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Posttranslational Removal of the Carboxyl-terminal KDEL of the Cysteine Protease SH-EP Occurs Prior to Maturation of the Enzyme*

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SH-EP is a cysteine protease from germinating mung bean (*Vigna mungo*) that possesses a carboxyl-terminal endoplasmic reticulum (ER) retention sequence, KDEL. In order to examine the function of the ER retention sequence, we expressed a full-length cDNA of SH-EP and a minus-KDEL control in insect Sf-9 cells using the baculovirus system. Our observations on the synthesis, processing, and trafficking of SH-EP in Sf-9 cells suggest that the KDEL ER-retention sequence is posttranslationally removed either while the protein is still in the ER or immediately after its exit from the ER, resulting in the accumulation of proSH-EP minus its KDEL signal. It is this intermediate form that appears to progress through the endomembrane system and is subsequently processed to form mature active SH-EP. The removal of an ER retention may regulate protein delivery to a functional site and present an alternative role for ER retention sequences in addition to their well established role in maintaining the protein composition of the ER lumen.

SH-EP is a thiol protease expressed in germinating mung bean (*Vigna mungo*) cotyledons (1–3) that is among a large number of similar cysteine proteases that are synthesized after germination in order to degrade seed storage proteins (2, 4–10). All cysteine proteases are initially synthesized as larger precursor proteins with NH_2-terminal prodomains of approximately 120 amino acids (11). The prodomain is an inhibitor of the protease (12) and is essential in the correct folding of the protein (13). Processing of the proenzyme occurs by self-catalysis (14) in post-Golgi compartments of the secretory system. With intracellular proteases, this processing occurs in the lytic compartment (lysosomes or vacuoles) (15); with extracellular proteases, the processing occurs during secretion (16). Targeting to the correct compartment for processing is mediated by a peptide (17) or phosphomannose (18) targeting signal on the NH_2-terminal precursor domain that is recognized by a specific receptor that is presumed to be localized in the Golgi apparatus (19, 20) to target the protein to the activating compartment.

SH-EP is unusual because it and a few closely related thiol proteases (21–23) are the only cysteine proteases known to possess a carboxyl-terminal ER[1] retention sequence KDEL (24–26). Although papain superfamily proteases are widely distributed among eukaryotes (27, 28), only these plant cysteine proteases have been identified as possessing a carboxyl-terminal ER retention sequence. Several other legume cysteine proteases have been cloned that do not possess a KDEL tail (28–34), indicating that even among legumes and more broadly in plants these KDEL-proteases appear to constitute a special class. Plant cells utilize both HDEL and KDEL as ER retention sequences (35–38). The presence of a carboxyl-terminal KDEL in SH-EP raises a question as to whether this sequence is functional *in vivo*. A papain superfamily cysteine protease would appear to be an unusual putative constituent of the ER lumen, which is the site of glycosylation and folding in the initial steps of protein assembly. SH-EP is a general protein hydrolase whose activity is lytic and not a protein that would be expected to be retained in the ER lumen. Moreover, although cysteine proteases are self-processed (39–42), other enzymes such as the asparaginyl endopeptidase (43, 44) or vacuolar processing enzyme (VPE) (45, 46) may also be required. The necessary acid/reducing conditions for activation are available in the vacuolar lytic compartment (47) and within secretory vesicles exiting the *trans*-Golgi; however, these conditions are absent in the ER lumen. Consequently, KDEL-mediated retention of SH-EP in the pre-Golgi endomembrane system would be expected to inhibit its processing and activation, resulting in the accumulation of the inactive precursor enzyme in the ER lumen.

In this paper, we describe the processing of SH-EP expressed in Sf-9 cells. We demonstrate that although the proSH-EP is synthesized with a KDEL sequence, this sequence is posttranslationally removed from the proSH-EP. The proSH-EP without KDEL is subsequently processed to form mature SH-EP in a multistep process as observed in seeds (2, 43) that results in a KDEL-minus mature 33-kDa active protease (48). We hypothesize the KDEL sequence on the inactive proSH-EP functions to permit the seed cells to build up an inventory of precursor protein in the ER that can then be delivered to the vacuole to rapidly mobilize the stored protein. We propose that the temporary sequestration of a KDEL-tailed protease precursor has

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[1] The abbreviations used are: ER, endoplasmic reticulum; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy.

been evolved to exploit the ER-retention system store proSH-EP as a "transient zymogen."

