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A C-Terminal Signal Prevents Secretion of Luminal ER Proteins

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Summary

Proteins that permanently reside in the lumen of the endoplasmic reticulum (ER) must somehow be distinguished from newly synthesized secretory proteins, which pass through this compartment on their way out of the cell. Three luminal ER proteins whose sequence is known, grp78 ("BiP"), grp94, and protein disulphide isomerase, share the carboxy-terminal sequence Lys-Asp-Glu-Leu (KDEL). We show that deletion (or extension) of the carboxyl terminus of grp78 results in secretion of this protein when it is expressed in COS cells. Conversely, a derivative of chicken lysozyme containing the last six amino acids of grp78 fails to be secreted and instead accumulates in the ER. We propose that the KDEL sequence marks proteins that are to be retained in the ER and discuss possible retention mechanisms.

Introduction

The lumen of the ER contains several proteins that appear to be soluble rather than membrane-bound. The most prominent of these are the glucose-regulated proteins grp78 and grp94, so called because their synthesis is stimulated when fibroblasts are deprived of glucose (for review see Pelham, 1986). We have recently reported the complete cDNA sequence of grp78 and have shown that it is related to the heat shock protein hsp70 and is identical with "immunoglobulin heavy chain binding protein" (BiP) (Munro and Pelham, 1986; Haas and Wabl, 1983; Bole et al., 1986). Grp94 is a glycoprotein that is related to another heat shock protein, hsp90 (Sorger and Pelham, 1987). It is one of the most abundant proteins of the ER and has been variously referred to as grp94 (Shiu et al., 1977; Lee et al., 1984), "100K" (Welch et al., 1983), "hsp108" (Kulomaa et al., 1986; see also Sorger and Pelham, 1987), "endoplasmin" (Koch et al., 1986), and "ERp99" (Lewis et al., 1985; M. Green, personal communication). Other proteins in the lumen include protein disulphide isomerase (Freedman, 1984; Edman et al., 1985) and prolyl-4-hydroxylase, one of the enzymes that modifies newly synthesized collagen molecules in the ER (Kivirikko and Myllyla, 1982).

These proteins occupy the same functional compartment as newly synthesized secretory proteins, but do not share their fate. A clear example is that of grp78 (BiP), which forms a complex with newly synthesized immunoglobulin heavy chains. Subsequently, the heavy chains associate with light chains and are secreted, while grp78 remains in the ER (Bole et al., 1986). There must therefore exist a mechanism by which the cell distinguishes resident luminal ER proteins such as grp78 from secreted proteins such as immunoglobulins. In principle, this could involve recognition of either a retention signal or a secretory signal on the appropriate proteins.

We have previously noted that the three soluble ER proteins whose sequences are known (grp78, grp94, and protein disulphide isomerase) share a common carboxy-terminal tetrapeptide sequence (Munro and Pelham, 1986; Sorger and Pelham, 1987). We show here that the carboxyl terminus of grp78 is crucial for its retention in the ER and that transfer of the last six amino acids to a secreted protein, chicken lysozyme, is sufficient to cause retention of lysozyme in the ER.

Results

An Intact Carboxyl Terminus Is Necessary for Retention of grp78 in Cells

The sequences of grp78, grp94, and protein disulphide isomerase show similarities at both their amino and carboxyl termini, although the proteins are otherwise quite different from each other. In the case of the grps, the similarities lie outside the regions that are homologous to the heat shock proteins (Figure 1). We reasoned that one or both of these terminal sequences might be important for retention of the proteins in the cell. To test this idea, we deleted portions of a rat grp78 cDNA clone and inserted the altered coding sequences into a COS cell expression vector. The amino acid sequences at each end of the mutant proteins are indicated in Figure 2. In the first mutant (SAGM2), the carboxy-terminal 60 amino acids of the protein were replaced with an 11 amino acid sequence derived from the human c-*myc* gene. The *myc* sequence was chosen because it is recognized by an existing monoclonal antibody and thus forms a convenient tag for detection of the expressed protein. In another mutant (SIG2), the leader peptide and first 11 amino acids of grp78, which are not homologous to hsp70, were replaced with the leader peptide and first six amino acids of an immunoglobulin heavy chain. A control plasmid (SAG1) encodes intact grp78. These constructs were transfected into COS cells, and 45 hr later the cells were labeled with [^{35}S]methionine for 2 hr. Labeled proteins in the cells and culture supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Transfection of cells with the wild-type construct (SAG1) produced no new labeled protein in the culture supernatant relative to the untransfected control (Figure 3A). The transfected cells, however, contained an abundance of labeled grp78 that could be immunoprecipitated with a polyclonal antibody raised against the purified rat protein (Figure 3B). This antibody also recognized the endogenous monkey grp78, but the amounts synthesized in untransfected COS cells were much lower than in the transfected cells. We were able to quantitate the accumulation of grp78 cells by probing protein blots with a monoclonal antibody (7.10) that recognizes most hsp70-related proteins (see Munro and Pelham, 1986). Such experiments showed

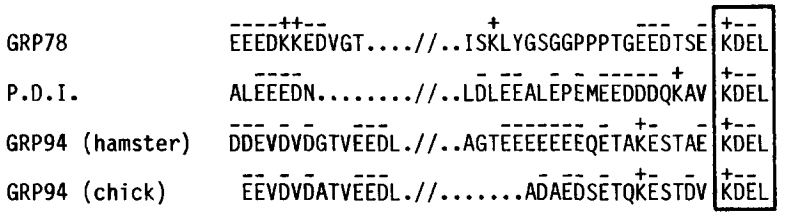


Figure 1. Comparison of Amino- and Carboxy-Terminal Sequences of ER Proteins

The sequences shown are from rat grp78 (Munro and Pelham, 1986), rat protein disulphide isomerase (P.D.I.; Edman et al., 1985), hamster grp94 (Sorger and Pelham, 1987), and chick grp94 (Kulomaa et al., 1986; these authors refer to the protein as hsp108, but comparison with the hamster sequence identifies it as grp94). For the grps, the sequences shown are from those regions that are not homologous to heat shock proteins. Charged residues are indicated by + or − above the sequence, and the conserved carboxy-terminal tetrapeptide is boxed. The one-letter amino acid code is used.

