et al.*, 1989; Horton *et al.*, 1989) were used to introduce a c-myc-epitope tag (Evan *et al.*, 1985) and a *N*-glycosylation site into ERGIC-53 cDNA (Schindler *et al.*, 1993). This construct, GM, was cloned into a pECE expression vector (Ellis *et al.*, 1986) and additional PCR-generated mutants were directly introduced as *AccI/XbaI* fragments. CD4 chimeras were constructed as described (C.Itin, R.Schindler and H.-P.Hauri, in preparation), and cloned into pCB6 for transfection studies (Brewer and Roth, 1991).

Antibodies

The following antibodies were used: G1/93 mAb against ERGIC-53 (Schweizer *et al.*, 1988), Fab fragments of G1/93 generated according to Matter *et al.* (1990), IgGs from HP2/6.1 mAb against human CD4 (Carrera *et al.*, 1987; a generous gift from F.Sanchez, Madrid), G1/221 mAb against human transferrin receptor (B.Sander and H.-P.Hauri, unpublished), and 9E10.2 mAb against a c-myc epitope (Evan *et al.*, 1985) from the American Type Culture Collection (ATCC CRL 1729).

Cell culture and transfection

COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 1 μg/ml fungizone. COS cells were transfected (2 μg DNA per 35 mm dish) using the DEAE–Dextran method (Cullen, 1987).

Endocytosis analyzed by immunofluorescence microscopy

All morphological assays were carried out with cells cultured on eight-well multi-chamber glass slides (Miles Labs, Naperville, IL, USA). Cells were cooled to 4°C, 42 h after transfection, and mAbs (G1/93 against ERGIC-53 diluted 1:10, or HP2/6.1 ascites fluid against human CD4 diluted 1:500 in COS medium) were allowed to bind to the cell surface for 30 min. After five washes with COS medium the cells were either fixed immediately or fixed after reculture at 37°C for various times to allow endocytosis. Immunofluorescence was as previously described (Schweizer *et al.*, 1988).

Quantification of endocytosis

Endocytosis of ^{125}I-labeled Fab fragments (mAb G1/93) or ^{125}I-labeled IgGs (mAb HP2/6 against CD4; Carrera *et al.*, 1987) was carried out according to Kappeler *et al.* (1994). Briefly, ^{125}I-labeled Fab fragments (5×10^6 c.p.m. per 500 μl PBS, 0.5% BSA per 35 mm dish) were incubated for 60 min at 4°C and unbound antibodies were removed by five rinses with PBS, 0.5% BSA. The cells were recultured in complete COS medium at 37°C for the indicated times and the ratio of acid-resistant to total counts was defined as uptake.

Immunoprecipitation and treatment with endo H

The protocol was according to C.Itin, R.Schindler and H.-P.Hauri (in preparation). Briefly, 42 h post-transfection, the cells were pulsed for 5 min at 37°C with 20 μCi [^{35}S]methionine (Trans-label, NEN-Dupont, France) in 200 μl labeling medium per 35 mm dish. The cells were washed once with cold DMEM containing 10 mM methionine and then chased in DMEM containing 10 mM methionine at 37°C. The cells were homogenized on ice in 1 ml solubilization buffer [100 mM sodium phosphate, 1% Triton X-100, pH 8, 1 μg/ml pepstatin, 1 μg/ml aprotinine, 0.5 μg/ml leupeptin, 1 μg/ml benzamidin, 1 μg/ml antipain, and 0.2 mM phenylmethylsulfonyl fluoride (SIGMA, St Louis, MO)]. After incubation for 1 h on ice the lysate was centrifuged at 100 000 *g* for 60 min. The resulting supernatant was incubated with 20 μl 9E10.2 for 1 h followed by an incubation with 2 μl rabbit anti-mouse IgG (Cappel, Belgium) for 30 min. Immunocomplexes were isolated by adding 10 μl protein A-Sepharose (Pharmacia, Uppsala, Sweden). After an overnight incubation at 4°C the beads were washed four times with solubilization buffer, once with 100 mM sodium phosphate, pH 8, and once with 10 mM sodium phosphate, pH 8. Immunoprecipitates were released from the protein A-Sepharose by boiling for 3 min in 30 μl 200 mM Na-citrate, pH 5.5, 2% SDS in the presence of protease inhibitors, frozen at –20°C for 30 min and boiled again for 3 min. The samples were then diluted with an equal volume of distilled water containing protease inhibitors and incubated with 4 mU endo H for 22 h at 37°C. The protein samples were run on 7–10% SDS–polyacrylamide gradient slab gels (Laemmli, 1970), the gels salicylated and the proteins visualized by fluorography using Kodak X-omat AR or Fuji RX films. The fraction of endo H-resistant protein was determined by laser densitometry.

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