EXPERIMENTAL PROCEDURES

Expression of SH-EP in Escherichia coli—SH-EP lacking putative signal peptide was expressed in *E. coli* BL21(DE3) and purified as described (44).

Preparation of Recombinant Baculovirus—The DNA insert of full-length SH-EP cDNA was cut out of pBLUESCRIPT II KS$^+$ vector by *Pst*I and *Bam*HI, and was subcloned into pVL1393 baculovirus transfer vector cut by the same enzymes. Using the pVL1393 vector harboring SH-EP cDNA and BaculoGold™ transfection kit (PharMingen), recombinant baculovirus containing KDEL-plus SH-EP was prepared and amplified according to manufacturer's instructions. To prepare recombinant baculovirus for KDEL-minus SH-EP, two kinds of mutagenic primers (TCCTAGGAACAGTAGAGGGATTCTACTTGA and CCCTCTACTGTTCCTAGGACAACCTAACAG) and pBLUESCRIPT II KS$^+$ vector harboring SH-EP cDNA as a template were used for recombination PCR. The PCR reaction was proceeded in 100 μl for 30 cycles (94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min), and following the last cycle, the reaction was incubated at 72 °C for 5 min. After gel electrophoresis, the amplified fragment was cut from the gel and transformed directly to *E. coli* JM109. The recombinant baculovirus containing KDEL-minus SH-EP was prepared as KDEL plus SH-EP. After isolation of plasmids, the introduce of mutation was checked by digestion with *Bam*HI, which is contained the in primer sequence and the mutant DNA was sequenced by dye termination cycle sequencing kit (ABI, Foster City, CA) and analyzed using an ABI 373A sequencer.

Active Site Cysteine Was Mutated to Glycine by PCR— Two primers (TGTGGTAGCGCTCTGGGCGTTTTC and AACGCCCAGCCGCTACCACATTG) were used for mutation of the cysteine residue to glycine (C26G) and the pBLUESCRIPT II KS$^+$ vector with SH-EP cDNA as template. The condition of PCR, isolation of mutant DNA, and the DNA sequencing were run as same as above. The SH-EP (C26G) cDNA was excised from the vector by *Pst*I and *Bam*HI and introduced into the *Pst*I-*Bam*HI site of pVL1393 vector. The sequence alteration was verified by DNA sequencing.

Production of KDEL Plus or Minus SH-EP and Cys-26 to Gly (C26G) Mutants in Sf-9 Cells—Sf-9 cells were grown at 27 °C in TNM-FH medium (49) supplemented with 10% fetal bovine serum. Cultures were grown in 25-cm^2 culture dishes. Cells at the density of 5×10^5 cells/cm^2 were infected with recombinant baculovirus at a multiplicity of infection of 2. One to 4 days after infection, the media were collected and centrifuged at 200 $\times$ *g* for 5 min, and supernatants were retained. Remaining cells attached to culture dishes were peeled off with serum-free Grace's medium (Life Technologies, Inc.). The resuspended cells were centrifuged at 200 $\times$ *g* for 5 min, and the pellet was washed with serum-free Grace's medium and centrifuged again. The pellet was then resuspended with 0.1 M Tris-Cl (pH 8.0) and subjected to sonication (5 $\times$ 30 s, 30 watts, UR-20P, Tomy Seiko Co., Ltd.). The sonicated cells were centrifuged at 20,000 $\times$ *g* for 5 min, and the supernatants were used as cell fractions. Protein content was measured with the Bradford method using bovine serum albumin as a standard.

SDS-PAGE, Immunoblots, NH_2-terminal Protein Sequencing, and Activity Staining—SDS-PAGE was conducted on 12.5% gel, and immunoblotting using a rabbit antiserum raised against SH-EP was performed as described elsewhere (2). A monoclonal antibody (1D3) was provided from Dr. S. Fuller, European Molecular Biology Laboratory, Heidelberg (50). Non-denaturing PAGE and activity staining was carried out as described previously (4). Automated NH_2-terminal protein sequencing was obtained by analysis of 43- and 42-kDa proSH-EP excised from Coomassie Blue-stained polyvinylidene difluoride membrane blot of SDS-PAGE as described (51).

