Abstract. The sequence Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) has been shown to be a signal which leads to retention of at least two proteins in the endoplasmic reticulum of animal cells (Munro and Pelham, 1987). In this study we tested the function of this signal by appending it to two secretory proteins, rat growth hormone and the α subunit of human chorionic gonadotrophin (hCG-α). We used oligonucleotide-directed mutagenesis and expression to generate proteins with SEKDEL added to the exact COOH termini and then carried out a detailed analysis of their transport in monkey COS cells. We found that transport was not blocked for either protein, but rather that the half-time for secretion was increased about sixfold for both proteins. Analysis of oligosaccharide processing on hCG-α-SEKDEL and indirect immunofluorescence microscopy on cells expressing both proteins was consistent with a retardation of transport between the endoplasmic reticulum and the Golgi apparatus. A change in the last amino acid of the SEKDEL sequence from Leu to Val abolished the retardation almost completely, suggesting a highly specific interaction of the sequence with a receptor. A change in the first amino acid had little or no effect on retardation. We conclude that the SEKDEL signal can have strong effects on reducing the rate of protein exit from the endoplasmic reticulum without generating absolute retention. Presumably other features of protein structure must be important to generate absolute retention.

Mechanisms exist for sorting of proteins within the exocytic pathway of eucaryotic cells. This sorting leads to retention of specific proteins within the ER and subcompartments of the Golgi apparatus, and is thought to be mediated by sorting signals in the proteins themselves and receptors in the pathway (reviewed by Rose and Doms, 1988). One of the simplest and clearest examples of a sorting signal is the tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL) which Munro and Pelham (1987) noted at the COOH termini of four proteins that are residents of the ER. Deletion of this sequence from one of these resident ER proteins, GRP78, resulted in slow secretion of the truncated GRP78, and addition of the SEKDEL sequence from GRP78 to the secretory protein lysozyme resulted in retention of lysozyme in the ER. Although the receptor for the KDEL sequence has not yet been identified, it was suggested that it might reside in an early Golgi compartment and function through a recycling process that retrieves resident ER proteins back from the Golgi apparatus. Evidence for such a mechanism has been obtained both in mammalian cells (Pelham, 1988) and in yeast which use the related retention signal HDEL (Pelham et al., 1988).

Because the function of the KDEL signal had only been tested on one secretory protein from mammalian cells, we decided to test it further by appending it to two well-characterized secretory proteins that were under study in our laboratory, rat growth hormone (rGH), and the α subunit of human chorionic gonadotropin (hCG-α). rGH is a nonglycosylated monomeric protein that is secreted efficiently and rapidly from monkey COS cells with a half-time of 30 min (Guan and Rose, 1984). Because the three-dimensional structure of growth hormone (Abdel-Meguid et al., 1987) showed that the COOH terminus is exposed, we felt that growth hormone was an excellent choice for testing the generality of the SEKDEL signal.

In contrast to rGH, hCG-α, is a glycosylated secretory protein that is secreted efficiently (even in the absence of the hCG-β subunit) with a half-time of 100 min in COS cells (Guan and Rose, 1988). The oligosaccharides on hCH-α are processed to a complex form (Pierce and Parsons, 1981) and thus acquire resistance to the enzyme endoglycosidase H (endo H) before secretion from monkey COS cells (Guan et al., 1988). The acquisition of processed oligosaccharides on hCG-α provided an additional marker which allowed us to assess the effect of SEKDEL addition on the rate of protein transport within the exocytic pathway before secretion. We show that the addition of COOH-terminal SEKDEL has dramatic effects on reducing the rate of transport of GH and hCG-α from the ER to Golgi apparatus, but that both pro-

1. Abbreviations used in this paper: endo H, endoglycosidase H; hCG-α, the α subunit of human chorionic gonadotropin; rGH, rat growth hormone.
proteins are still secreted with high efficiency even with SEKDEL at their COOH termini.

