A molecular specificity code for the three mammalian KDEL receptors

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Introduction
During the early stages of the secretory pathway, soluble ER-resident proteins must be sorted and retrieved back to the ER from the intermediate compartment of cis-Golgi by a coat protein (COP) I–mediated transport mechanism (Pelham, 1996; Ellgaard et al., 1999). To be recognized by this system, soluble ER-resident proteins either require a KDEL-like motif at their extreme C terminus or must form a complex with other ER-resident proteins that have this motif or with ER-resident transmembrane proteins (Lewis et al., 1990; Semenza et al., 1990). Proteins with a KDEL motif interact with a KDEL receptor in the intermediate compartment or in the cis-Golgi. This interaction is thought to cause a conformational change in the receptor, resulting in the sequestration of the complex to vesicles that are retrieved back to the ER. Higher pH in the ER results in dissociation of the KDEL motif from the receptor, with the empty receptor then being recycled.

The receptor in this process was first identified in yeast as ER retention-defective complementation group (ERD) 2 (Semenza et al., 1990). This receptor mainly interacts with proteins that have a C-terminal HDEL motif. It was shown later that different organisms have homologues of ERD2 receptors. Two human KDEL receptors, ERD21 (Lewis and Pelham, 1990) and ERD22 (Lewis and Pelham, 1992a), were identified experimentally and a third human KDEL receptor, ERD23, appeared in the Swiss-Prot database in 2000. The potential role of ERD23 in ER localization of soluble secretory pathway proteins has not been reported. Furthermore, to our knowledge, any difference in function between ERD21 and 22 has not been reported.

It is known that variants of the KDEL motif also work to keep proteins ER resident, with 24 possible variants being listed as the Prosite motif for localization of soluble proteins ([KRHQSA]-[DENQ]-E-L; Hulo et al., 2006). However, there are several human proteins that are ER located and contain variants of the KDEL motif that do not fit the Prosite motif (Alanen et al., 2003b, 2006). Hence, it is possible that other motifs might also work as ER-retrieval signals and this information could help define other ER-resident proteins.

In this study we report the Golgi localization of the third human KDEL receptor, identify 35 variants of KDEL that do not match the current Prosite motif for ER localization ([KRHQSA]-[DENQ]-E-L), and all were found in the ER. A subsequent screen by bimolecular fluorescence complementation determined the specificities of the three human KDEL receptors. Each KDEL receptor has a unique pattern of motifs with which it interacts. This suggests a specificity in the retrieval of human proteins that contain different KDEL variants.
Results

Mammalian cells have three KDEL receptors

Proteins containing C-terminal KDEL-like motifs are thought to be retrieved back to the ER from the intermediate compartment and the cis-Golgi by KDEL receptors (Lewis et al., 1990; Semenza et al., 1990). Although yeast have only a single KDEL receptor, many multicellular eukaryotes have more than one and two human KDEL receptors have been reported to date (Lewis and Pelham, 1990, 1992a). There are, however, three human KDEL receptors in the Swiss-Prot database, ERD21, 22, and 23 (Swiss-Prot accession nos. P24390, P33947, and O43731; chromosomal locations 19q13.32, 7p22.1, and 22q13.1, respectively). These three KDEL receptors show an amino acid sequence pairwise identity of 73.0–83.5% (Fig. 1 A). All three are predicted to have seven transmembrane-spanning regions with their N termini in the ER lumen, their C termini in the cytoplasm, and no N-terminal cleavable signal sequence. Based on Genecard data (Rebhan et al., 1997; http://www.genecards.org/) for the receptors, all three appear to be widely expressed in a range of human tissues, with ERD21 being generally more highly transcribed than ERD22, which in turn is more highly transcribed than ERD23 in most tissue types. The similarity in structure and widespread tissue expression suggests that ERD23, the uncharacterized human KDEL receptor, may also play a role in ER localization of soluble secretory pathway proteins.

To ensure that we were using a cell line in which all three receptors were expressed, RT-PCR was used to examine for the presence of mRNA. The results (Fig. 1 B) indicated that all three receptors were transcribed in HeLa cells. Quantitative real-time PCR showed that the ratio of ERD23 to 21 mRNA in HeLa was constant, which was comparable with the relative levels in different tissues. In contrast, the ratio of ERD22 to 21 mRNA levels was more variable between different preparations of RNA, but the ERD22 mRNA levels from HeLa were consistently significantly higher than those of ERD21 (Fig. 1 C). To further complicate the issue of multiple mammalian KDEL receptors,
there are two alternative transcripts in the databases for human ERD23. The second of these (Isoform O43731-2), which has an alternative C terminus, has not been experimentally validated. Using primers specific for this transcript, we were unable to detect an RT-PCR product using total RNA from HeLa cells. In addition to HeLa cells, Cos7 cells were also used in our studies because they have been widely used to study human KDEL receptors (Lewis and Pelham, 1992b; Townsley et al., 1993); however, we were unable to confirm that all three receptors are expressed in this monkey cell line.

