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Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants

(retention motif/cytoplasmic domain/C-terminal sequence)

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ABSTRACT **A mutant of CD4 (CD4.Q421stop), in which the cytoplasmic C-terminal 13 amino acids were truncated, was not expressed on the surface of HeLa cells after transfection but was retained in the endoplasmic reticulum (ER). Seven other truncation mutants of CD4 were expressed well on the cell surface, thus suggesting that the C-terminal amino acids of CD4.Q421stop (-Ser-Glu-Lys-Lys-Thr-Cys) may have the sequence information for ER retention. Further mutational study has revealed that two consecutive lysine residues at the third and fourth positions from the C-terminal end are sufficient for ER retention. Lysine at the fourth position, but not at the third position, from the C terminus can be replaced by arginine without disturbing ER retention. Furthermore, two lysine residues at the third and fifth positions from the C terminus also resulted in ER retention. Thus lysine at the third position and a positively charged amino acid either at the fourth or fifth position from the C terminus are sufficient for ER retention of this CD4 mutant, and possibly all transmembrane proteins. In addition to the requirement of specific amino acids at specific positions, the ER retention signal -Lys-Lys-Xaa-Xaa also requires a transmembrane region for function. By contrast -Lys-Asp-Glu-Leu, which targets soluble proteins to the lumen of the ER, does not function in the presence of a transmembrane region.**

Secreted soluble proteins and some suborganelle-specific proteins (e.g., lysosomal and plasma membrane proteins) are synthesized and transported to their destinations through the endoplasmic reticulum (ER) and Golgi apparatus (1). Transport of proteins from the ER to the Golgi apparatus has been hypothesized to follow the bulk flow of materials, whereas ER-resident proteins must have a signal to escape from this pathway. Endoplasmic luminal proteins, such as heavy chain binding protein (BiP) and disulfide isomerase, indeed share the same amino acid sequence -Lys-Asp-Glu-Leu (-KDEL) at their C-terminal ends that is responsible for their ER retention (2). It is not clear how this sequence provides escape from the bulk flow transport from ER to Golgi, but binding to a hypothetical ER-retention receptor or selective retrieval transport from a pre-Golgi compartment (salvage compartment) back to the ER (3, 4) are possibilities. Recently, proteins of the salvage compartment have been described that bind to those soluble proteins containing the -KDEL sequence (5, 19), thus supporting a mechanism of selective retrieval transport.

ER-resident transmembrane proteins that do not have the -KDEL motif for luminal retention may have a different C-terminal sequence for ER retention. An adenoviral early gene product (E3/19-kDa protein) and UDPglucuronosyltransferase (UDP-GT) have both been shown to be retained in the ER through their C-terminal amino acid sequences (6). However, these two proteins in addition to other ER-resident proteins [e.g., hydroxymethylglutaryl CoA reductase (7)] do not share an identical sequence at their C-terminal ends except for an apparent similarity in charge distribution. The required sequence for their ER retention, therefore, appears to be less exact than that for luminal proteins.

During a study of the endocytosis of CD4 molecules, a series of mutations in the cytoplasmic domain of CD4 were made. CD4, a cell surface marker of helper T cells, is a glycoprotein composed of four extracellular domains, a single transmembrane region, and a 40-amino acid long cytoplasmic region (8). Wild-type CD4 (CD4.wt) and more than 30 cytoplasmic region mutant CD4 molecules were all well expressed on the surface of transfected HeLa cells (ref. 9 and unpublished results). The single exception was a mutant CD4 in which the cytoplasmic domain was truncated from Gln-421 to the C-terminal end. This mutant, CD4.Q421stop, was completely localized in the ER. Additional mutational analysis of the C-terminal region of the CD4.Q421stop molecule presented in this report reveals common features required for ER retention of transmembrane proteins. During preparation of this manuscript, two related, although not identical, reports (10, 20) have appeared.