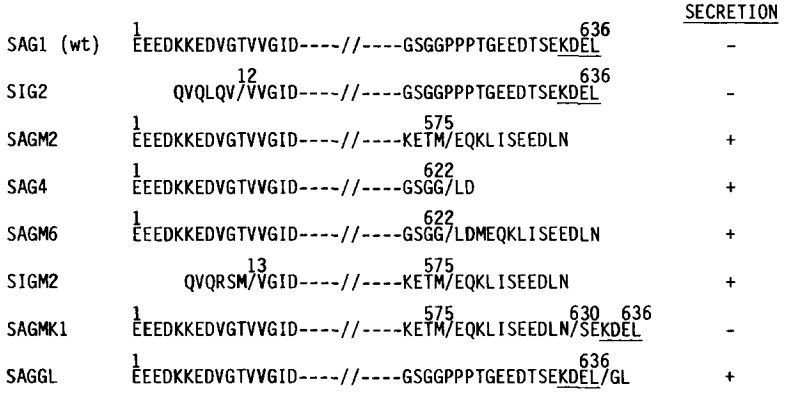


Figure 2. Terminal Sequences of grp78 and Mutants

Plasmid names are at the left, and the relevant amino acid sequences are shown in the one-letter code. Slashes indicate junctions between grp78 sequences and others. The KDEL sequence is underlined. The sequence EQKLISEEDL, present in SAGM2, SAGM6, SIGM2, and SAGMK1, is derived from the human c-*myc* gene and is recognized by monoclonal antibody 9E10. Numbers refer to residues of the mature wild-type grp78 protein.

that the transfected cell population contained about 3-fold more grp78 than untransfected cells (data not shown). Since the transfection efficiency (measured by immunofluorescence) was typically 20%–40%, individual cells must have contained at least 7-fold more grp78 than normal. Despite this considerable overproduction, the protein was efficiently retained within the cells.

The amino-terminal deletion mutant SIG2 behaved in the same way as the wild-type protein, being present in the cells but absent from the medium (Figures 3A and 3B). In contrast, transfection of cells with the carboxy-terminal deletion mutant SAGM2 resulted in a new labeled band in the culture supernatant with the expected mobility of the truncated grp78 (Figure 3A). The altered protein was not recognized efficiently by the polyclonal antiserum (Figure 3B), but subsequent experiments with a monoclonal antibody identified this band as the product of SAGM2 (see below). Similar results were obtained with two other carboxy-terminal deletion mutants of grp78 (SAGM6 and SAG4), both being secreted from transfected COS cells (Figure 3C). These mutants lack only 15 amino acids from the grp78 sequence; in SAG4 they are replaced with the dipeptide Leu-Asp, while SAGM6 contains the c-*myc* sequences in addition (see Figure 2). Thus a variety of changes to the carboxyl terminus of grp78 have the effect of converting the protein from a resident of the ER to a secretory protein.

Figure 4 shows the effect of simultaneously removing both the amino and carboxyl termini of grp78. In this experiment, the mutant proteins were detected by protein blotting, using the monoclonal antibody that recognizes the c-*myc* peptide tag. The proportion of the expressed protein that reached the medium was the same, whether both termini were deleted (SIGM2) or only the carboxyl terminus (SAGM2). From this and the previous results, we conclude that the amino terminus has little effect on the retention of grp78 in the cell.

Experiments such as that shown in Figure 4 give an indication of the efficiency with which the carboxy-terminal deletion mutants are secreted. From such experiments, we estimate that an amount equivalent to the steady state intracellular level of the mutant SAGM2 is released into the supernatant in 4–5 hr, giving an approximate half-life in the cell of 3 hr for this protein. Similar half-lives were obtained for the other secreted mutants. Although quite slow, this rate of release is within the range observed for proteins that are secreted via the constitutive pathway (Gebhart and Ruddon, 1986).

The behavior of the three carboxy-terminal mutants indicates that deletion of part of grp78 is sufficient to cause its secretion. This strongly suggests that retention of the intact protein in the ER is an active process, requiring recognition of a specific structural feature. Conversely, the successful negotiation of the secretory pathway by the truncated proteins supports the idea that transit from the ER to the outside of the cell requires no special signal, since there is no reason why grp78 should contain such a signal.