If ERD23 is to act to retrieve proteins with KDEL-like motifs to the ER, it must, as per ERD21 and 22, be located in a post-ER secretory pathway compartment. The commercially available antibody against the human KDEL receptor was raised against a peptide representing the cytoplasmic C terminus of ERD21, but this sequence is >90% identical between all three receptors (Fig. 1 A) and hence the antibody is cross-reactive between receptors. The more variable regions of the three receptors are limited, but there are potential specific antigenic regions. However, despite trying to raise multiple antipeptide antibodies, we were unable to generate an antibody that specifically recognized only one receptor. Thus, to confirm the subcellular localization of ERD23, a tagged receptor was required. Human ERD23 was cloned from IMAGE clone 3462392, with a Myc tag at either the N or C terminus of the protein. Each variant was then transiently transfected into HeLa or Cos7 cells and localized by immunofluorescence. N- (ER lumen) and C-terminally (cytoplasmic)–tagged variants of ERD23 constructs both showed localization in a compact perinuclear structure (Fig. 1 D), suggesting that ERD23 is normally located in the Golgi. This localization was confirmed by staining the transfected cells against known ER and Golgi markers (calreticulin [CRT] and giantin). This post-ER localization is consistent with the putative retrieval function of ERD23.

**Development of localization reporter constructs**

With reports of ER-localized proteins with KDEL-like motifs that do not match the Prosite motif at their C termini, such as ERP18 (EDEL; Alanen et al., 2003b) and ERP27 (KVEL; Alanen et al., 2006), it is possible that other, as yet unreported KDEL variants could result in ER localization. When examining the efficiency of variants of the KDEL motif in maintaining the ER localization of a soluble protein, other variables, such as the rest of the protein sequence and hence possible interaction partners, should be minimized. To this end, a reporter construct was developed onto which variants of the KDEL motif could be added at the C terminus. Ideally, this reporter construct should be based on a secreted human protein that folds in the ER, has not been reported to interact with other ER proteins, is structurally stable, has no free thiol groups, and has minimal association with ER-resident protein-folding catalysts and molecular chaperones, i.e., no cis-proline peptide bonds, no disulphide bonds, and no N-glycosylation or other posttranslational modifications. Because we were unable to find a human-secreted protein that matched these criteria, we started examining other human secretory pathway proteins. The construct selected for testing was based around the isolated b domain of protein disulphide isomerase (PDI). This domain of PDI is thought to play a structural role in this ER-resident folding catalyst (Ellgaard and Ruddock, 2005). The structure has been solved (Kemmink et al., 1999) and it meets all of the other selection criteria. The test versions of the reporter construct had the N-terminal ER signal sequence of human CRT to target it to the ER, a HA tag for immunological detection, the b domain of PDI, and either no retention motif or AKDEL added to the C terminus (Fig. 2 A).

Immunofluorescence analysis of the test reporter constructs transiently transfected into HeLa or Cos7 cells predominantly showed localization of the AKDEL-containing construct in a fine reticular network and the nuclear envelope (Fig. 2 B), suggesting that the fusion protein localizes mainly in the ER. In contrast, the construct with no C-terminal retention motif predominantly showed localization in a compact perinuclear structure (Fig. 2 D), suggesting that it is not retained in the ER and hence is observed in the Golgi en route to being secreted. These localizations were confirmed by staining the transfected cells against known ER and Golgi markers (CRT and giantin).
The cellular retention of the AKDEL construct and secretion of the construct with no retention motif was subsequently confirmed by Western blotting (see KDEL variants found...). These results imply that the reporter construct selected was suitable to be used and is retained in the ER only via KDEL and not via interactions with other ER-resident proteins.

**Figure 3. Immunofluorescence-based localization of the reporter constructs.** For each construct, transient transfection of HeLa cells was performed in at least two independent experiments and the subcellular localization of the reporter construct was determined by examining at least 150 cells costained with a CRT antibody (ER marker) and a giantin antibody (Golgi marker). For each transfection, KDEL and no-motif controls were performed in parallel. Cells showing abnormal cell morphology or undergoing nuclear division were not included in the analysis. [A–F] The black bar represents the percentage of cells with ER-only localization, the gray bar the cells with mixed ER–Golgi localization, and the white bar the cells with Golgi-only localization. A, XDEL motifs; B, KXEL motifs; C, KDXL motifs; D, KDEX motifs; E, all other motifs found on human proteins ending L; F, other motifs found on human proteins ending F or M; G, linear correlation ($R^2 = 0.880$) between HeLa and Cos results for 27 of the KDEL variants (note the marked YCEL and EDEL outliers).

**Systematic examination of retention variants**

To systematically examine the role of each position in the KDEL motif in ER localization, all 77 variants of AXDEL, AKXEL, AKDXL, and AKDEx (where X is any amino acid) were made on the reporter construct.
After optimization of the transfection and expression conditions to get reproducible and reasonably uniform transfection with low but detectable expression levels, immunofluorescence analysis revealed that for all constructs examined, there was variability in the subcellular localization between different cells after transient transfection in all cell types tested, even in those tested at low expression levels. For all transient transfections, >80% of HeLa cells showed ER localization of the AKDEL-containing construct, with <1% of cells showing Golgi localization for any single transfection, and the remainder showed mixed ER–Golgi localization, as determined by costaining with anti-CRT and anti-giantin antibodies. The mixed ER–Golgi localization possibly results from saturation of the KDEL receptor system. In contrast, for all transfections >80% of HeLa cells transfected with the construct with no C-terminal retention motif showed Golgi localization, whereas the rest showed mixed ER–Golgi localization. No cells showed only ER localization of the construct with no retention motif.