Materials and Methods

Plasmid Constructions and Oligonucleotide-directed Mutagenesis

Plasmids used to express GH and hCG-α in COS cells (pGH and pCG-α) have been described previously (Guan and Rose, 1984; Guan et al., 1988) and were based on the pJC119 vector (Sprague et al., 1983). For in vitro mutagenesis, cDNAs encoding rGH and hCG-α were cloned into the Xho I site of M13mp8X (Guan et al., 1985) and Bluescript SK+ (Stratagene, San Diego, CA), respectively. Single-stranded DNA was prepared as described previously (Guan et al., 1985; Shaw et al., 1988). The oligonucleotide shown in Fig. 1 was used to introduce the DNA encoding SEKDEL and a stop codon into the GH cDNA sequence and 5′ATTATACAAATCTACG-GAAAAGGAGCAGCTCTATTCATTCTGACTTGTG 3′ was used to introduce the same coding sequence into the DNA encoding hCG-α. The procedure of Zoller and Smith (1983) was followed with the modifications described by Machamer et al. (1985). Mutated genes were identified by stability of hybridization to 5′ 32P-labelled primer at 69°C and by the presence of a new Sst I restriction site introduced in both cases. The oligonucleotide 5′-GAAAAGGAGCAGCTCTATTCATTCTGACTTGTG-3′ was used to introduce the Leu (CTC) to Val (GTG) change in the GH-SEKDEL construct.

Results

Construction and Expression of cDNA Clones Encoding Secretory Proteins with Potential ER Retention Signals

To examine the effect of COOH-terminal sequences on retention of secretory proteins, we wanted to generate constructs encoding rat growth hormone or the hCG-α subunit with COOH-terminal extensions of the amino acid sequence SEKDEL. This was done in a single step for each protein using oligonucleotide-directed mutagenesis to replace sequences in the cDNA clones encoding rGH or hCG-α. The mutagenesis eliminated the normal translation termination codon for each protein and added sequences encoding SEKDEL as well as a new termination codon (Fig. 1).

The mutated genes were then cloned into the SV40-based vector pJC119 in which mRNA synthesis is directed by the SV40 late promoter (Sprague et al., 1983). Plasmids encoding GH and GH-SEKDEL were designated pGH and pGH-SEKDEL, respectively. Those encoding hCG-α and hCG-SEKDEL.

Figure 1. (A) Schematic diagrams of rGH, rGH-SEKDEL, hCG-α, and hCG-α-SEKDEL. The COOH-terminal sequences of the wild-type and mutated molecules are indicated in the single-letter amino acid code. (B) Method of addition of the SEKDEL coding sequence. The primer used to generate the addition of SEKDEL to rGH by oligonucleotide-directed mutagenesis is illustrated above the sequence in the rGH clone. Incorporation of the mismatched nucleotides resulted in addition of the hexapeptide sequence SEKDEL to the COOH terminus of rGH expressed from the mutated DNA.
Figure 2. Indirect immunofluorescence showing staining of wild-type and mutant GH. COS cells (5 x 10^5) plated on coverslips were transfected with pGH (A), pGH-SEKDEL (B), or pGH-SEKDEV (C). After 40 h, cells were fixed, permeabilized with detergent, and then incubated with monkey anti-GH serum, followed by FITC-conjugated rabbit anti-monkey IgG. The presumed Golgi region is indicated by an arrow in A and C.
α-SEKDEL were designated phCG-α and phCG-α-SEKDEL. We next transfected these DNAs onto COS cells and after 40 h labeled the cells with [35S]methionine. Immunoprecipitates prepared from cell lysates were analyzed by SDS gel electrophoresis. The apparent molecular weights of GH-SEKDEL and hCG-α-SEKDEL were slightly larger than the parent proteins as would be expected from the addition of the six amino acids (not shown).

**Indirect Immunofluorescence Shows Strong ER Localization of GH and hCG-α Containing COOH-terminal SEKDEL**

To compare the intracellular distribution of GH and GH-SEKDEL, we first examined the intracellular distribution of the two proteins by indirect immunofluorescence. Transfected COS cells expressing both proteins were fixed, permeabilized with detergent, and incubated with monkey anti-GH polyclonal antibody, followed by FITC-conjugated mouse anti–monkey IgG. All cells expressing wild-type GH exhibited intense Golgi staining typified by the cell shown in Fig. 2 A. This pattern is characteristic of proteins such as rat growth hormone which move rapidly from the ER to the Golgi apparatus, and the identity of the putative Golgi region has been verified previously (Guan and Rose, 1984). The pattern obtained with cells expressing GH-SEKDEL was strikingly different as shown by the cell in Fig. 2 B. There was prominent staining of the nuclear envelope as well as a lattice of fine tubular structures (Fig. 2 B) but there was no obvious staining of the Golgi apparatus. This pattern is typical of that obtained for proteins which are retained in the ER or are relatively slow to exit the ER (Rose and Bergmann, 1983; Munro and Pelham, 1987).