METHODS AND MATERIALS

Site-Directed Mutagenesis, DNA Construction, and Expression. Site-directed mutagenesis was performed in the single-stranded bacteriophage M13 containing several uracil residues in place of thymidine (9, 11). To construct the CD4 cDNA containing the codons for -KDELstop or -KKTSstop (where -KKTS is -Lys-Lys-Thr-Ser) at various positions as the C-terminal sequences, six pairs of coding and complementary oligonucleotides (21-mer to 35-mer) were synthesized, phosphorylated, annealed, and inserted in the *Ava* I site [1200 base pairs (bp) from 5′ end for CD4.351KDEL or -KKTS], *Bsp*HI site (1240 bp from 5′ end for CD4.365KDEL or -KKTS), or *Nar* I site (1354 bp from 5′ end for CD4.403KDEL or -KKTS). The *Bsp*HI site at bp 1240 of the CD4 cDNA insert was created by mutating ACA-TGG (Ser-364 and Trp-365) to TCA-TGA (Thr-364 and stop), which was used as a truncation mutant sCD4.W365stop. Mutant CD4 cDNA inserts were transferred to the *Hin*dIII–*Bam*HI cloning site of the expression vector pcDNA1 (InVitrogen, San Diego) and transfected into HeLa cells using calcium phosphate (12). Expression was monitored by immunofluorescence microscopy 48–72 hr after transfection; >50% of cells were positive in most cases.

Immunofluorescence Microscopy, Immunoprecipitation, and Endoglycosidase (endo H) Digestion. Experiments were done 48–72 hr after transfection. Anti-CD4 antibody Leu3a was used for both immunofluorescence and immunoprecip-

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Abbreviations: ER, endoplasmic reticulum; UDP-GT, UDPglucuronosyltransferase; endo H, endoglycosidase H; NBRF, National Biomedical Research Foundation.

itation (ref. 9 and unpublished results). Endo H digestion was performed with the immunoprecipitated samples from cells pulse-labeled with [^{35}S]methionine (100 μCi/ml) in methionine-free Dulbecco's modified Eagle's medium (DMEM) for 15 min followed by a 1-hr chase in complete DMEM.

C-Terminal Sequence Search from the Natlional Biomedical Research Foundation (NBRF) Protein Data Base. All human, mouse, rat, and yeast sequences were retrieved from the NBRF protein data base (release 63.0) by using the Sequence Analysis Software Package (version 6.2) of the University of Wisconsin Genetics Computer Group under Vax VMS, transferred to a Unix machine and then edited to remove all spaces from the sequence. The end of the last line of each file was searched for each of the four motifs: -KKXX, -RKXX, -KXKXX, and -RXKXX (where X is any amino acid), by using the Unix operating system routines GREP and EGREP on a Convex C2 computer. The NBRF data base used contained 12,476 sequences in total, of which 1636 were human, 953 were mouse, 735 were rat, and 447 were yeast sequences. Transmembrane sequences identified from the NBRF header included 81 human and 69 mouse, rat, or yeast protein sequences.

The probability of any of the sequence motifs appearing randomly is determined as follows: The appearance of -KXX randomly would occur 7% of the time, since lysine accounts for 7% of amino acids in unrelated proteins. The occurrence of either arginine or lysine at position 4 or 5 from the end is equal to 1− (the probability of neither occurring at position 4 or 5). This latter is 0.883 × 0.883 = 0.78. Since lysine accounts for 7% and arginine accounts for 4.7% of the amino acids in proteins, the amino acids that are neither occur 88.3% of the time. The probability of having arginine or lysine at position 4 or 5 is then 1 − 0.78 or 0.22. The likelihood of both -KXX and either arginine or lysine at position 4 or 5 is the product of their probabilities [0.07 × 0.22 or 0.015 (1.5%)].