So that a direct comparison of the ER localization efficiency of each KDEL motif variant could be undertaken, the subcellular localization of each variant was examined from at least 150 cells in each of at least two independent experiments, with AKDEL-containing and no-motif controls performed in parallel for each set of constructs tested. In each case, abnormal-looking cells or those undergoing nuclear division were discounted from the analysis. The analysis of this set of 77 variants plus the no-motif control (Fig. 3, A–D) reveals that there is a graduation in the efficiency of ER localization, ranging from motifs that were the equivalent of AKDEL with >80% of HeLa cells showing ER localization, to motifs that were the equivalent of the no-motif construct and >80% of HeLa cells showing Golgi localization. Between these two extremes there was a continuum of constructs that showed <80% ER localization and <80% Golgi localization, implying that these motifs act as inefficient ER-localization motifs.

The results of these 77 variants also reveal that many non-Prosite motifs are able to result in the efficient ER localization of the reporter construct. In particular, for ER localization by an AKDEL variant, the −4 position (i.e., the K position) is not limited to the six possible amino acids suggested by the Prosite motif (i.e., KRHSQ) but rather the aromatic amino acids F, W, and Y are equally as effective as K, R, H, or N and more effective than the Prosite motif options S or Q. The −3 position (i.e., the D position) extends far beyond the Prosite motif of E, D, Q, or N and includes all possible amino acids, but not the cyclic imino acid proline. The −2 position strongly favors E, but D also results in >20% of cells showing ER localization of the AKDDL construct. The −1 position, i.e., the L position, equally favors F and the Prosite motif L and to a lesser extent M, with I also resulting in >20% of cells showing ER localization of the AKDEL construct.

KDEL variants found on soluble human proteins

Because there are 160,000 combinations for the C-terminal four amino acids of a protein, a systematic examination of all possible combinations for ER localization is unfeasible. Instead, a bioinformatics approach was adopted to identify motifs found on soluble human proteins that enter the secretory pathway, i.e., motifs that might function in a natural system to ER localize human proteins. Based on the initial screen of AXDEL, AKXEL, AKDXL, and AKDEX variants, the bioinformatics analysis was restricted to human proteins that contained the motif XX[DE][FLM] at their extreme C terminus. Proteins containing these motifs were identified using Protein Prospector (Chalkley et al., 2005) and their sequences were analyzed by BLAST searches, Signal-P (Bendtsen et al., 2004), and PSORT II (Horton and Nakai, 1997) to confirm that the sequence was the full-length protein, that it contained an N-terminal ER signal sequence, and that it was not predicted to be a transmembrane protein. This analysis identified 113 proteins that had 63 KDEL-like variants at their C termini (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1).

The 37 KDEL-like variants ending in L that came out of the bioinformatics screen and had not previously been analyzed were made in the reporter construct, and the subcellular localization of the transiently transfected constructs was determined by immunofluorescence microscopy. The results revealed that 15 of these motifs resulted in efficient ER localization (>80% of HeLa cells showing ER localization), whereas many others resulted in at least partial ER localization (Fig. 3 E). In addition, the effects of 10 additional variants found on human proteins ending in F or M were analyzed and three of these motifs resulted in efficient ER localization (Fig. 3 F). Because two yeast transmembrane proteins have been reported to be localized to the ER by C-terminal KDEL-like motifs (Hardwick et al., 1992; Sweet and Pelham, 1992), eight KDEL variants, which were found on human transmembrane proteins whose C terminus was predicted to be in the ER lumen and had not been previously tested, were also tested, and of these, two showed efficient ER localization (Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1). 17 other KDEL variants were also tested (Fig. S1 B).

Overall, the analysis of 152 different potential ER localization motifs revealed that 46 were able to efficiently result in the ER localization of the reporter (>80% of cells showing only ER localization of the construct), whereas 60 resulted in at least partial ER localization and 46 were comparable to the construct with no motif added. Of the 46 motifs that result in efficient ER localization, 35 do not match the Prosite motif for ER localization, whereas others that do match the Prosite motif, such as ASNEL, show limited ER localization or, such as ASDEL or AQEEL, show a significantly increased Golgi localization compared with that of AKDEL.

Because Cos7 cells have been widely used to study the human KDEL receptors, 27 of the constructs were expressed in Cos7 cells and their localization was determined by immunofluorescence to look for cell type–specific effects. An excellent correlation was observed between the results obtained in HeLa and in Cos7 cells for 93% of the motifs tested (Fig. 3 G; \( R^2 = 0.880 \)).

To confirm that the motifs resulted in ER localization or secretion (Golgi localization visible in cells en route to being secreted), the degree of secretion of 88 constructs was compared with that of the no-motif control. Each of these constructs was...
results previously obtained but with significant results (Fig. S1 C) showed a good correlation with the immunofluorescence, and transit time through the secretory pathway. To control for the effects of transfection efficiency, expression, and protein levels, it migrates faster in SDS-PAGE and is thus a good internal construct is slightly shorter than the constructs containing motifs. It was also the only motif to show a statistically significant difference, showing a high degree of ER localization by immunofluorescence in HeLa cells (Fig. 3 E) but not in Cos7 cells (Fig. 3 G) and also a secretion efficiency greater than that of the no-motif construct by Western blot for both cell lines (Fig. 3 G) and also a secretion efficiency greater than that of the no-motif construct by Western blot for both cell lines (Fig. 4 A). However, because soluble proteins may be expected to be ER localized, whereas we have shown (Fig. 2) that the reporter construct does use this motif to be ER localized.