![Figure 3. Secretion of GH, GH-SEKDEL, and GH-SEKDEV proteins.](image-url)

COS cells plated on 35-mm dishes were transfected with 51 μg of pGH, pGH-SEKDEL, or pGH-SEKDEV. At 40 h posttransfection, the cells were incubated in DME lacking methionine and serum for 15 min and then pulse-labeled for 15 min with 50 μCi [35S]methionine in 0.5 ml of the above medium. After removal of the labeling medium the cells were incubated in DME containing 2 mM unlabeled methionine and 1% FCS for the indicated times. Immunoprecipitates from the cell lysate (C) and the medium (M) were analyzed by SDS-PAGE. Fluorograms of gels for GH (A), GH-SEKDEL (B), and GH-SEKDEV (C) are shown. Positions of marker proteins of 30 and 14 kD are indicated. Preflashed films from these and other experiments were quantified by densitometry and the fraction of protein secreted was calculated (D). Each point represents the average of at least two experiments.
We also carried out indirect immunofluorescence on COS cells expressing hCG-α and hCG-α-SEKDEL. The patterns of fluorescence were nearly identical to those shown in Fig. 2, A and B for GH and GH-SEKDEL except that the Golgi fluorescence relative to ER was not as striking as that for GH, presumably because hCG-α is transported more slowly from the ER. However, like GH-SEKDEL, the hCG-α-SEKDEL protein was only visible in ER-like structures (data not shown). Although these data suggested that addition of SEKDEL might have generated absolute retention in the ER, the following experiments measuring secretion showed that both proteins were in fact secreted.

Kinetic Analysis Shows that SEKDEL Retards Secretion but Does Not Retain GH and hCG-α

To obtain a quantitative measurement of the effects of SEKDEL on secretion we proceeded to examine the kinetics of secretion of pGH and pGH-SEKDEL. Fig. 3 A shows the results of a pulse-chase experiment in which COS cells transfected with pGH were labeled with [35S]methionine for 15 min and then incubated in chase medium containing excess unlabeled methionine. Proteins were then immunoprecipitated from cell lysates (C) and media (M) and analyzed by SDS-PAGE. Quantitation of the gels showed that GH was...
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found to be secreted rapidly from COS cells with a half-time of 30 min as reported earlier (Guan and Rose, 1984). In contrast (Fig. 3 B) GH-SEKDEL was steadily secreted with a half-time of ~3 h, or about sixfold slower than GH itself (Fig. 3 D). Thus the addition of SEKDEL did not block the secretion of GH completely, but did slow it down significantly. Quantitation of the autoradiograms from which the half-times were calculated is shown in Fig. 3 D.

We next examined the effect of SEKDEL on the kinetics of secretion of hCG-α. This protein has two N-linked oligosaccharides, both of which are processed in the Golgi apparatus to the complex form which is resistant to cleavage with endo H. In addition hCG-α is secreted from COS cells about threefold more slowly than GH with a half-time of ~100 min (Guan et al., 1988). The results of the pulse–chase experiment are shown in Fig. 4, A, B, and D. The half-time of secretion for hCG-α was found to be ~2 h while the half-time of secretion for hCG-α-SEKDEL was ~11 h (Fig. 4 B).

Again, as for GH, absolute retention of hCG-α was not observed. Instead, the rate of secretion of hCG-α-SEKDEL was approximately sixfold slower than that of the wild-type protein. As can be seen in Fig. 4, hCG-α immunoprecipitated from the medium had an electrophoretic mobility slower than the intracellular protein. This is probably due to a higher degree of processing of the two N-linked oligosaccharide chains in the secreted protein. We show below that the intracellular hCG-α contains mainly high mannose oligosaccharides while those present on the secreted form are processed to complex carbohydrates.

hCG-α-SEKDEL Is Transported through the Golgi Apparatus before Secretion

We next considered an alternative explanation for our results showing that SEKDEL did not function to retain proteins in the ER. Conceivably the release of both GH-SEKDEL and hCG-α-SEKDEL might have resulted from cell death and lysis releasing the protein directly from the ER into the medium. This seemed unlikely because the two proteins were secreted at very different rates. To determine if hCG-α-SEKDEL immunoprecipitated from the medium was transported via the normal secretory pathway, we examined the degree of oligosaccharide processing on hCG-α and hCG-α-SEKDEL present in both cells and medium. This was accomplished using endo H, which cleaves high mannose oligosaccharides added in the ER, but will not cleave those processed further in the Golgi compartment. Endo H resistance is probably acquired in the medial Golgi (Dunphy and Rothman, 1983).