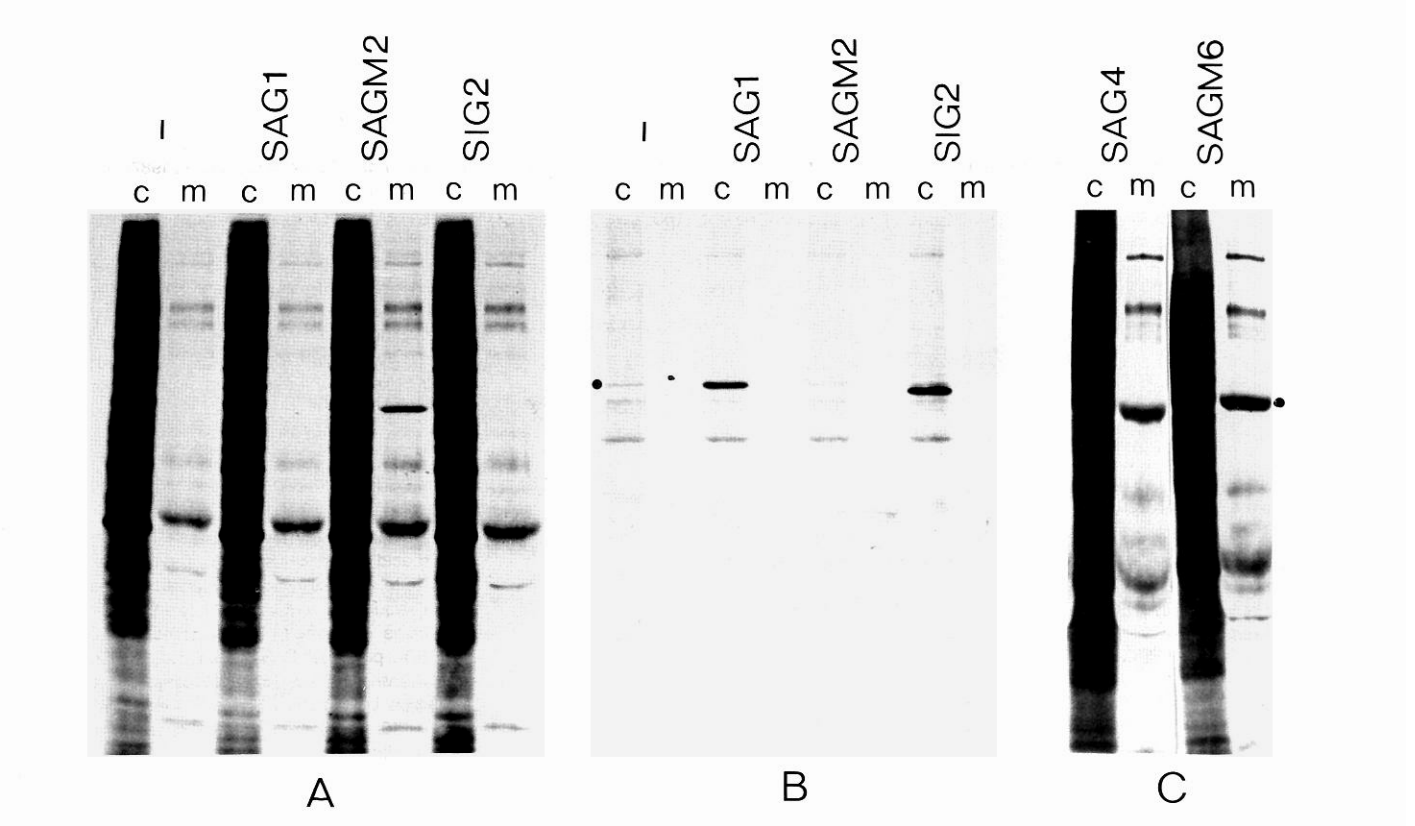


Figure 3. Expression of Amino- and Carboxy-Terminal Deletion Mutants of grp78

(A and C) COS cells that were untransfected (–) or transfected with the indicated plasmid were labeled with [^{35}S]Met for 2 hr, and samples of the cells (c) and culture medium (m) were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. (B) Samples from the same experiment as (A) immunoprecipitated with a polyclonal anti-grp78 serum; equivalent amounts of the cell and medium samples were analyzed. Dots indicate the position of migration of grp78. SAG1 expresses wild-type protein; SAGM2, SAG4, and SAGM6 are carboxy-terminal deletions, and SIG2 is an amino-terminal deletion. The SAGM2 product is not recognized by the polyclonal antiserum.

The Last Six Amino Acid Residues of grp78 Contain an Important Signal

Although the above experiments show that removal of 15 amino acids from grp78 causes its secretion, they do not prove that the putative retention signal is within that sequence; the deletions may simply disrupt the folding of an entire carboxy-terminal domain. However, comparison of the four luminal ER proteins whose sequence is known shows that they have an identical carboxy-terminal tetrapeptide Lys-Asp-Glu-Leu (KDEL in the one-letter code) but diverge further into the protein (Figure 1). To test whether it is the presence of this conserved sequence that is important for retention, we made a construct (SAGMK1) in which the last six amino acids of grp78 were added back to the 60 amino acid deletion mutant, with the c-*myc* peptide present at the junction (Figure 2). The protein expressed from this construct was not secreted into the medium (Figure 5A: SAGMK1), and probing of a protein blot with the anti-*myc* monoclonal antibody confirmed that it remained intact in the cells (Figure 5B). This strongly suggests that the putative signal is encoded in the carboxy-terminal hexapeptide and does not require an elaborate tertiary structure to be recognized.

We next asked whether the conserved sequence had to be present at the extreme carboxyl terminus. The construct SAGGL encodes the entire grp78 sequence, but with a two-residue extension (Gly-Leu) at the carboxyl terminus (Figure 2). This protein was secreted from COS cells with about the same efficiency as the carboxy-terminal deletion mutants (Figure 5C). Thus the presence of the conserved sequence at an internal position is not sufficient for retention; to be recognized, the signal must be at the carboxyl terminus. Moreover, since the extended protein has the same carboxy-terminal residue as grp78 (leucine), we can conclude that this residue itself is not a sufficient signal.

Addition of a Hexapeptide Sequence to Lysozyme Prevents Its Secretion

Soluble ER proteins share features other than their carboxy-terminal sequences; for example, the proteins mentioned in the introduction are all quite strongly acidic. To see whether the carboxyl terminus alone is sufficient to prevent secretion, we made a series of constructions based on the chicken lysozyme gene. Lysozyme is a small, highly basic protein that is constitutively secreted from cells (Cutler et al., 1981; Kondor-Koch et al., 1985). If the KDEL sequence is a sufficient retention signal, addition of these amino acids to lysozyme should cause it to accumulate in the ER rather than be secreted.

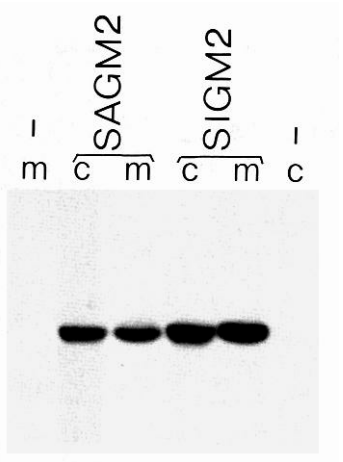


Figure 4. Blot Analysis of Expressed Proteins

Cells (c) or medium (m) from untransfected (–) or transfected cultures were analyzed by protein blotting. Expressed proteins were detected with the anti-*myc* monoclonal antibody 9E10. SAGM2 has the carboxyl terminus deleted, while SIGM2 has both the amino and carboxyl termini removed.