To further examine the potential role of the C-terminal KDEL-like motif of each protein, a stop codon was inserted at the end of the tag before the putative ER-retention motif and the transiently transfected construct was localized by immunofluorescence. Four of the proteins with motifs that gave inconsistent results remained in the ER even after the motif was deleted. This indicates that the motif is not required for the ER localization of these proteins. Further studies on the fifth motif EDEL revealed that for some motifs, positions −5 and −6, as well as the C-terminal four amino acids, play an important role in the efficiency of ER localization (unpublished data). Three of the 16 constructs, ERp18, Hag3, and GP7R, changed their localization from the ER to the Golgi when the putative ER-retention motif was not present (Fig. 4 A). Furthermore, others, such as ERp27, showed an increased tendency to show ER–Golgi mixed localization when their KDEL-like motif was not present on the C terminus of the protein.

These results suggest that many proteins with KDEL variants at their C termini may not require them to be ER localized. Because this dataset was based on proteins with motifs that do not match the Prosite motif, we decided to also analyze a small set of proteins with KDEL motifs that matched the Prosite motif. To this end, two previously uncharacterized human proteins, along with the testes-specific CRT3 and the peptidyl-prolyl isomerases FKBP7 and FKBP14, were cloned with a tag located 5 aa before the C terminus and with a stop codon inserted at the end of the tag. When transiently transfected in HeLa or Cos7 cells and cotransfected with the no-motif construct and 48 h after transfection, the degree of secretion was examined by Western blotting samples taken from cell-free media. Because the no-motif construct is slightly shorter than the constructs containing motifs, it migrates faster in SDS-PAGE and is thus a good internal control for the effects of transfection efficiency, expression, folding, and transit time through the secretory pathway. The results (Fig. S1 C) showed a good correlation with the immunofluorescence results previously obtained but with significant quantitative variability between different experiments. The motif AYCEL was unusual in that it showed a reproducibly significant difference, showing a high degree of ER localization by immunofluorescence in HeLa cells (Fig. 3 E) but not in Cos7 cells (Fig. 3 G) and also a secretion efficiency greater than that of the no-motif construct by Western blot for both cell lines (Fig. S1 C). It was also the only motif to show a statistically significantly lower signal for interaction than the no-motif–containing construct with all three human KDEL receptors (for assay details, see Specificities of the human...). These results hint at a secretion route for the YCEL-containing construct that does not go via the Golgi or transits the Golgi much faster. It may be significant that the three human proteins that have this motif are all associated with high density lipoprotein.

ER localization of human proteins

Many of the KDEL variants tested are found on human proteins that are annotated as being secreted or post-ER, either by electronic annotation or by using constructs in which a tag has been fused to the C terminus of the protein. To examine if any of these human proteins are ER located, 13 such proteins with non-Prosite motifs were cloned. All of these proteins, along with the previously cloned ERp57, ERp18, and ERp27 (Alonen et al., 2003a,b, 2006), which also have non-Prosite motifs, were cloned with a tag located 5 aa before the C terminus, i.e., before the putative KDEL variant ER-retention motif. Transient transfection of Cos7 or HeLa cells followed by immunofluorescence revealed that all of these proteins were located in the ER (Fig. 4 and Table I). Of the 14 KDEL variants tested by these 16 human proteins, 9 resulted in predominantly ER localization, 3 in predominantly Golgi localization (EDEL, PEGL, and QEDL), and 2 in predominantly mixed ER–Golgi localization (NEDL and PDEL) of the reporter construct. Hence, five motifs apparently did not give consistent results between the natural protein and the reporter construct localization. However, because soluble proteins may be expected to be ER localized in the ER either by having a C-terminal KDEL-like motif or by interacting with a protein with such a motif, it is possible that the protein with this five motifs may not use the motif to be ER localized, whereas we have shown (Fig. 2) that the reporter construct does use this motif to be ER localized.

Figure 4. Localization of tagged human proteins with and without their C-terminal retention motifs. HeLa cells were transiently transfected with the plasmid for ERp18–GFP–LEDEL (A), ERp18–GFP–stop (B), GP7R–HA–KKEDL (C), GP7R–HA–stop (D), CRT3–HA–RRNEL (E), or CRT3–HA–stop (F), fixed, and stained with antibodies against CRT (A or giantin (B) or double-stained with the antibodies against HA and CRT (C), HA and giantin (D), or HA and PDI (E and F). Merged images show good colocalization of the examined proteins with ER markers (A, C, E, and F) and Golgi markers (B and D). The examples shown are of ERp18 and GP7R changing localization and CRT3 staying in the ER after removing their putative ER-retention motif. Bars, 10 μm.
visualized by immunofluorescence, all five proteins were located in the ER and all remained localized to the ER upon deletion of the KDEL-like motif (Table I), presumably because of their interaction with other proteins with KDEL-like motifs.

**Specifities of the human KDEL receptors**

With three different human KDEL receptors present in the same cell and showing widespread tissue distribution, the possibility arises that each receptor acts to retrieve different subgroups of proteins based on the KDEL variant they have at their C terminus.