RESULTS

ER Retention of CD4.Q421stop Is Due to Its C-Terminal Amino Acid Sequence. A series of cytoplasmic domain truncation mutants of CD4 were constructed by changing the codons for Phe-426, Gln-421, Arg-412, or Gln-403 to stop codons utilizing CD4 cDNA inserted in M13 plasmids (Fig. 1*A*, CD4.F426stop, CD4.Q421stop, CD4.R412stop, or CD4.Q403stop, respectively). Expression of these CD4 variants was measured by cell surface staining, permeabilized cell staining, or immunoprecipitation (Fig. 1 *B* and *C*). Wild-type and all mutant CD4s except for CD4.Q421stop were stained well on the cell surface and in punctate organelles inside the cells, representing transport vesicles. Cells transfected with CD4.Q421stop, however, were not stained on the cell surface at all, but rather on a tubular network throughout the cytoplasm, a typical pattern of ER staining. The staining pattern of this mutant was clearly demonstrated by using confocal fluorescence microscopy; the entire network was connected as one tubule originating from the nuclear membrane, confirming the localization to the ER (data not shown).

CD4 has two N-glycosylation sites. N-linked glycosylation initially occurs in the lumen of ER as the high-mannosyl oligosaccharide, and modification to the complex form occurs in the cis-Golgi compartment. Endo H digestion, which only cleaves high-mannose oligosaccharides, therefore, was used to discriminate ER-retained molecules from those processed through the Golgi apparatus (Fig. 1*C*). CD4.Q421stop (53-kDa band) digested with endo H produced a single 46-kDa band that represents the backbone polypeptide, thus providing evidence of the ER retention of this molecule. In contrast, after endo H digestion, wild-type and the other truncated

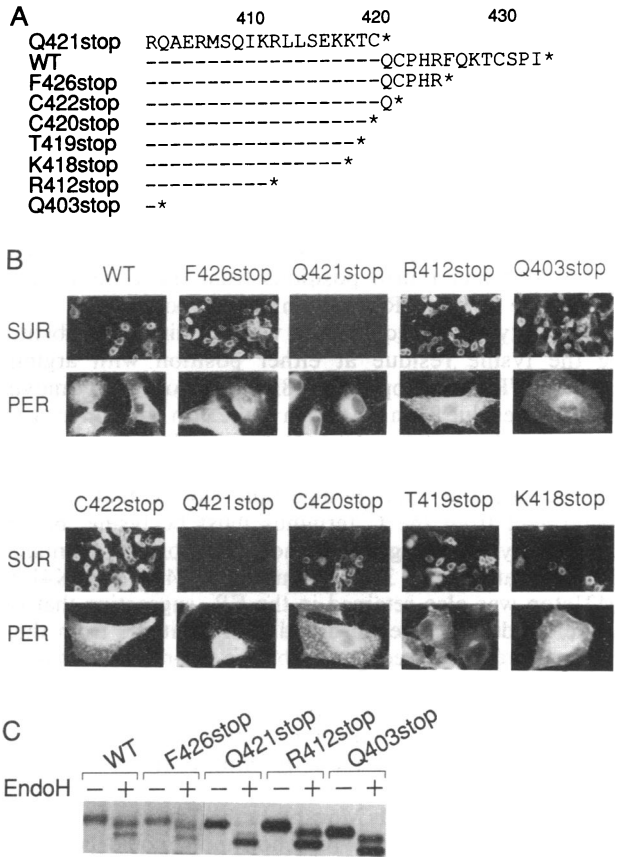

FIG. 1. ER retention of truncation mutants of CD4. Specific localization of CD4 wild type (WT) and the mutants was examined by cell surface staining, permeabilized cell staining, and endo H digestion of immunoprecipitates. (*A*) Amino acid sequences of cytoplasmic region from Arg-402. Identity of amino acids to the reference sequence is indicated by a dash and stop codons are indicated (*). (*B*) Cell surface (rows SUR) and permeabilized cell (rows PER) staining patterns are shown. (×20 and ×110, respectively.) (*C*) Immunoprecipitates were incubated with (+) or without (−) endo H for 12 hr and analyzed by SDS/PAGE using 10% gels.