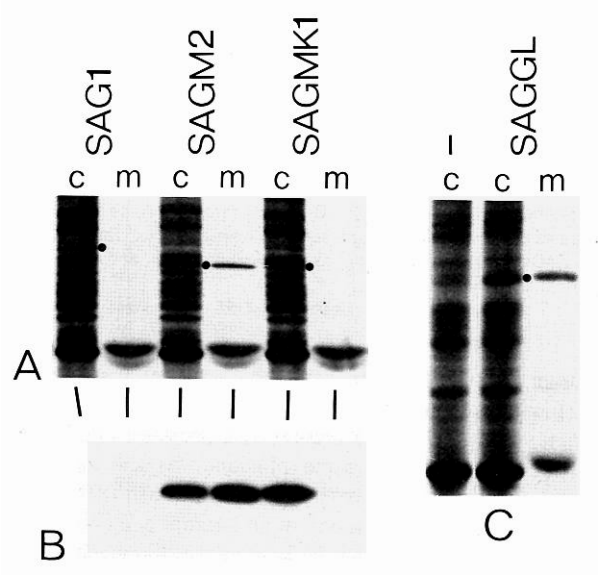
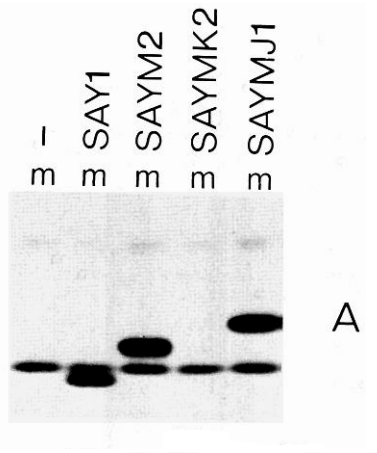
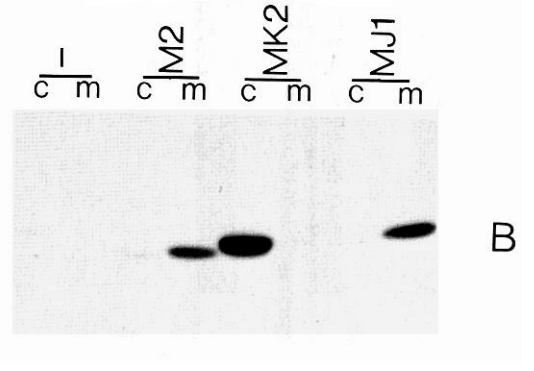


Figure 5. Requirements for Retention of grp78 in Cells

(A and C) [^{35}S]Met labeling. SAG1 is wild type, SAGM2 lacks 60 amino acids from the carboxyl terminus, and SAGMK1 is the same as SAGM2 but with the last 6 residues added back to the carboxyl terminus. SAGGL encodes grp78 with a two-amino-acid extension at the carboxyl terminus. Dots indicate the expressed proteins. (B) Blot analysis of samples like those in (A) using the anti-*myc* monoclonal antibody. Lanes correspond to those above them, as indicated. SAG1 lacks the *myc* sequences and thus serves as a control.





SAY1 (wt)	---RGCRL
SAYM2	---RGC/PCMEQKLISEEDLN
SAYMK2	---RGC/PCMEQKLISEEDLN/SEKDEL
SAYMJ1	---RGC/PCMEQKLISEEDLN/SEKDAS

Figure 6. Expression of Lysozyme Derivatives

The carboxy-terminal amino acid sequences of the proteins encoded by the various plasmids are shown at the bottom. (A) Medium from [^{35}S]Met-labeled transfected cells. (B) Blot analysis of cells and medium from cultures transfected with no DNA (–), SAYM2 (M2), SAYMK2 (MK2), and SAYMJ1 (MJ1), probed with the anti-*myc* monoclonal antibody. Equivalent amounts of cells and medium were analyzed.

The carboxy-terminal sequences of the proteins encoded by the various constructs are shown in Figure 6, together with the results of the transfection experiments. Figure 6A shows the analysis of [^{35}S]Met-labeled proteins in the culture supernatants from transfected cells. Wild-type lysozyme, encoded by SAY1, was readily detectable in the medium; it migrates slightly faster on the gel than a prominent protein that is secreted by untransfected COS cells. SAYM2, which contains the c-*myc* tagging sequences, produced a correspondingly larger protein that was also efficiently secreted. Blotting analysis with the *myc* monoclonal antibody showed that almost all the SAYM2 protein made during a 3 hr incubation was present in the culture supernatant (Figure 6B). In contrast, the product of SAYMK2, which has both the *myc* sequence and the last six amino acids of grp78, was only just detectable in the supernatant (Figure 6A), and blotting showed that it had accumulated to very high levels in the cells (Figure 6B).

We were concerned that the addition of the grp78 sequences to lysozyme might interfere with its folding, and that this alone might be sufficient to prevent secretion. To minimize this possibility, a construct was made that was identical to SAYMK2, but had the last two codons of the grp78 sequence changed from Glu–Leu to Ala–Ser. This plasmid produced a protein that was secreted as effi-