To test this hypothesis, a BiFC assay was established. Similar constructs to those used to study the interaction of ERp57 and ERp27 (Alanen et al., 2006) were made with parts of YFP fused to human ERD21, 22, and 23 and variants of the reporter with either AKDEL or no retention motif at the C terminus (Fig. 5 A).

Upon expression in HeLa cells, negligible fluorescence was observed by flow cytometry with the ER-targeted YFP fragments Y1 alone or Y2 alone. Expression of any combination of Y1- and Y2-tagged constructs in the same subcellular compartment results in an increase in fluorescence because of nonspecific interactions. Hence, coexpression of the Y1-tagged KDEL receptors with the Y2-tagged variant of the reporter, which contained no retention motif, or the coexpression of Y1 and Y2 resulted in a small increase in fluorescence. However, a significantly greater fluorescence was observed when any of the Y1 fragment–tagged KDEL receptors were coexpressed with the Y2 fragment–tagged AKDEL variant of the reporter construct (Fig. 5 B). This implies that there are specific interactions between the three KDEL receptors and the KDEL motif that can be detected by an increase in the BiFC fluorescence signal intensity. The signal obtained with ERD21 was significantly greater than that obtained with ERD23, which in turn was significantly greater than that obtained by ERD22, suggesting that ERD22 and 23 may favor interaction with motif variants other than KDEL.

Because statistically significantly (P < 0.01) larger signals were observed by BiFC in the BiFC fluorescence intensities for all three human KDEL receptors interacting with the AKDEL-containing reporter compared with the no-motif–containing reporter, 84 other KDEL-like variants were cloned into the BiFC system and their interactions with the three human KDEL receptors analyzed. The results showed a continuum of intensity signals ranging from signals comparable to that obtained with the no-motif construct up to, and beyond, the signal obtained with the KDEL motif. This continuum is consistent with the results obtained by immunofluorescence analysis of ER localization efficiency, which showed that as well as efficient motifs and motifs that had no effect on ER localization, there were also a wide range of weak localization motifs.

Overall, there was a very good correlation between the magnitude of the BiFC fluorescence signal obtained and degree of ER localization previously determined by immunofluorescence (Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1), especially for ERD21 and 23 (Fig. 5 C). This is consistent with the direct role of these receptors in mediating ER localization. However, there were clear differences between the three KDEL receptors. 43 of the AXXXXKDEL-like motifs tested had previously been shown to be efficient signals for ER localization (>80% of cells showing only ER localization; Fig. 3). All of these motifs showed a statistically significantly higher signal (P < 0.01) than the no-motif construct for interaction with ERD21, whereas only 41 of the 43 motifs showed the same correlation for ERD23. In contrast, 18 of the 43 motifs did not show a statistically significantly higher (P < 0.01) BiFC signal than the no-motif construct for ERD22. Indeed many motifs, including KAEL, NDEL, and REDL, which are found on soluble proteins, showed no significant BiFC signal.
human proteins that enter the secretory pathway, showed a signal that was statistically indistinguishable from that of the no-motif construct. Hence, ERD22 appears to be a specialist.

More detailed examination of the BiFC results from the 85 KDEL variant constructs showed very clear differences in the specificities of the three human KDEL receptors. ERD21 appears to be a generalist (Fig. 5 D for motifs found on human proteins and Table S2), with AHAEL, AHDEF, AHEEF, AHEEL, AHDEL, ARDEL, and AREEL showing no significant difference in signal compared with AKDEL (at 5% significance level) and 63 out of 85 motifs showing a significantly larger signal ($P < 0.05$) than the no-retention–motif construct, including all KXEL variants.

In contrast to ERD21, ERD22 does not favor KDEL or closely related motifs and it appears to be more specialized (Fig. 5 E for motifs found on human proteins and Table S2). Although ERD22 gives only a weak signal when coexpressed with the AKDEL-retention motif, it gives significantly stronger signals with motifs of the sequence HXEL.

ERD23, like ERD21, appears to be a generalist (Fig. 5 F for motifs found on human proteins and Table S2), giving a statistically significant signal ($P < 0.01$) with 41 motifs versus 43 motifs for ERD21. Closer examination of the data, however, reveals several subtle differences in the specificities of the two receptors. Furthermore, there is one very clear difference. Although ERD21 favors KDEL over HDEL ($P < 0.01$), ERD23 favors
HDEL very markedly over KDEL (P < 0.01). Because KDEL and HDEL are the most common ER-retrieval motifs found on human proteins (Table S1), this marked difference is probably physiologically significant.

In addition to allowing quantification of the relative efficiency of interaction, BiFC also allows the steady-state localization of the complex. For all combinations tested of the three receptors with motifs that gave a strong BiFC signal, the fluorescence observed by fluorescence microscopy showed a punctate distribution consistent with potential vesicular localization (Fig. 6 A). Costaining with an antibody against β-COP revealed considerable overlap between the BiFC signal and COP-I localization. This result is consistent with a functional interaction between the receptor and the motif resulting in recycling of the complex back to the ER. However, because of BiFC complex formation, the complex of the receptor and KDEL motif reporter is unable to dissociate in the ER, which leads to constant recycling. In contrast to this result, BiFC localization of all three receptors with the no-motif reporter showed a weaker, more diffuse fluorescence signal, which colocalized with the signal from an anti-giantin antibody (Fig. 6 B). This result is consistent with a nonfunctional nonspecific association between the receptor and the reporter construct.