We transfected COS cells with the appropriate plasmids, labeled the cells with [35S]methionine for 30 min and then incubated in chase medium for 4 h. Immunoprecipitates of the proteins from the medium and cell lysates were then divided equally and treated or not treated with endo H to determine the degree of oligosaccharide processing. As can be seen in Fig. 5, the intracellular forms of both hCG-α and hCG-α-SEKDEL are endo H sensitive, while the secreted forms show endo H resistance. This result indicates that the proteins found in the medium are true secretory products which have traversed the secretory pathway, not products released directly from the ER because of cell death.

Effects of Minor Sequence Changes on the Function of the SEKDEL Signal

It was shown previously that when the SEKDEL hexapeptide is changed to SEKDAS at the COOH terminus of chicken lysozyme, it no longer prevents secretion of this protein into the medium (Munro and Pelham, 1987). This result indicated that the last two amino acids are critical to the function of the signal. To perform a more subtle test of the specificity of this signal we altered only the last amino acid, changing it from leucine to valine. This change retains the hydrophobic character of the last amino acid and removes only a single methylene group.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Endo H digestion of intracellular and secreted forms of hCG-α and hCG-α-SEKDEL. COS cells plated on 35-mm dishes were transfected with ptCH-α and ptCH-α-SEKDEL and labeled as described in Fig. 4. After incubation for 4 h in medium containing 2 mM excess unlabeled methionine and 1% PCS, the immunoprecipitates from the cell lysate (C) and medium (M) were incubated in the presence (+) or absence (−) of endo H and analyzed by SDS-PAGE.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Time course of secretion of GH, GH-SEKDEL, and GH-SEKDEV in HeLa cells. HeLa cells were infected with a recombinant vaccinia virus (vTF7-3) encoding T7 RNA polymerase and transfected with the appropriate plasmids. The cells were labeled with [35S]methionine for 15 min, at 4 h postinfection, incubated in chase medium containing excess unlabeled methionine for the indicated periods of time. Immunoprecipitates of the medium and cell lysates were then analyzed by SDS-PAGE and quantitated by densitometry. The percentage of total protein secreted at each time point was calculated. The results shown are averages of at least two experiments.
Kinetic analysis of the secretion of GH-SEKDEV showed that the protein was secreted efficiently with a half-time of 30 min, essentially the same as for GH (Fig. 3 C). This demonstrates clearly that even a small change in the COOH-terminal amino acid eliminates the effect of the signal and suggests a very high degree of specificity in the putative receptor. As expected from the kinetic data, examination of cells expressing GH-SEKDEV by indirect immunofluorescence showed intense Golgi staining, similar to the pattern observed in cells transfected with plasmid expressing the wild-type GH cDNA (Fig. 2 C).

The only sequence conserved at the COOH termini of several resident ER proteins is KDEL, although GRP78 contains SEKDEL. Also SEKDEL was the sequence appended to lysozyme by Munro and Pelham (1987) in the experiment showing that it could function to retain lysozyme. In the process of generating mutations in hCG-α we fortuitously obtained one which contained NEKDEL instead of SEKDEL. We also examined the secretion of this protein and found it to be very similar to hCG-α-SEKDEL (Fig. 4 C), indicating that the first amino acid in SEKDEL is not a critical part of the signal.

**COOH-terminal SEKDEL Also Retards Protein Secretion in HeLa Cells**

Because the previous experiments on the function of SEKDEL in mammalian cells (Munro and Pelham, 1987; Pelham, 1988) were performed in COS cells, we wanted to test our findings on another cell line using a different expression system. We chose HeLa cells and the vaccinia T7 system to obtain high level transient expression (Fuerst et al., 1986). This system is based on infection of the cells with a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase and subsequent transfection with the gene to be expressed cloned into the vector PAR2529 under control of the T7 promoter (Fuerst et al., 1986). Transcription of the gene is mediated by the T7 polymerase produced in the cytoplasm by the recombinant vaccinia virus.