CD4 molecules produced two protein bands smaller in size by 2 kDa and 7 kDa. A 2-kDa decrease in size after endo H (or endo F, data not shown) digestion of the transported CD4 molecules suggests that one of the glycosylation sites in these mature molecules is biantennary and not converted to the complex type in the Golgi compartment, whereas the other site is endo H-resistant. The molecules decreased by 7 kDa in each would have no complex oligosaccharide and, presumably, would represent CD4 molecules still resident in the ER. The ratio of the endo H-resistant form to the sensitive form indicates the relative rates of processing. CD4.wt was processed most efficiently (70% in 1 hr), and the mutant molecules were processed at slower rates in the order CD4.F426stop (50%), CD4.R412stop (30%), CD4.Q403stop (30%), and CD4.Q412stop (<1%).

To define the ER-retention signal precisely, the cytoplasmic tail of CD4 was truncated one amino acid at a time from Cys-422 to Lys-418 (Fig. 1*A*, CD4.C422stop, Q421stop, C420stop, T419stop, and K418stop). All of these additional CD4 mutants except for CD4.Q421stop were well expressed on the cell surface and in transport vesicles inside the cells (Fig. 1*B*). Therefore, ER retention of CD4.Q421stop is precisely due to its C-terminal amino acid sequence, -SEKKTC.

A Lysine Residue at the Third Position and a Lysine or Arginine at Either the Fourth or Fifth Position from the C Terminus Is an ER-Retention Signal. Substitution mutations

of the C-terminal amino acids of CD4.Q421stop were constructed next. Glu-416, Lys-417, Lys-418, Thr-419, and Cys-420 were changed to Gln-416 or Val-416, Gln-417, Gln-418, Ile-419, and Trp-420, respectively (Fig. 2*A*, CD4.E416Q/Q421stop or E416V/Q421stop, K417Q/Q421stop, K418Q/Q421stop, T419I/Q421stop, and C420W/Q421stop). Substitution of either Lys-417 or Lys-418 resulted in cell surface expression, whereas substitution of Glu-416, Thr-417, or Cys-420 did not change ER residency of CD4.Q421stop (Fig. 2 *B* and *C*). Thus, a specific localization of two lysine residues at the third and fourth positions from the C terminus is responsible for the ER retention of this molecule.

Flexibility of the requirement was examined by substituting the lysine residue at either position with arginine (CD4.K417R/Q421stop or K418R/Q421stop) or by moving the lysine residue from the fourth to the fifth position from the C terminus by mutating Glu-416 to Lys-416 and Lys-417 to Gln-417 (CD4.E416K/K417Q/Q421stop) (Fig. 3*A*). Immunofluorescence and endo H digestion showed that the third amino acid from the C terminus must be lysine, but the change of lysine to arginine at the fourth position maintained ER localization (Fig. 3*B*). The mutant CD4.E416K/K417Q/Q421stop was also retained in the ER, suggesting that two lysine residues at the third and fifth positions from the C terminus were also recognized by the ER-retention machinery (Fig. 3). The less-strict requirement at the fourth position as well as the C-terminal amino acid sequence of the retained 53-kDa sarcoplasmic reticulum protein (-RYKKH, refs. 10 and 13) indicate that lysine at the fifth position can also be replaced by arginine.

ER-Retention Motif Is Movable in the Cytoplasmic Domain. From these data the ER-retention motif could be a short C-terminal stretch of amino acids or part of a more complex tertiary structure in a specific region of CD4.Q421stop. The location of this motif was changed by mutating Arg-425 and Gln-427 to Gln-425 and Lys-427, and by either leaving the three C-terminal amino acids (-SPI) or removing them (CD4.R425Q/Q427K and R425Q/Q427K/S431stop). The exposed C-terminal sequences were -QFKKTCSPI or -QFKKTC (Fig. 4*A*). A truncation mutant CD4.S431stop (C-terminal sequence -RFQKTC) was also constructed.