**Discussion**

Compartmentalization is essential for cellular function. It is especially relevant for soluble proteins with an N-terminal signal sequence that targets them to the ER. Such proteins are secreted when correctly folded unless there is active partitioning to the correct subcellular compartment. This partitioning requires a motif to signal to the appropriate machinery that this protein should be located in the ER, Golgi, lysosome, etc. The initial work on ER localization for soluble proteins in *Saccharomyces cerevisiae* (Pelham et al., 1988; Lewis et al., 1990; Semenza et al., 1990) identified a C-terminal motif, HDEL, which interacted with a Golgi-located HDEL receptor, an interaction that resulted in the retrieval of the complex to the ER. Subsequently, the first human KDEL receptor was identified (Lewis and Pelham, 1990) followed by the second (Lewis and Pelham, 1992a). Although some work was reported toward the specificity of the KDEL receptor (Semenza and Pelham, 1992), there is a noticeable absence of discussion as to why mammals require two receptors to perform an identical function. This question became more pertinent with the appearance in the databases of a third human KDEL receptor. To our knowledge, this is the first paper regarding the localization or functional characterization of this receptor. In addition, here, we demonstrate that the Prosite motif for ER localization needs revision. Of the 152 different potential retention motifs tested, 46 resulted in efficient ER retention with 35 of these not matching the Prosite motif. Furthermore, several motifs that did match the Prosite motif did not result in efficient ER localization. The motifs that result in efficient ER localization are found on 70 human proteins, many of which are of currently unknown or poorly defined function.

With three distinct KDEL receptors in mammals showing ∼20% difference in amino acid sequence, there may be some difference in function. Because all three receptors are widely expressed in a range of human tissues and are expressed at the same time in a human cell line, the most likely functional difference would be differences in substrate specificity. Here, we show for the first time the specificity of a KDEL receptor. Furthermore, we show that the three human KDEL receptors ERD21, 22, and 23 have different specificities, with ERD21 and 23 being generalists and ERD22 being a specialist.

There are three generally recognized methods for ER localization of folded soluble proteins: via the KDEL receptors, by noncovalent interactions with proteins that are retained by the KDEL receptors, e.g., the P4H α-subunit is retained by PDI (Vuori et al., 1992), or by thiol-mediated retention, e.g., ERo1α by ERp44 (Anelli et al., 2002). The excellent correlation between immunofluorescence-based localization and the interactions of the constructs with the human KDEL receptors (Fig. 5 C and Table S2) implies that, for the 152 variants of the reporter tested, ER localization is mediated only via the KDEL receptors. There is no evidence from our data for a general thiol-based retention
mechanism for folded proteins because constructs with the motifs ACSDL, ACTDL, AKDCL, and AKDEC are secreted, whereas CDEL and KCEL, which are retained, interact with the KDEL receptors (Fig. 3 and Table S2). In contrast, there is indirect evidence for large-scale retention via noncovalent complex formation. Some complexes have previously been reported (Meunier et al., 2002). Here, we report that deleting the KDEL-like motif from 21 ER-localized human proteins resulted in the secretion of only three of them (Table I). Although some of the others are known to form complexes, e.g., ERp57 with CRT, calnexin, or ERP27 (Oliver et al., 1997; Zapun et al., 1998; Alalen et al., 2006), the rest are generally not well characterized, but complex formation can be hypothesized. In a separate incomplete large-scale screen for interactions in the ER, all 18 proteins that do not change localization upon removal of the C-terminal motif were found to interact with at least one other ER-resident protein with a KDEL-like motif (unpublished data).

In addition to soluble proteins, it has been previously reported that two yeast transmembrane proteins are retained in the ER by a luminal C-terminal HDEL motif (Hardwick et al., 1992; Sweet and Pelham, 1992). Here, we find that four human transmembrane proteins have C-terminal KDEL variants (Fig. S1 A and Table S3, available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1) that act as efficient ER localization motifs and that three of these four motifs are conserved between human and mouse, which implies functionality. Because transmembrane proteins were not the focus of our bioinformatics search, it is possible that there are many more yet to be identified. This question needs addressing: why use these motifs rather than the widely used cytoplasmic C-terminal KKXX or RXR motifs?

What is the functional significance of the three KDEL receptors having different specificities? This question is not easy to answer because the three receptors have overlapping specificities and because RNAi studies are not viable without receptor-specific antibodies. An analysis based on the function of the proteins with motifs that are recognized by the different receptors is also difficult because for many of these proteins either the function is poorly understood or they belong to a family of proteins with a known function but where the exact physiological role of each of the multiple family members in the ER is unclear. However, some generalizations can be made. All of the carboxylesterases with KDEL-like motifs have a motif that is recognized by ERD22 better than KDEL is. Other proteins with motifs recognized by ERD22 include proteins, such as sulfamidase, which are thought to be located in other subcellular compartments (for review see Anson and Bielicki, 1999). ERD21, in contrast, recognizes motifs that are found on all of the molecular chaperones and protein-folding catalysts that have been reported to interact early with nascent protein chains entering the ER. These results suggest that the exact KDEL-like motif a protein has may be of functional importance, i.e., that KDEL, a strong ERD21 interaction motif, is not the same as HVEL, a strong ERD22 motif, or HDEL, a strong ERD23 motif.