Fig. 6 shows quantitation of the results of a secretion experiment in which HeLa cells were expressing GH, GH-SEKDEL, and GH-SEKDEV in this system. The half-times of secretion for the three proteins were the same as in COS cells, indicating that the effects of the SEKDEL sequence on the secretion of the proteins were independent of the cell type and expression system.

**Discussion**

The experiments described here were designed to test the effects of addition of the hexapeptide sequence SEKDEL to the COOH termini of two secretory proteins, hCG-α and rGH. This COOH-terminal sequence has been shown to function as a signal which retains proteins in the ER of monkey COS cells (Munro and Pelham, 1987; Pelham, 1988). In our experiments we found that after addition of SEKDEL, the half-times for secretion of both proteins increased dramatically (about sixfold) from 30 min to 3 h for rGH and from 2 to 11 h for hCG-α. We also observed an identical effect on rGH in Hela cells. Although the secretion of hCG-α-SEKDEL was very slow, it did result from passage through the exocytic pathway because the oligosaccharides on the secreted form were processed. The effect we observed is clearly different from the generation of absolute retention in the ER reported by Munro and Pelham (Munro and Pelham, 1987) when they added the sequence SEKDEL to the COOH terminus of chicken lysozyme. Although kinetic analysis was not performed in the lysozyme-KDEL experiments, the experiments suggested greater retention than we observed here. What is the explanation for this difference?

Because all molecules of hCG-α-SEKDEL or GH-SEKDEL appear to be greatly retarded in transport to the Golgi apparatus compared to wild-type proteins, we assume all are binding to the putative KDEL receptor. Binding might be of lower affinity for these molecules than for lysozyme-SEKDEL, resulting in slow release from the receptor. Certainly the neighboring sequences in these molecules might affect this affinity. If indeed the mechanism of retention requires multiple rounds of protein interaction with the receptor and recycling from the Golgi apparatus (Munro and Pelham, 1987), then the degree of exposure to or the affinity of receptor for the SEKDEL sequence would be expected to determine the probability of protein retrieval during each successive round of transport. Proteins with a high degree of SEKDEL exposure would then be secreted more slowly than those with a lesser degree of exposure. Although the three-dimensional structures of both GH and lysozyme show free COOH termini, one would need to determine the three-dimensional structure of each molecule containing SEKDEL to assess the degree of exposure.

Another explanation for the lack of retention might be that the receptor was saturated by overexpression of protein. However, the vector used in the earlier studies on expression of lysozyme-SEKDEL in COS cells (Munro and Pelham, 1987) should give comparable or higher levels of expression than the one we used here. We also examined this question using the vaccinia/T7 system where it was possible to vary the levels of synthesis greatly. We found identical effects of SEKDEL on secretion of rGH with as much as 10-fold differences in expression, suggesting that receptor saturation is not the explanation.

In yeast the comparable ER retention signal is HDEL instead of KDEL and its effect on retention of invertase is only observed when cells are in stationary phase (Pelham et al., 1988). Also, addition of FEHDEL to the COOH terminus of yeast invertase did not result in any retention of invertase in the ER (Pelham et al., 1988). It was argued that in the first construct the FEHDEL sequence was not sufficiently exposed to be recognized by the receptor. Evidence for this model was obtained by adding a long "spacer" sequence between FEHDEL and invertase. In this context FEHDEL did function as a retention signal. The situation we have observed here in which SEKDEL addition generates retardation rather than retardation might thus reflect an "intermediate" degree of SEKDEL exposure or some other effects of neighboring sequences on affinity for the receptor.

The earlier studies of Munro and Pelham (1987) had suggested that the last two amino acids were important in the SEKDEL signal because a change to SEKDAS abolished retention. We performed a more subtle test of the specificity by changing only the last amino acid to valine. This change removes a single methylene group and abolishes the retardation effect suggesting a highly specific interaction with a receptor. Our analysis of another mutant with the first amino acid of the hexapeptide changed (NEKDEL) showed little effect on retardation suggesting that this amino acid is not important.
Our results provide confirmation that the SEKDEL functions as a signal that is recognized by a receptor, although we show that it can cause retardation in export from the ER rather than complete retention in the ER. Our experiments also suggest caution in the interpretation of experiments in which SEKDEL addition to a protein appears to generate an exclusively ER pattern as judged by indirect immunofluorescence. This was the case for both of the molecules we examined, yet both must be passing through the Golgi compartment to be secreted. Apparently the level of protein in the Golgi was insufficient to be detected or was obscured by intense labeling of the ER.

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