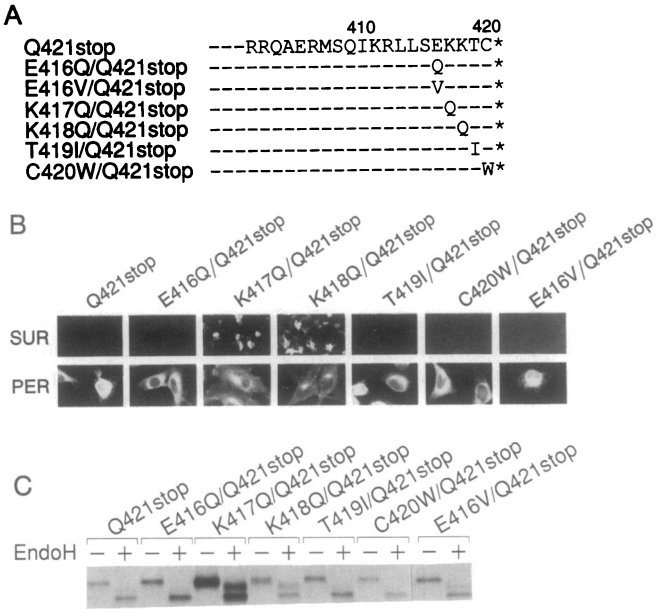

FIG. 2. Effect of single mutations of CD4.Q421stop at five positions (*A*) on ER retention examined by cell surface (SUR) immunofluorescence and permeabilized cell (PER) staining (*B*) and by endo H sensitivity (*C*).

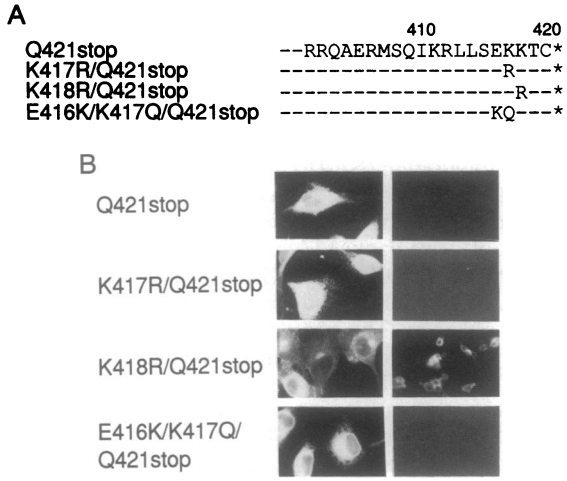

FIG. 3. Alteration of the positively charged amino acids and their position in the ER-retention motif of CD4.Q421stop (*A*). Each mutant was tested for its ER retention by immunofluorescence of the cell surface (on the right) and permeabilized cell (on the left) staining (*B*).

CD4.wt, CD4.S431stop, and CD4.R425Q/Q427K were expressed on the cell surface. However, CD4.R425Q/Q427K/S431stop was completely retained in the ER (Fig. 4*B*). In addition, attachment of -SEKKTS to the C-terminal end of CD4.Q403stop, which then has only a 9-amino acid long basic cytoplasmic region, also resulted in complete ER retention (Fig. 5, CD4.403KKTS). Thus, the ER-retention signal can be in short primary structures and must be at the C terminus but can be preceded by cytoplasmic domains of variable length containing other charged amino acids. The strict requirements of certain specific amino acids and their positions and the flexibility of cytoplasmic tail length and positions of other charged residues suggest that the ER-retention mechanism is mediated by an intermolecular event rather than by simple environmental differences [e.g., local charge