Sucrose Gradient Centrifugation of Sf-9 Cell Lysates—Isopycnic sucrose centrifugation was accomplished by lysing 3 day postinfection Sf-9 cells in 17% w/v sucrose in 25 mM HEPES pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride with a glass hand homogenizer. The lysate was centrifuged at 5,000 rpm for 5 min in a Sorvall RC-2b centrifuge. The supernatant was layered on a continuous 17–45% sucrose gradient and centrifuged for 16 h at 7 °C in a Beckman SW-41 rotor at 30,000 rpm. After the run was completed, the gradient was fractionated into 0.7-ml fractions and to an aliquot of each an equal volume of 20% trichloroacetic acid was added to precipitate total proteins. The resulting precipitate was washed with cold acetone and processed for SDS-PAGE immunoblot as described above.

Electron Microscopy and Immunocytochemistry—Sf-9 cell samples (3 day postinfection) of KDEL-plus and KDEL-minus SH-EP for electron microscopy were fixed in 2% glutaraldehyde, 4% formaldehyde, 50 mM potassium phosphate, pH 7.4. The fixed cells were dehydrated in a graded ethanol series and embedded in LR White resin. Immunogold labeling of thin sections was accomplished by blocking in 10% fetal bovine serum in Tris-buffered saline with Tween (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% Tween 20) for 15 min at room temperature. The sections were then labeled in primary anti-SH-EP sera diluted 1:25 in blocking solution for 30 min at room temperature. The bound IgGs were indirectly labeled by 10-nm goat anti-rabbit IgG-colloidal gold (Ted Pella, CA) solution diluted 1:1 in the blocking solution for 5 min at room temperature. After labeling the sections were washed with Tris-buffered saline with Tween and distilled water and then stained with 5% (w/v) uranyl acetate for 20 min. The samples were visualized in a Phillips 400 electron microscope and the images captured by Photometerics Sensys 1400 CCD camera interfaced to a Power Macintosh computer.

Sucrose Gradient Centrifugation of Microsomes from Cotyledon Cells—*V. mungo* seeds were germinated on layers of wet filter paper at 27 °C in darkness, and cotyledons were collected on day 3 post-imbibition. Day 3 cotyledons (25 g) of *V. mungo* seedlings were gently ground in a mortar and pestle with 62.5 ml of 0.2 M Tris-Cl, pH 7.4, containing 0.44 M sucrose, 1 mM EDTA, and 0.1 mM $MgCl_2$. The homogenate was centrifuged at 800 $\times$ *g* for 10 min and then at 4,500 $\times$ *g* for 30 min. The supernatant was again centrifuged at 100,000 $\times$ *g* for 60 min, and the precipitate was used as a microsomal fraction. The fraction was washed twice with the homogenization buffer and resuspended with 1.5 ml of the buffer. The dissolved solution was centrifuged at 200 $\times$ *g* for 5 min, and the supernatant was layered on a continuous 0.6–1.6 M sucrose gradient and centrifuged at 100,000 $\times$ *g* for 17 h. After the run was completed, the gradient was fractionated into 0.9-ml fractions and each fraction was analyzed by SDS-PAGE immunoblotting with antiserum against SH-EP or maize BiP (52).

RESULTS

Heterologous Expression of SH-EP in Sf-9 Cells—Prior research has demonstrated that papain can be expressed in insect Sf-9 cells using the baculovirus system (53). All members of the papain superfamily of cysteine proteases are highly homologous, which suggests that Sf-9 expression would be a suitable heterologous system to elucidate the processing steps and would allow us to compare the process of SH-EP activation with observations on the papain archetype. Other plant proteins including seed storage proteins have been successfully produced in baculovirus (54). The exposure of KDEL sequences has been evaluated by expression of auxin-binding protein in Sf-9 cells (55), indicating that heterologous expression would be useful to evaluate the exposure of SH-EP's KDEL sequence. cDNAs of the full-length plus-KDEL SH-EP and a minus-KDEL mutant were cloned into a transfer vector and used to form a recombinant infective particle *in vivo* using vectors and protocols from a commercial vendor (PharMingen). The minus-KDEL control consisted of a mutating the wild type cDNA by site-directed mutagenesis using PCR to change the Lys residue in the carboxyl-terminal KDEL from AAA (Lys) to TAA (stop).