Because the KDEL receptors are very similar between human and mouse, showing 99.5, 98.6, and 92.5% identity for ERD21, 22, and 23, respectively, further evidence for the potential importance of the specificity of the KDEL-like motif used by a single protein can be found from a cross-comparison between human and mouse proteins. BLAST searches using the sequences of the 116 human proteins previously identified to be soluble proteins that entered the secretory pathway (Table S1) allowed for the identification (including the C-terminal motif) of 111 homologous mouse proteins. For this dataset, the mean percentage identity between the human and mouse proteins is 86.0%, which implies that for any four-amino stretch, the expected chance that all four amino acids are identical would be 0.86^4, or ~55%. For those regions that are functional, e.g., active sites, this probability would be expected to be higher, whereas for nonfunctional regions this probability would be expected to be lower. For the 42 proteins with motifs that did not act as an efficient ER-localization motif in the immunofluorescence-based reporter assay system and for which a mouse homologue was identified, 21 proteins (50% of the total) showed conservation of the KDEL-like motif between mouse and human proteins. This result, 50 versus 55% random chance, suggests that the KDEL-like motifs at the C terminus of these proteins were nonfunctional, which is in line with the results from the reporter assay system. In contrast to the 69 proteins with KDEL-like motifs that were efficient in ER localization of the reporter and for which a mouse homologue was identified, 58 (84% of the total) showed conservation of the KDEL-like motif between the human and mouse proteins, whereas a further 5 (7%) had a very similar motif that would be recognized by the same KDEL receptor. Of the remaining six proteins, two, ERP27 and CALR3, formed part of the protein screen for ER localization reported here, and although both were ER located, when the KDEL-like motif was deleted they remained in the ER, indicating that the KDEL-like motif was not the primary affector of ER localization for these proteins. These results, 84 ± 7% versus 55% random chance, strongly suggest that the KDEL-like motif at the C terminus of this grouping of proteins is functional and that it is important which KDEL-like motif a protein has or at least that it is a motif that is recognized by the same receptor. A similar analysis across different species grows increasingly complicated, not only because of the positive identification of the KDEL motif–containing proteins, especially because many ER proteins exist in families, e.g., there are at least 17 human PDI family members (Ellgaard and Ruddock, 2005), but also because of the growing dissimilarity of the KDEL receptors between species. However, as a generalization, KDEL-like motifs are conserved between human and the African clawed frog (Xenopus laevis; 24/29 or 83% of proteins would use the same human receptor most efficiently), which also has three KDEL receptors, whereas yeast (S. cerevisiae), which has only one KDEL receptor, exclusively uses HDEL, and fruitflies (Drosophila melanogaster), which also have only one KDEL receptor, show a greatly increased propensity to use motifs commencing with histidine.

Although there are still many unanswered questions, such as what determines the specificities of the three receptors and their interactions with vesicular transport proteins, this study, by determining that the three human KDEL receptors have different specificities, opens up new possibilities for the subcompartmentalization of the secretory pathway.
Materials and methods

Plasmid construction

Plasmids encoding for ERp18 (including GFP tagged), ERp27 (including myc tagged), ERp57, and PDI were generated previously (Alonen et al., 2003b, 2006). The following plasmids encoding for full-length human proteins were generated by cloning PCR products from IMAGE clones into pET23 (EMD): ERD21 (5214794), ERD22 (5183647), ERD23 (3462392), FBKP7 (3187713), FBKP14 (4042173), CS021 (4398813), MSR38 (6025918), NENF (1895972), LOC493689 (7472098), FOXRED2 (3873448), AGR2 (3852448), PCYOX1 (5207140), KTEL1C (4796951), PATE (3574008), TNRC5 (2959532), SUMF2 (6599083), AG6R (4694757), GX7 (3628580), CRT3 (4822108), ARMET (3354774), and FKBP2 (3049222). These plasmids were then used as templates to generate tagged proteins by PCR in mammalian expression vectors (for a list of constructs see Table S4, available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1).

The control plasmid for real-time PCR analysis of ERD22 mRNA levels, whose sequence, including part of the nondocking region of the mRNA, was cloned using the primers 22 forward and 22 reverse (see below) in pCNA3.1 using total RNA from HeLa cells as the template. The control plasmid for real-time PCR analysis of ERD23 isoform 2 was cloned using annealed synthetic complementary primers into pJK18.

The reporter construct (see Fig. 2 A for a schematic) was constructed with and without a C-terminal AKDEL motif by cloning the b domain of PDI by PCR in frame into a pET23 vector, to which the signal sequence of CRT and a HA tag had already been added using annealed synthetic complementary primers.

BIFC vectors targeted to the ER were made previously (Alonen et al., 2006) according to the design of Nyfeler et al. (2005), with the Q69M mutation introduced into the Y1 fragment to reduce environmental sensitivity of fluorescence (Griesbeck et al., 2001). Constructs expressing ERD21, 22, and 23 and 86 KDEL variants of the reporter construct were subcloned into these vectors (see Fig. 5 A for schematic).

Point mutations in plasmids were performed as recommended by the manufacturer using the QuikChange kit (Stratagene). All 486 generated plasmids were sequenced to ensure that there were no errors in the cloned genes.