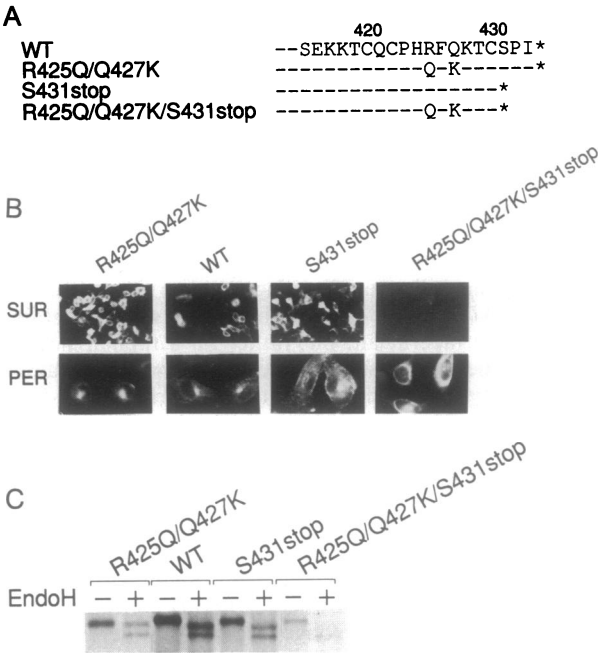

FIG. 4. Relocation of -Lys-Lys-Thr-Cys (-KKTC) to another region of the CD4 cytoplasmic domain (*A*). ER retention was examined by immunofluorescence of the cell surface (SUR) and permeabilized cell (PER) staining (*B*) and by endo H sensitivity (*C*) (see also Fig. 5, CD4.403KKTS).

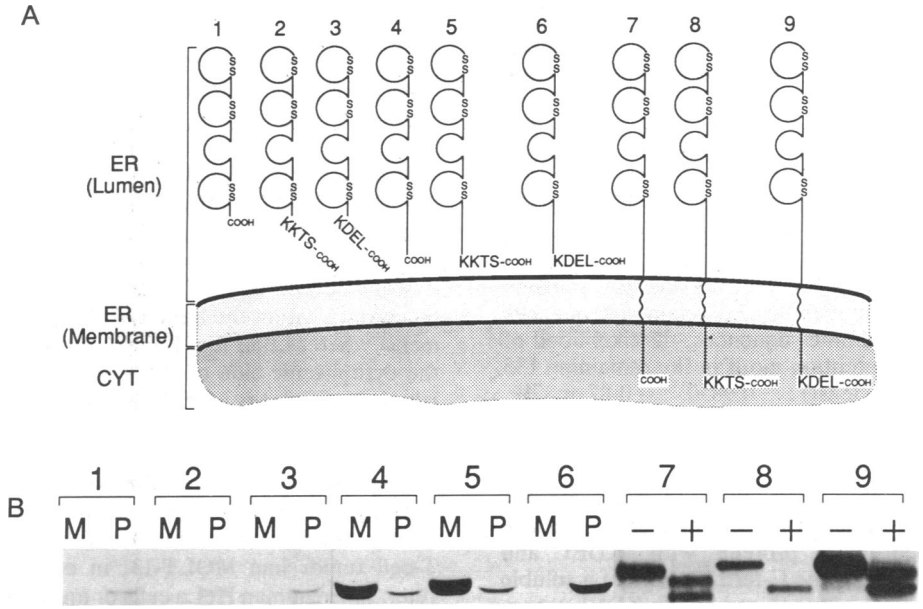

FIG. 5. Effect of transmembrane regions on the ER-retention motifs -KDEL and -KKXX. (*A*) Introductions of the motifs at Gly-351 (lanes 1–3), Trp-365 (lanes 4–6), or Gln-403 (lanes 7–9). As the result of different restriction sites used for the constructions, the sequences added to Ser-350 were -VKDEL or -VEKKTS, to Thr-364 were -WSKDEL or -WSEKKTS, and to Arg-402 were -LVKDEL or -LSEKKTS. Cyt, cytoplasm. (*B*) ER retention of soluble proteins was measured by secretion into the medium after a 15-min pulse labeling and a 4-hr chase [lanes 1–6, immunoprecipitates of medium (M) or cell pellet lysate (P)]. Endo H sensitivity was used for transmembrane proteins [lanes 7–9, with (+) or without (−) endo H digestion].

differences in related compartments (ER, salvage compartment, etc.)].