Time Course of Expression of Plus-KDEL and Minus-KDEL cDNAs of SH-EP—The synthesis and processing of plus-KDEL and minus-KDEL SH-EP was monitored by infecting Sf-9 cells with the recombinant baculovirus and then conducting a time-course assay of the Sf-9 cells and the surrounding medium by SDS-PAGE immunoblot. Fig. 1*A* shows a comparison of the distribution of SH-EP immunoreactive polypeptides in the cells and medium for both plus-KDEL and minus-KDEL. The cells expressing plus-KDEL at 1 day postinfection contain a prominent 43-kDa immunoreactive polypeptide that continued to accumulate through day 3 postinfection, and decreases slightly in abundance by day 4. The 43-kDa band appeared to broaden to a doublet on day 3 postinfection, and by day 4 only the lower half of the doublet remained. On the second day, the 39-kDa intermediate polypeptide was detected. By the fourth day, little of the 39-kDa polypeptide remained, whereas the amount of

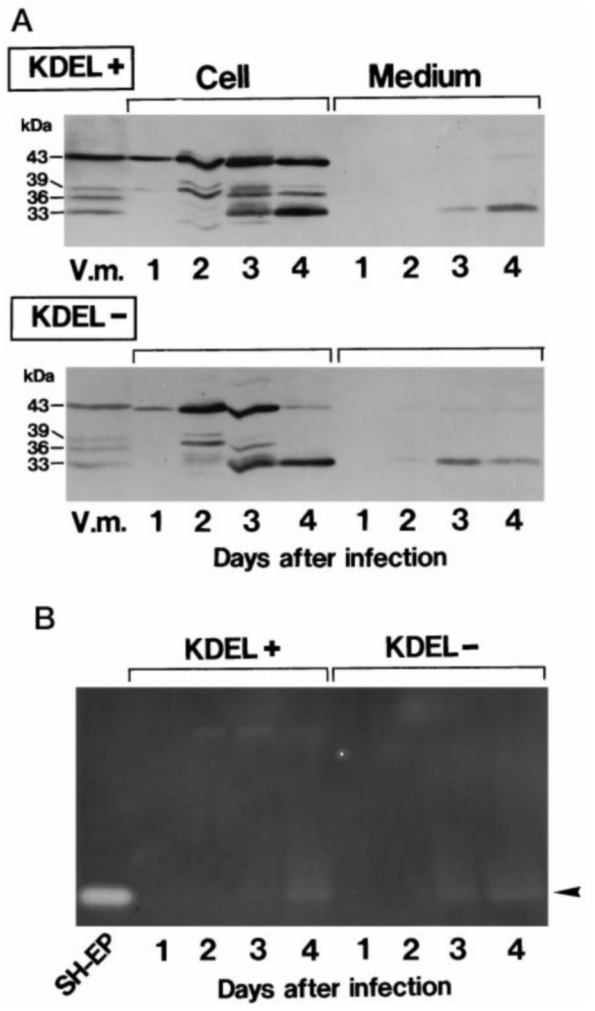

FIG. 1. **Changes with time in molecular mass and endopeptidase activity that accompany expression of plus- or minus-KDEL SH-EP in Sf-9 cells.** Sf-9 cells were infected with plus- or minus-KDEL SH-EP baculovirus, and cell and medium fractions were prepared after one to four days postinfection. *A*, cellular fraction (10 μg of protein) and medium fraction (5 μl) were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with an antiserum against SH-EP. *V.m.*, lysate from *V. mungo* seeds which provides control polypeptides for SH-EP and its processing intermediates. *B*, cellular fraction (10 μg of protein) was separated by 10% nondenaturing-PAGE, and endopeptidase activity in the gel was visualized with gelatin plate method (2). Purified mature SH-EP from *V. mungo* cotyledons was loaded on SH-EP lanes.