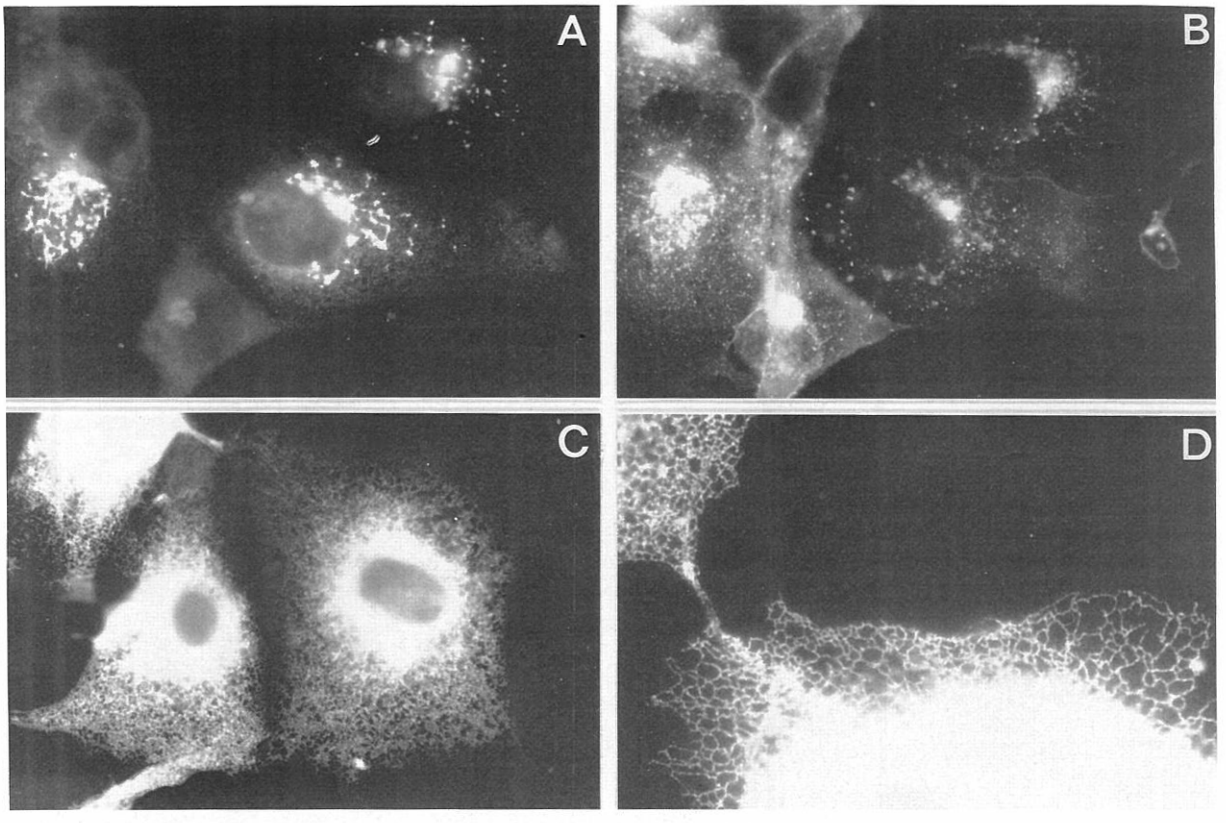


Figure 7. Immunofluorescent Staining of Transfected COS Cells

(A and B) Cells transfected with SAYM2 (lysozyme tagged with the c-*myc* peptide) double stained with the anti-*myc* monoclonal (A) and rhodamine-conjugated wheat germ lectin (B). The protein is mostly concentrated in the Golgi region, which stains brightly with the wheat germ lectin. (C and D) Cells transfected with SAYMK2 (same as SAYM2, but with six amino acids containing the KDEL signal added) and stained with the anti-*myc* monoclonal. (D) An enlargement of the periphery of a cell in which the tubular ER network is clearly visible.

ciently as normal lysozyme (Figure 6: SAYMJ1). Replacement of the last six amino acids with five other unrelated heptapeptide sequences also failed to block secretion (data not shown; the peptides were SAEAARL, SREKAWL, SPNHSMV, SGPREWL, and SGPWEWL). These results argue strongly that the failure of the SAYMK2 protein to be secreted is not simply due to some problem of folding or aggregation, but is a specific consequence of the presence of the carboxy-terminal retention signal. The successful transfer of this signal to the lysozyme molecule indicates that no features of grp78 other than its carboxyl terminal sequence are required to prevent secretion.

The Retained Protein Is in the ER of Transfected Cells

If the signal identified in the previous experiments is solely responsible for the intracellular location of luminal ER proteins, then the lysozyme molecules bearing the grp78 carboxyl terminus should accumulate in the ER and not in the Golgi or elsewhere in the cell. To see whether this was true, we examined the intracellular distribution of the various altered proteins by indirect immunofluorescence, using the anti-*myc* monoclonal antibody. Untransfected cells were not detectably stained with this antibody.

Figure 7A shows the distribution of the *myc*-tagged, secreted lysozyme made from SAYM2. There is relatively little protein in the cells, and it is mostly concentrated in the Golgi apparatus, which was identified by double labeling with wheat germ lectin (Figure 7B) (Virtanen et al., 1980). Low levels of protein are also present in the ER; this appears to represent newly synthesized material, since the ER staining was much reduced when cells were incubated for 1 hr with cycloheximide prior to fixation (data not shown). An identical pattern of bright Golgi staining was obtained when cells expressing wild-type lysozyme were stained with the anti-lysozyme monoclonal antibody D1.3 (Mariuzza et al., 1983) (data not shown). It appears that passage through the Golgi is the rate-limiting step in secretion of this protein, as has been found for other rapidly secreted proteins (Fries et al., 1984).

The lysozyme derivative containing the retention signal (SAYMK2) showed a completely different distribution (Figure 7C). At the periphery of the cells a network of tubules is clearly visible (Figure 7D). This pattern is very similar to that obtained when the ER is visualized with lipophilic fluorescent dyes (Pagano et al., 1981; Terasaki et al., 1984), which strongly suggests that the protein is indeed present in the ER. However, because of the bright staining in the perinuclear region, it was difficult to tell whether it is also present in the Golgi complex.