Only ER-Resident Transmembrane Proteins Have the -KKXX Sequence at the C-Terminal End. If the retention signals -KKXX, -RKXX, -KXKXX, and -RXKXX are general in mammalian cells, one should expect that only mammalian transmembrane proteins that are to be retained in the ER should contain one of these C-terminal motifs, and all other mammalian transmembrane proteins should not. A search of all human, rat, mouse, and yeast protein sequences in the NBRF data base was performed to confirm this conclusion. All transmembrane proteins from the search with C-terminal sequences with one of the four ER-retention motifs are listed in Table 1, along with their C-terminal sequences (the last line of their sequences from the NBRF files). The results confirm the hypothesis: of known mammalian sequences (in the NBRF data base), all proteins with the C-terminal sequences -KKXX, -RKXX, -KXKXX, and -RXKXX are either soluble proteins (lacking a transmembrane region) or transmembrane proteins known to be retained in the ER. One might expect that ≈1.5% of all proteins would have such a sequence if they appeared randomly. In a search of 5066 human, mouse, rat, and yeast proteins containing 150 transmembrane proteins, ≈75 proteins of any type and perhaps 2 or 3 transmembrane proteins would likely have one of the ER motifs. Of the 5066 sequences searched, 112 proteins had one of the four motifs: 97 soluble proteins and 15 transmembrane proteins. All 15 transmembrane proteins

Table 1. C-terminal sequences of transmembrane proteins in NBRF data base containing one of the four ER-retention motifs

NBRF no.	Species	Name	C-terminal sequence
b25777	Human	TCRβ	SALVLMAMVKRKDF
a02133	Human	TCRβ	SALVLMAMVKRKDF
a00356	Human	HMG CoA reductase	NLQDLQGACTKKTA
a27878	Human	UDP-GT	CWKFVRTGKKGKRD
g27579	Rat	TCRβ	SALVLMAMVKKKNS
a24324	Rat	UDP-GT 2F	CCRKTANMGKKKKE
a26064	Rat	UDP-GT R23	YRFFVKKEKKMKNE
a24600	Rat	UDP-GT K39	GGKGRVKKSHKKTH
a28460	Rat	UDP-GT R38	YRLFVKKEKKMKNE
a02134	Mouse	TCRβ	SGLVLMAMVKKKNS
a02135	Mouse	TCRβ	STLVVMAMVKRKNS
a02246	Mouse	CD3 δ chain	YSRLGGNWPRNKKS
a22515	Human adenovirus 5	E3/19 kDa	YKSRRSFIEEKKMP
a03821	Human adenovirus 2	E3/18.5 kDa	YKSRRSFIDEKKMP
—	Rabbit	Ser 53 kDa	KTGCGTPKNRYKKH

Only human, mouse, rat, and yeast sequences were searched for the C-terminal motifs -KKXX, -KXKXX, -RKXX, and -RXKXX. Transmembrane proteins were identified from their NBRF file headers. Each sequence is unique; proteins listed that have identical C-terminal sequences have different N-terminal sequences. Rabbit Ser 53 kDa (10, 13), which contains the -RXKXX motif, is also listed although rabbit sequences were not part of the NBRF data base search. TCRβ, T-cell receptor β chain; HMG, hydroxymethylglutaryl.

are ER-retained proteins (Table 1). There are more proteins with one of the motifs than predicted by chance, probably because charged amino acids are favored on the surface of globular domains. Although only 2 or 3 transmembrane proteins with one of the motifs were expected by chance [or perhaps 3 or 4 might be expected because of the higher probability of C-terminal charged amino acids (2 or 3 × 112/75)] and 15 were found, it is nevertheless reassuring that all such proteins are known to be retained in the ER. This includes the T-cell receptor β chain, which, unless coexpressed with other components of the CD3 complex, remains in the ER (14). If one removes the ER-retained sequences from the list of transmembrane sequences, the likelihood of finding at least one ER retention motif in the remaining 135 sequences by chance is (at least) $1 - 0.9850^{135} = 0.87$ or 87%. Since none was found, it appears that the ER-retention motifs were selected against during evolution in proteins not meant to be retained in the ER.