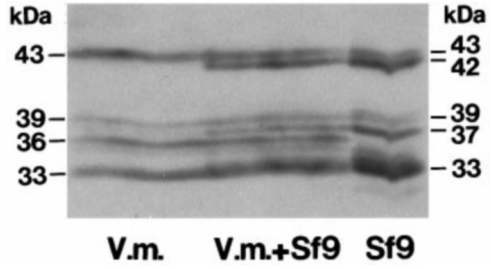

FIG. 2. **Comparison of molecular masses of SH-EP related polypeptides expressed in Sf-9 cells with those in *V. mungo* cotyledon.** Extracts prepared from 3-day-old cotyledons (0.2 mg of protein) and 3-day postinfection Sf-9 cells after infection with plus-KDEL SH-EP baculovirus (10 μg of protein) were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with an antiserum against SH-EP. *V.m.*, extract from cotyledons; *Sf9*, extract from Sf-9 cells; *V.m.+Sf9*, mixture from extracts from cotyledons and Sf-9.

37-kDa polypeptide appeared unchanged. Mature 33-kDa SH-EP was not observed until the third day postinfection and increased by the fourth day. Parallel analysis of the culture supernatant indicated that secretion of SH-EP was delayed until the third day, with increasing quantities of the enzyme observed by the fourth day. Only mature 33-kDa SH-EP was secreted, although a faint band corresponding to the position of proSH-EP can be discerned. The presence of this protein may result from lysis of Sf-9 cells that occurs at increasing rate by the fourth day.

Parallel expression of the minus-KDEL resulted in a similar pattern of initial accumulation of the precursor, followed by progressive processing and accumulation of the mature form during the 4-day time course. However, the minus-KDEL was processed more quickly than the plus-KDEL. By the fourth day postinfection, little proSH-EP or intermediate forms remained in the cell; all of the SH-EP appeared to be either intracellular or secreted 33-kDa mature SH-EP. Enhanced processing and secretion of minus-KDEL indicates that the presence of the KDEL functions to retard SH-EP in the endomembrane system in location(s) where it is unable to be processed to the mature active enzyme. In controls using uninfected Sf-9 cells, no immunoreactive polypeptides were detected using anti-SH-EP antiserum in either cellular or medium fractions (data not shown). Time course experiments of expression of KDEL-plus and -minus SH-EP in Sf-9 cells were independently performed in triplet, and the results of synthesis and processing of SH-EPs were almost same (data not shown).

The Presence of the KDEL Sequence Does Not Influence Maturation and Activity of Mature SH-EP—In order to test whether the KDEL is necessary for folding the SH-EP to result in an active mature protein the activity of the recombinant SH-EP initially synthesized with and without KDEL was compared. Cellular fractions were prepared from Sf-9 cells from 1 to 4 days postinfection, and the samples were assayed using a gel-based activity staining for proteolysis (Fig. 1*B*). This shows that the control purified 33-kDa mature SH-EP is visualized as a single digested band on the activity gel (*SH-EP lane*). The cDNA clones encoding both the plus- and minus-KDEL proteins also yielded active proteolytic enzymes with the same mobility as the purified protein (*plus-KDEL lanes 1–4*; *minus-KDEL lanes 1–4*). The accumulation of activity is directly in accord with the pattern of accumulation of immunoreactive 33-kDa mature SH-EP. The SH-EP protein initially synthesized as minus-KDEL yields proteolytically active mature SH-EP on days 3 and 4, whereas the SH-EP protein initially synthesized as plus KDEL yields a proteolytically active protein only weakly in day 3 and at the same level as the minus-KDEL protein on day 4. Thus, the KDEL is not required to produce a correctly folded protein that undergoes maturation to an enzymatically active form.