Cell transfections

HeLa (American Type Culture Collection CCL2) or Cos7 cells (American Type Culture Collection) were grown on 6-well plates with or without glass coverslips in DMEM-high glucose medium supplemented with Glutamax (Invitrogen), 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (100× penicillin-streptomycin solution; Sigma-Aldrich). Cells seeded one day earlier were transfected according to the manufacturer’s protocol with 3 μl of Fugene6 transfection reagent (Roche) and 0.1–1 g of plasmid DNA. After 10 min of optimization, 0.3 μg of reporter construct plasmid of 0.5 μg of human KDEL receptor was used for all transfections reported. For each transfection, KDEL and no-motif controls were performed. Cells showing abnormal cell morphology or undergoing nuclear division were not included in the analysis. The cotransfection efficiency was 40–55% and control experiments with ERD21/22/23/Myc and the HA-tagged reporter construct with AKDEL showed that only ~1% of cells showed a single with either only Myc or only HA.

Immunofluorescence

After 24 to 48 h, the transfected cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min at RT, and processed for indirect immunofluorescence as described previously (Alonen et al., 2006) using Immuno-Mount medium (Thermo Fisher Scientific). The following antibodies were used: monoclonal antibody against PDI (Dako), polyclonal antibody against CRT (Affinity BioReagents), polyclonal antibody against ε1-G (Abcam), polyclonal antibody against β-COP (Affinity BioReagents), C-Myc monoclonal (Sigma-Cruz Biotechnology, Inc.) and polyclonal (Abcam) antibodies, and HA monoclonal (Sigma-Aldrich) and polyclonal (Abcam) antibodies. The Alexa Fluor 488 (ab1/2 fragment of goat anti-mouse IgG (Invitrogen) and Alexa Fluor 594 (ab2/2 fragment of goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Fixed and stained cells were examined with an epifluorescence microscope (BX61; Olympus) fitted with oil immersion objectives (100×/1.35 NA). Images were recorded with a charge-coupled device (CCD) camera (Soft Imaging System) controlled by Analysis software (Soft Imaging System), converted to TIFF files, adjusted for brightness and contrast, and assembled into montages with Photoshop 8.0 (Adobe).

BIFC measurements

For flow cytometric analysis, HeLa cells were grown on 24-well plates. After extensive optimization, 0.2 μg/well of each plasmid and 1.5 μl of Fugene6 transfection reagent were used. Furthermore, because the Y1-KDEL receptor–Y2-reporter construct gave larger and more reproducible fluorescence signals than the Y2-KDEL receptor–Y1-reporter construct combination, it was used for all of the transfections reported here. After 24 h, the cells were rinsed with PBS and then detached from the plate by incubating them with 500 μl Trypsin-EDTA solution (0.5 mg/ml Trypsin and 0.02% EDTA in PBS) for 5 min at 37°C. The cells were then collected by centrifugation at 400 g for 5 min at 4°C and resuspended in 1 ml PBS containing 2% FCS. For each sample, the yellow fluorescence of 3,000 cells was then analyzed using a CyFlow flow cytometer (Partec) with appropriate filter sets. Gating to analyze the mean fluorescence intensity of transfected cells was performed using FlowMax software (Partec). On all days on which samples were analyzed, controls with the appropriate Y1-KDEL receptor–Y2-reporter construct with AKDEL and the Y1–Y2 control were performed at least in duplicate along with a nontransfected control. Transformation with the control Y1–ERD1 or Y2 reporter constructs resulted in transfected cells whose fluorescence intensity overlapped with that of the nontransfected cell population, and therefore the mean fluorescence intensity of these controls could not be determined accurately, although it was <1% of that of the Y1–ERD21 + Y2 reporter construct with AKDEL sample.

Real-time quantitative PCR

Real-time quantitative PCR for cell transfections was performed with the AccessQuick RT-PCR system (Promega) according to the manufacturer’s protocol, using total RNA isolated from nontransfected HeLa cells using the E.Z.N.A Total RNA kit (Omega Bio-tek) according to the manufacturer’s protocol. The following primers were used for RT-PCR to show the presence of mRNA in nontransfected cells: ERD21, forward, CACAGCATTCTGGCCGCTTCG, and reverse, CGTCGCGTAAACGCGCTAGCC; ERD22, forward, GGCGCTTTGAGCCTAATG, and reverse, TGCGTCCGTGTTGAAGGA; ERD23, forward, GCTTCTCCTCTGGCCGTATGT, and reverse, AGCGCAGTAGGTCGCGGTTAC; and ERD23.2, forward, GCTTCTCCTCTGGCCGTATGT, and reverse, CCTTAGGAGTCGAACGATCGTCACTCC.

Online supplemental material

Table S1 is a list of human gene products, and their Swiss-Prot identification numbers, that are predicted to enter the secretory pathway and have a predicted transmembrane region and which have a motif [DE][FLM] at the C terminus. Table S2 represents correlations between immunofluorescence localization of the reporter constructs and the BIFC fluorescence signal for interactions with the three human KDEL receptors. Table S3 is a list of human gene products, and their Swiss-Prot identification numbers, that are predicted to enter the secretory pathway and have a predicted transmembrane region and a motif [XDE][FLM] at the C terminus. Table S4 is a list of constructs used. Fig. S1 shows immunofluorescence and Western blot-based localization of the reporter constructs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200705180/DC1.

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