ER Luminal and Transmembrane Proteins Use Separate ER Structures for Their Retention. Distinct mechanisms may be involved in ER retention of proteins with -KDEL and -KKXX motifs. In one case, the C-terminal end of a soluble ER-resident protein may be recognized by the KDEL receptor (5, 19) at the luminal side of the compartment (ER or salvage compartment). Conceivably, however, the C-terminal -KDEL sequence may be recognized during synthesis in the cytoplasm, resulting in its subsequent ER retention. In that case, -KDEL at the C-terminal end of a transmembrane protein might also be used as an ER-retention signal. Similarly, the question of whether the KKXX motif will function in the presence or absence of a transmembrane region is also of importance. -KDEL or -KKTS sequences were, therefore, conjugated to the C-terminal end of truncation mutants of CD4 at the following several positions: (*i*) in the extracellular region far above the transmembrane region (Fig. 5*A*, lanes 1–3), (*ii*) just above the transmembrane region (lanes 4–6), and (*iii*) at the end of a truncated intracytoplasmic region (lanes 7–9). In the second set both the soluble CD4 (sCD4.W365stop) and the same molecule with the -KKTS sequence attached (sCD4.365KKTS) were secreted >90% into the medium in 4 hr, whereas -KDEL conjugated at the same position (sCD4.365KDEL) produced a molecule associated with the cell pellet (Fig. 5*B*, lanes 4–6). sCD4.365KDEL was actually retained in the ER as judged by its fluorescence staining pattern and endo H sensitivity (data not shown). In contrast, in the third set, both cytoplasmic-domain-truncated transmembrane CD4 (CD4.Q403stop) and -KDEL-conjugated CD4.Q403stop (CD4.403KDEL) were expressed on the cell surface very well (data not shown) and both were resistant to endo H digestion (Fig. 5*B*, lanes 7 and 9). However, addition of the -KKXX motif to transmembrane CD4 (CD4.403KKTS) resulted in a molecule not expressed at the surface (data not shown) and sensitive to the endo H digestion (Fig. 5*B*, lane 8), thus suggesting ER retention. Therefore, the -KKXX motif is recognized by its receptor only in the presence of a transmembrane region. Its binding is presumably in the cytoplasmic side of the ER, whereas the -KDEL motif must be recognized by the -KDEL receptor only inside the lumen of the ER or salvage compartment and thus only in the absence of a transmembrane region.

In the first set, truncation at Gly-351 (sCD4.G351stop) resulted in a small amount of protein retained in the ER. Truncation at this position may result in a misfolded state, ER retention, and fast degradation (Fig. 5, lanes 1–3). Since Gly-351 is very near to Cys-345, this mutation may prevent proper folding and formation of a disulfide bond with Cys-303 in the fourth extracellular domain of CD4. In this situation, addition of either -KDEL or an -KKXX motif to the C-terminal end had little effect.

DISCUSSION

The mechanism of retention of proteins in the ER is not fully understood. The present results contribute to that discussion (e.g., refs. 5, 12, 15, 16, and 19) by defining more precisely the motif for retention of transmembrane proteins in the ER. This motif (C-terminal -KKXX, -KXKXX, -RKXX, or -RXKXX) functions only in the presence of a transmembrane region and the -KDEL motif for retention of proteins within the lumen of the ER does not function in the presence of a transmembrane region. Thus, it is likely that the -KDEL receptor is within the lumen of the ER (or salvage compartment) whereas that for -KKXX and related sequences is on the cytoplasmic side of the ER. It is also intriguing to ask whether sequences as different as -KKXX and -RKXX, on one hand, and -KXKXX and -RXKXX, on the other, would result in exactly the same destiny within the ER.

One exception to the ER retention should be noted. Strikingly, CD4.Q421stop was well expressed on the cell surface of either a murine T-cell hybridoma or the human T-cell tumor line MOLT-13, in contrast to complete ER retention in human HeLa cells or murine L cells (unpublished observations). Thus the retention is not species-specific, but tissue-specific. It is possible either that T cells lack the receptor for the -KKXX motif or that a T-cell-specific molecule binds to CD4.Q421stop in the context of this retention motif and results in transport of CD4.Q421stop to the surface by preventing the motif from being recognized by the ER-retention machinery. One possibility is that the T-cell-specific protein-tyrosine kinase lck associates weakly with the truncated CD4 in the ER even though its lck binding domain at amino acids 421–429 (17) is absent. Similarly, each single subunit of the T-cell receptor/CD3 complex remains in the ER until complete association and only then is this complex transported to the cell surface (14, 18). Therefore, ER retention appears reversible if the retention motif is paired with another protein.

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