Posttranslational Processing of SH-EP in Sf-9 Cells Occurs in a Series of Steps Similar to That in Mung Bean Cells—To determine whether heterologously expressed SH-EP would be processed analogously to SH-EP from *V. mungo*, we compared the molecular mass of SDS-PAGE fractionated lysates from each using immunoblots (Fig. 2). *V. mungo* SH-EP is initially observed as a 43-kDa precursor that is processed through intermediate forms of 39 and 36 kDa to a final product of 33-kDa. Sf-9 cell-expressed SH-EP is also initially observed as a 43-kDa polypeptide; it is then observed as 42-, 39-, and 37-kDa intermediates and finally as a mature 33-kDa form. These differences between the *V. mungo* and Sf-9 processing pattern are shown in the *middle lane* of Fig. 2, which shows cofractionation of mixed lysates of *V. mungo* and Sf-9 cells. The 43-, 39-, and 33-kDa bands clearly merge showing an apparent identity of the processing forms. However, the 36-kDa band from mung bean lysate and the 42- and 37-kDa band from the Sf-9 stand

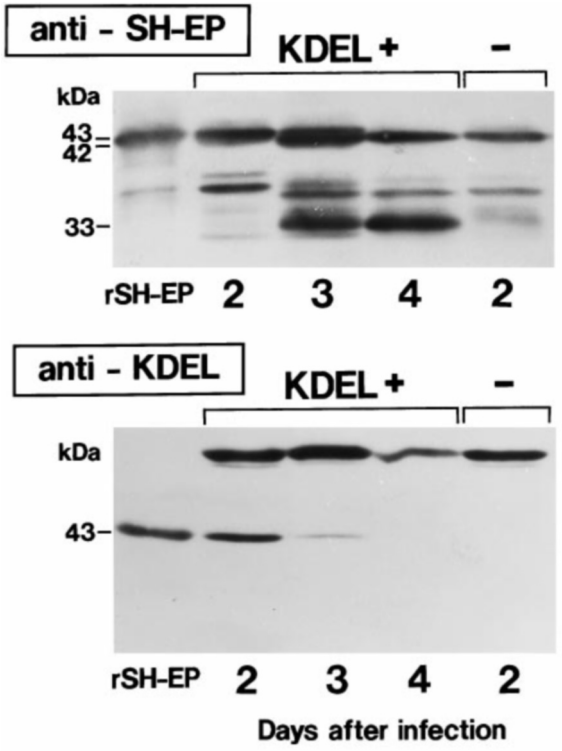

FIG. 3. **Immunoreactivity of SH-EP and its intermediates expressed in Sf-9 cells to the monoclonal antibody 1D3 against the KDEL epitope.** Sf-9 cells were infected with plus- or minus-KDEL SH-EP baculovirus and the cellular fraction was prepared after 2–4 days postinfection. The cellular fraction (10 μg of protein) was separated by 12.5% SDS-PAGE, followed by immunoblotting with an antiserum against SH-EP or a monoclonal antibody 1D3. rSH-EP is *E. coli*-produced proSH-EP to provide a control.

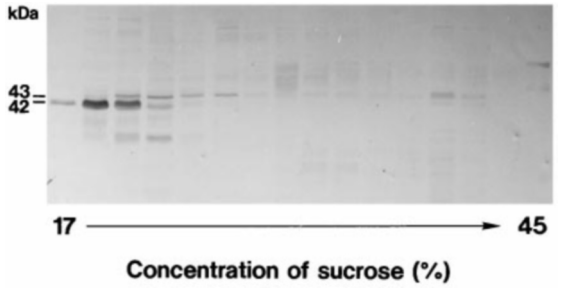

FIG. 4. **Sucrose density gradient analysis of the distribution of 43/42-kDa proSH-EP doublet in Sf-9 cells.** The extract prepared from the 3-day Sf-9 cells after infection with plus-KDEL SH-EP baculovirus was separated with 12 ml of 17–45% (w/v) sucrose gradient. The gradient fractions were analyzed by SDS-PAGE immunoblotting with an antiserum against SH-EP.

out as specific forms.