We were able to overcome the staining problem by

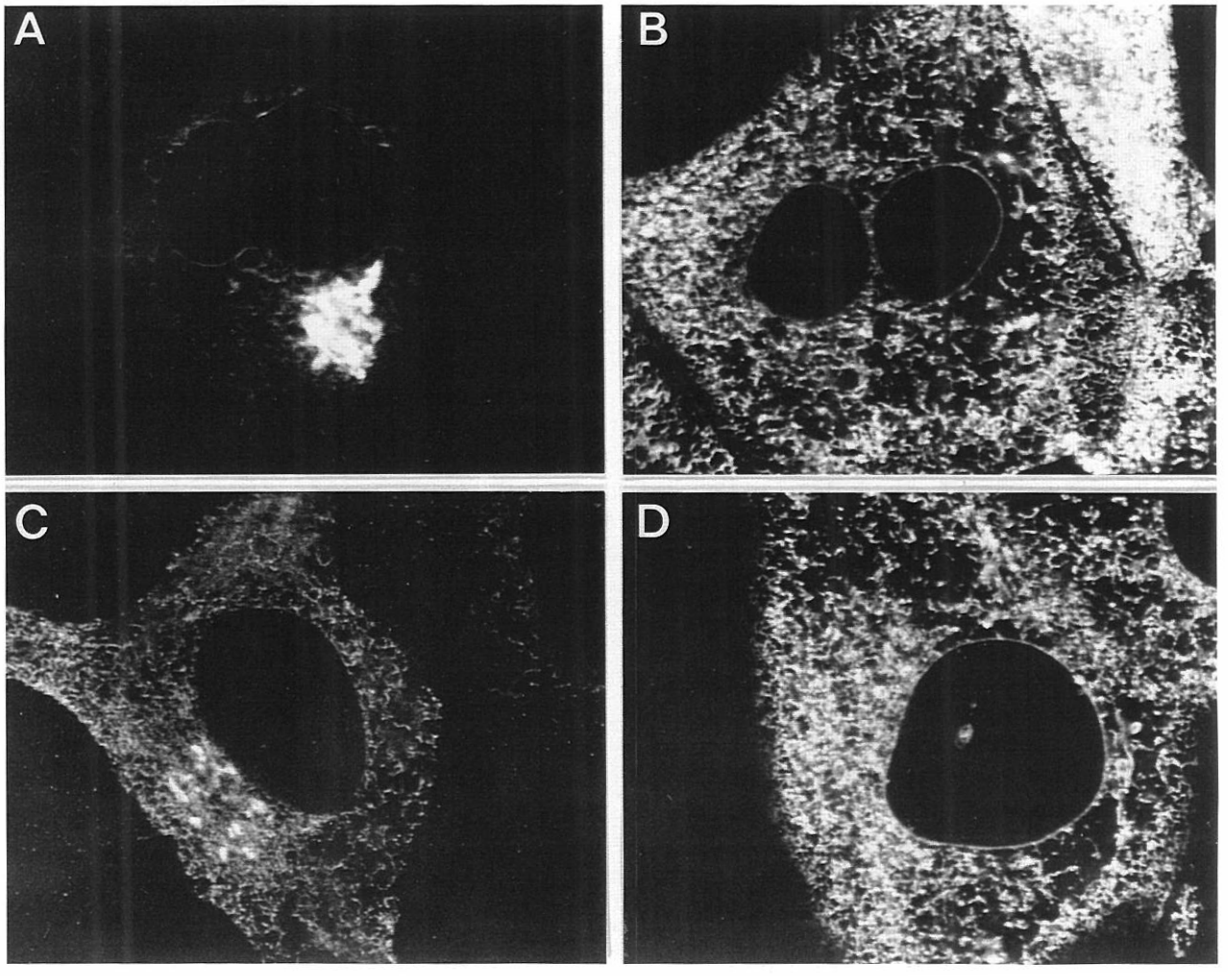


Figure 8. Stained COS Cells Viewed with the Confocal Scanning Laser Microscope

All samples were stained with the anti-*myc* monoclonal, and the microscope was focused about midway through the nucleus. Cells were transfected with the following constructs: (A) SAYM2 (secretable lysozyme, equivalent to Figure 7A); (B) SAYMK2 (lysozyme with the retention signal, equivalent to Figure 7C); (C) SAGM6 (grp78 with the retention signal deleted); (D) SAGMK1 (a derivative of grp78 that retains the KDEL signal). Golgi staining is visible in (A) and (C), but not in (B) or (D).

using a confocal scanning laser microscope developed at the MRC Laboratory of Molecular Biology by John White (to be described elsewhere). This microscope uses a scanning laser beam to illuminate the specimen, thus reducing the glare from out-of-focus material and allowing the visualization of an optical section of the stained cells that is about 1 μm thick. Examples of the images obtained are shown in Figure 8. Figures 8A and 8B show the distribution of lysozyme with and without the retention signal, respectively. The images confirm the results obtained by conventional microscopy and allow detailed examination of the perinuclear distribution of the protein containing the retention signal (Figure 8B). There is no sign that this protein is concentrated in the Golgi.

Figure 8C shows a cell transfected with SAGM6, which encodes a grp78 mutant lacking the carboxy-terminal 15 amino acids. This protein is secreted much more slowly than lysozyme, and the ER staining is much more prominent than in Figure 8A, indicating that transport from the ER to the Golgi is rate-limiting. Nevertheless, Golgi staining is also clearly visible. In contrast, cells transfected with SAGMK1, a grp78 derivative containing the retention signal (Figure 2), show intense staining of the ER and nuclear envelope, but not of the Golgi (Figure 8D). An identical though fainter pattern was obtained when the monoclonal antibody described by Bole et al. (1986) was used to visualize the endogenous grp78 in COS cells (data not shown). Taken together, the immunofluorescent images strongly support the conclusion that the retention signal contained in the last six amino acids of grp78 prevents net movement of proteins from the ER to the Golgi.

Discussion

A Signal That Prevents Soluble ER Proteins from Being Secreted

We have shown that a short sequence at the carboxyl terminus of GRP78 is both necessary to prevent loss of this protein from the ER and sufficient to cause retention of a normally secreted protein in the ER. Since there are presumably fewer types of soluble proteins resident in the

ER than there are secreted proteins, the existence of a constitutive secretion mechanism and a specific retention signal seems an economical way for cells to solve this particular sorting problem. The signal itself has to be at the carboxyl terminus and must comprise more than the terminal leucine residue but not more than the last six residues of grp78. Comparison of the sequences of different ER proteins suggests that the tetrapeptide Lys-Asp-Glu-Leu (KDEL in the one-letter code) is the signal (Figure 1).