The KDEL Is Apparently Removed from ProSH-EP—Mature 33-kDa SH-EP isolated from *V. mungo* cotyledons lacks the KDEL sequence (48), suggesting that the KDEL sequence of SH-EP is removed during the maturation process. A time course of plus-KDEL SH-EP baculovirus expression in Sf-9 cells was analyzed by probing parallel SDS/PAGE-immunoblots with anti-SH-EP antibody and with a monoclonal antibody 1D3 specific for the KDEL (50). As Fig. 3, recombinant 43-kDa proSH-EP produced in *E. coli* reacts with both anti-SH-EP and anti-KDEL. On the second day postinfection with the plus-KDEL SH-EP baculovirus, a prominent band at 43 kDa is immunolabeled by both SH-EP and KDEL antibodies, indicating that the proprotein possesses the KDEL retention sequence. In contrast, the 39-kDa partial processing step of SH-EP observed with the anti-SH-EP is not labeled by the anti-KDEL antibody. A parallel control expression of the minus-KDEL baculovirus at day 2 postinfection is labeled only by the anti-SH-EP antibody. On day 3 postinfection with the plus-KDEL baculovirus, the anti-SH-EP antibody labels a doublet at 42/43 kDa as well as the partial and mature processing products of 39, 37, and 33 kDa. The parallel blot labeled with the anti-KDEL antibody exhibited only a weak immunoreaction that is associated with the upper band of the 43/42-kDa proSH-EP doublet. The transition from the 43- to 42-kDa band within the doublet is associated with the loss of the carboxyl-terminal KDEL, and this appears to be initial step in the maturation of plus-KDEL SH-EP expressed in Sf-9 cells. NH_2-terminal sequencing of both the 43- and 42-kDa polypeptides purified from Sf-9 lysates from the third day postinfection by SDS-PAGE and blotting onto a polyvinylidene difluoride membrane showed that both polypeptides had identical FDFHE sequences, which result from cleavage of the signal sequence after amino acid 22. This is identical to the NH_2-terminal sequence of 43-kDa proSH-EP purified from *V. mungo* cotyledons (data not shown). This indicates that the processing of proSH-EP from 43 to 42 kDa does not result from alteration of the protein at the NH_2-terminal end and that the difference between the 43 and 42 kDa must be elsewhere on the polypeptide. On the fourth day postinfection with the plus-KDEL SH-EP baculovirus, the anti-SH-EP antibody labels 42-, faint 39-, 37-, and 33-kDa polypeptides representing the proSH-EP and intermediate steps resulting in maturation of the protease. In contrast, the anti-KDEL antibody does not label any of the SH-EP polypeptides on day 4, indicating that the KDEL sequence was removed from all of the polypeptides. The anti-KDEL-labeled blot also shows a prominent band at approximately 60 kDa in lanes analyzed from Sf-9 extracts from both plus- and minus-KDEL SH-EP baculovirus infection. Although we did not identify the protein labeled by the anti-KDEL antibody, its molecular mass is consistent with its being a reticuloplasmin that serves as an internal control for the immunological cross-reactivity of the anti-KDEL antibody.

ProSH-EP with and without KDEL Is Localized in a Different Subcellular Fractions—To examine the cellular localization of proSH-EP and intermediates Sf-9 cells infected with the plus-KDEL SH-EP baculovirus were lysed 3 days after infection and fractionated on a 17–45% continuous sucrose gradient by isopycnic centrifugation. The resulting fractions were analyzed by SDS-PAGE immunoblot using the SH-EP antibody as a probe (Fig. 4). The 42-kDa proSH-EP band was located at in fractions 2 and 3 and was well separated from the 43-kDa proSH-EP that retains the KDEL in fractions 3–6. The isopycnic density of the 43-kDa peptide-containing fraction is consistent with it containing ER derived microsomes. The 43-kDa SH-EP has been found in ER fractions prepared from lysates of cotyledon cells (48). The separation of the 43- and 42-kDa proSH-EP based on isopycnic density may indicate the possibility that the processing of the 43-kDa proSH-EP to remove the KDEL is coordinated with the protein's exit from the ER and its transfer to another cellular compartment. The 43-kDa proSH-EP is also observed in the fraction near the bottom of the gradient. Whether this fraction constitutes a dense cellular fraction or aggregates of cellular materials remains to be determined.