It is hard to rule out the possibility that changes in one region of a protein have a global effect on its folding and thus influence its behavior in an indirect fashion. Such problems can be minimized by comparing several different mutants, and by examining the effects of minimal changes. Five different alterations involving the KDEL sequence of grp78 caused its secretion, whereas a similar change that leaves KDEL intact did not. Only one of eight different additions to the carboxyl terminus of lysozyme resulted in its retention—the one that adds KDEL to the protein. In each case, changes involving only two amino acids were sufficient to convert a secreted protein to a retained one or vice versa. The simplest interpretation of these results is that the carboxyl terminus is recognized directly, and any indirect effects on protein folding are negligible. Although recognition of KDEL may well be influenced by its accessibility or by local features such as a net negative charge in the region, but it appears that the signal is a linear sequence rather than a complex three-dimensional structure.

The absence of a KDEL signal does not guarantee that a protein will leave the ER, and those that do leave do so at very different rates (compare truncated grp78 and lysozyme in Figure 4 and Figure 6; for review see Gebhart and Ruddon, 1986). One reason why some are not transported efficiently may be that they bind to other proteins that have a retention signal. For example, a number of mutant or unfolded proteins are known to bind tightly to grp78 ("Binding Protein") (e.g., Gething et al., 1986; Sharma et al., 1985), and as we have suggested previously, this may provide a mechanism by which they are held in the ER until their assembly is complete (Pelham, 1986).

Our experiments do not address the question of how transmembrane proteins that are restricted to the ER are distinguished from membrane proteins destined for other cellular compartments. At least some of these proteins have their carboxyl termini on the cytoplasmic side of the ER membrane and thus cannot be sorted by the KDEL system (unless they bind to a protein in the lumen). Indeed, deletion of the membrane anchor results in secretion of two viral proteins that are normally found in the ER membrane, one of which has its carboxyl terminus in the lumen (Paabo et al., 1986; Poruchynsky et al., 1985). The existence of a cytoplasmic domain on such proteins and their restriction to a two-dimensional compartment may allow quite different sorting mechanisms to operate.

Possible Mechanisms for Retention in the ER

What recognizes the KDEL sequence? One simple possibility would be that the ER proteins that appear to be soluble when cells are lysed are normally anchored to the membrane via an integral membrane protein that binds the KDEL sequence. A precedent for this would be the retention of β-glucuronidase, a portion of which is held in the ER by tight stoichiometric interaction with a membrane protein termed egasyn (for references see Medda and Swank, 1985). Such a mechanism seems unlikely for two reasons. First, although the β-glucuronidase/egasyn complex can readily be identified by immunoprecipitation, no such complexes have been identified for grp78, grp94, or protein disulphide isomerase; on the contrary, they are readily soluble and rapidly released upon homogenization of the ER (Bole et al., 1986; Koch et al., 1986; Freedman, 1984). In the case of grp78, complexes with newly synthesized secreted proteins (such as immunoglobulin heavy chains) can be found, but no anchoring protein has been detected. Indeed, the postulated roles of proteins such as grp78 and the disulphide isomerase in protein assembly (Pelham, 1986; Freedman, 1984) may require that they are free to reach their substrates in the lumen. Second, grp78 and grp94 are two of the most abundant proteins in the ER, and there is no obvious candidate for a protein that is sufficiently abundant to bind all of them. Moreover, overproduction of grp78 by at least 7-fold does not result in its secretion from COS cells (Figure 3). This apparent inability to saturate the retention system argues against simple stoichiometric binding.

An alternative possibility might be that the KDEL sequence is recognized by an enzyme that modifies the protein in some way, for example by addition of a lipid molecule. Such catalytic action would be relatively resistant to saturation. However, the modified protein would still have to be sorted. Moreover, there is no evidence for such modification; the proteins do not appear membrane-bound, and the lysozyme derivative with KDEL at its carboxyl terminus, which is retained, has exactly the same mobility on SDS-polyacrylamide gels as the version with KDAS, which is secreted (Figure 6 and unpublished observations).

The data could be explained if the proteins were not held in the ER, but instead were continually retrieved from some later point on the secretion pathway and returned to it. The contents of the ER is thought to be delivered to the *cis* Golgi by a rapid and efficient process of vesicle budding and fusion (Palade, 1975). Since there is no evidence for membrane traffic to the ER from the *trans* side of the Golgi complex or from the plasma membrane, it seems likely that there is a reverse flow from the *cis* Golgi (or from something close to it in the pathway) to the ER. In principle, a specific receptor could recognize soluble ER proteins that had been delivered to the *cis* Golgi along with secreted proteins and carry them back to the ER. Such a receptor would bind KDEL in the Golgi environment, but not in the ER. It would be difficult to saturate because it would act catalytically and be concentrated in a relatively small area; if retrieval were efficient, there would be no accumulation of the proteins in the Golgi. This model is very similar to the one proposed by Rothman (1981) for sorting of ER membrane proteins.

Whatever the retention mechanism, there must be

some cellular component that recognizes the KDEL sequence. It should be possible to test various sorting models by identifying and characterizing this component.

Experimental Procedures

Plasmids

The COS cell expression vectors are based on plasmid AH5. This plasmid contains the SV40 origin, the adenovirus major late promoter, and the Drosophila hsp70 gene between unique HindIII and SalI sites. A derivative, AHP2, has the SalI site converted to an EcoRI site (Munro and Pelham, 1984). SAG1 was formed by insertion of a NaeI–SspI fragment containing the complete grp78 coding sequence from clone R76 (Munro and Pelham, 1986) between the HindIII and EcoRI sites of AHP2, using intermediate cloning between the PstI and XbaI sites (blunted) of pUC12 to generate the appropriate sites at either end of the gene (the XbaI site, at the 3′ end of the gene, was reformed). The gene was truncated in SAG4 by fusion of the DraII site at amino acid 622 to the SalI site of AH5.

The amino-terminal deletion in SIG2 was formed by replacing the fragment in SAG1 between the HindIII site and the FokI site at amino acid 13 with an AvaII–PstI fragment that encodes the leader sequence and first five amino acids of an immunoglobulin heavy chain (Neuberger, 1983). The PstI–FokI junction contains sequences from the pUC12 polylinker (PstI to filled-in AccI). The amino-terminal region of SIGM2 is similar, but the polylinker-derived sequences are slightly different (blunted PstI to filled-in SalI), generating a different amino acid sequence at this point (see Figure 2).

SAGGL was made using a Bal31 deletion mutant of the grp78 cDNA generated during sequencing. This removed the termination codon, but a new one is formed by an adjacent polylinker-derived XbaI site. The mutated carboxyl terminus (KpnI–XbaI) was used to replace the corresponding region in SAG1.

Sequences encoding the c-*myc* epitope were originally defined by Bal31 deletion of the human c-*myc* gene and are flanked by a 5′ NcoI site and a 3′ EcoRI. Clones containing this sequence were constructed using a derivative of AHP2 from which other NcoI sites had been removed. In SAGM2, SIGM2, and SAGMK1 the NcoI site was joined to the NcoI site in the grp78 gene. In SAGM6 the (filled-in) NcoI site was first fused to a (filled-in) SalI site, which was then cut, filled in, and fused to the (filled-in) DraII site at amino acid 622. In SAGM2, SIGM2, and SAGM6 the EcoRI site at the 3′ end of the *myc* sequence was filled in to generate an in-frame termination codon. For SAGMK1, a double-stranded synthetic oligonucleotide with EcoRI sticky ends that encoded the last six amino acids of grp78 and a termination codon (AATTCGGAGAAGGATGAGCTCTAG/AATT) was cloned into the EcoRI site at the 3′ end of the *myc* sequence.

The chick lysozyme cDNA sequence with a HindIII site introduced just upstream of the ATG was obtained from Alan Colman (Krieg et al., 1984). A HindIII–NaeI fragment encoding all but the last two amino acids of the protein was cloned between the HindIII and HincII sites of pUC19; this results in a sequence that fortuitously encodes the wild-type carboxyl terminus, termination occurring within the adjacent XbaI site. The HindIII–XbaI fragment was then used to replace the grp78 gene in SAG1, forming SAY1. SAYM2 was formed by inserting the original HindIII–NaeI fragment between the HindIII and NcoI (filled) sites of SAGM2, and then filling in the (reformed) NcoI site to adjust the reading frame. SAYMK2 was formed similarly from SAGMK1. The synthetic oligonucleotide (see above) encoding the SEKDEL sequence of SAYMK2 was designed such that the last two codons and the termination codon consist of overlapping SacI and XbaI sites. The carboxyl terminus of SAYMK1 was modified to form SAYMJ1 by filling in the XbaI site and then trimming the overhanging ends of the SacI site with Klenow, which has the effect of changing the last two codons from Glu-Leu to Ala-Ser.

The amino acids encoded by the critical regions of these plasmids are shown in Figure 2 and Figure 6. Junction sequences and the orientation of synthetic oligonucleotide inserts were confirmed by restriction enzyme digestion and/or by DNA sequencing of appropriate plasmids.

Transfection of Cells and Analysis of Proteins

COS cells were transfected in the presence of 0.5 mg/ml DEAE-dextran, followed by a 3 hr treatment with 100 μg/ml chloroquine in complete medium, as previously described (Pelham, 1984). After 40–50 hr they were washed in serum-free medium and then incubated in the absence of serum for 2–4 hr. For labeling, this incubation was in methionine-free medium to which 100 μCi/ml [^{35}S]Met had been added. The medium was then removed from the cells, ovalbumin was added to 30 μg/ml, and the protein was precipitated with 10% trichloroacetic acid.

Samples of cells or culture medium were analyzed on SDS-polyacrylamide gels and either fluorographed using Amplify (Amersham) or transferred to nitrocellulose for reaction with antibodies as previously described (Munro and Pelham, 1984). Usually equivalent amounts of the cells and culture medium were analyzed, but in Figures 3A and 3C, where labeling was for only 2 hr, five times as much material from the medium as from the equivalent cell pellet was loaded on the gel.

For immunoprecipitation, samples of culture medium or the post-nuclear supernatant of Nonidet P-40-lysed cells were incubated with rabbit anti-grp78 serum. Immune complexes were recovered using protein A-Sepharose as described previously (Munro and Pelham, 1986).

Antibodies

Polyclonal rabbit anti-rat grp78 prepared against purified, denatured protein was provided by M. Lewis. A mouse monoclonal antibody (9E10) raised against a synthetic peptide comprising residues 409–439 of human c-*myc* (Evan et al., 1985) was a gift from G. Evan. A rat monoclonal anti-hsp70 (7.10), which cross-reacts with denatured grp78 (see Munro and Pelham, 1986), was provided by S. Lindquist. A mouse monoclonal that recognizes native (but not denatured) chick egg-white lysozyme (D1.3; Mariuzza et al., 1983) was a gift from J. Foote. A rat monoclonal antibody raised against mouse grp78 (BiP) (Bole et al., 1986) was provided by D. Bole. The anti-*myc* antibody was detected on blots with ^{125}I-labeled F(ab′)$_2$ sheep anti-mouse Ig (Amersham). For immunofluorescence, it was visualized with FITC-labeled rabbit anti-mouse IgG (Miles) that was affinity-purified by J. Kilmartin. Wheat germ lectin (Pharmacia) was conjugated to rhodamine by M. Robinson (Robinson and Pearse, 1986).

Immunofluorescence

Cells were transferred to slides and processed for immunofluorescence as described previously (Pelham, 1984), except that the cells were fixed with 2% paraformaldehyde/0.1% glutaraldehyde, permeabilized in 1% Triton X-100 in phosphate-buffered saline, and treated for 10 min with 1 mg/ml $NaBH_4$ (BDH, freshly dissolved in phosphate-buffered saline). Inclusion of glutaraldehyde in the fixation proved essential for good preservation of ER structure. Stained cells were photographed on a Zeiss Standard microscope fitted with epifluorescent illumination, using Kodak Technical Pan film. Images from the same slides were generated by the confocal scanning laser microscope on a video monitor, and this was photographed using Ilford FP4 film. The laser microscope was designed by John White and built at the MRC Laboratory of Molecular Biology; it will be described in detail elsewhere (J. White and B. Amos, personal communication).

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