Immunogold Localization of SH-EP in Sf-9 Cells—To determine what compartments the different forms of SH-EP localize to, immunogold EM localization assays were conducted on plus- and minus-KDEL SH-EP expressed in Sf-9 cells. To maximize the levels of protein, the assays were conducted on 3-day cells. At this point, the lysis of cells is minimized and the population of Sf-9 cells exhibits intact subcellular structures that have not

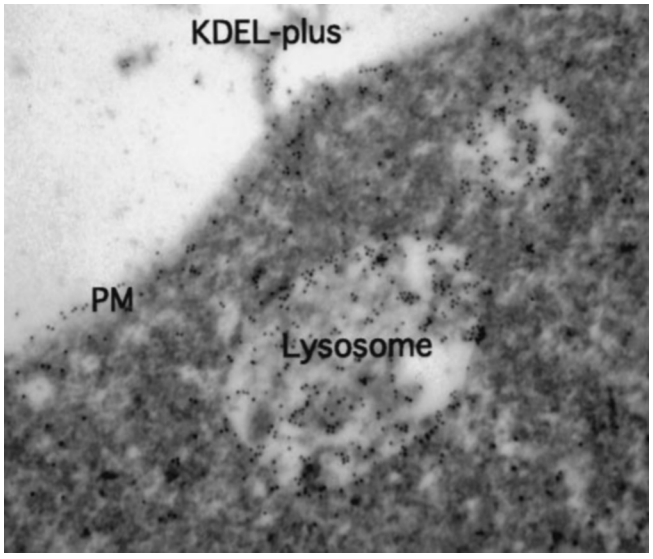

FIG. 5. **EM immunogold analysis of the cellular distribution of plus-KDEL SH-EP in Sf-9 cells.** Immunogold labeling is primarily localized within lysosomes and associated with the exterior of the plasma membrane (*PM*).

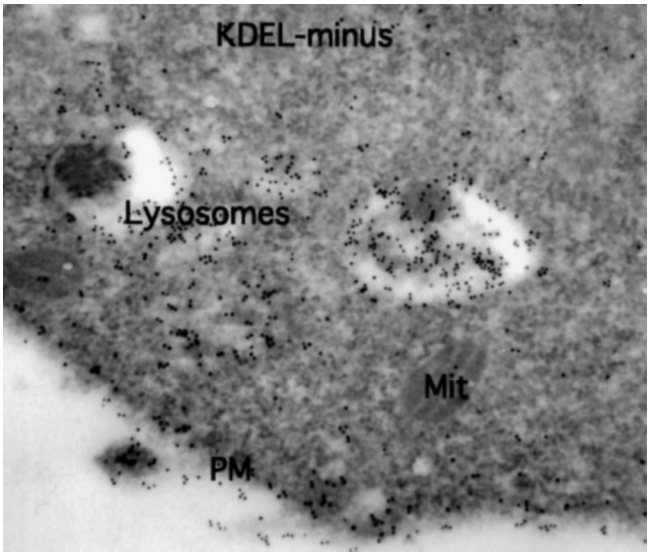

FIG. 6. **EM immunogold analysis of the cellular distribution of minus-KDEL SH-EP in Sf-9 cells.** Immunogold labeling is primarily localized within lysosomes and associated with the exterior of the plasma membrane (*PM*), which appears to be similar to the distribution of SH-EP expressed as plus-KDEL. *Mit*, mitochondria.

yet been disrupted. Expression of the plus- and minus-KDEL SH-EP in the Sf-9 cells results in similar intracellular distribution of the accumulated gene products. Most of the intracellular immunogold labeling was observed to be associated with large lytic compartments (lysosomes) containing disperse protein deposits (Fig. 5 for KDEL-plus and Fig. 6 for KDEL-minus). The presence of abundant SH-EP cross-reactive protein within lytic compartments is consistent with the pattern of processing observed in time-course immunoblots and the accumulation of cellular 33-kDa mature SH-EP. The immunocytochemical observations also provide evidence on the mechanism of mature 33-kDa secretion from the Sf-9 cells. Immunogold labeling of SH-EP associated with the plasma membrane appears to be specific for a surface-associated protein, possibly that adhered to the cell after secretion. The contents of lytic compartments appear to be expelled from the cell by fusion of exosomes to the plasma membrane that appear to be derived from lysosomes (Figs. 7 and 8).

The 42-kDa SH-EP Was Also Detected in Cotyledon Cells and Localized in Subcellular Fraction Distinct from ER—In order to test for the presence of the 42-kDa SH-EP in cotyledon cells, microsomes fractionated by continuous sucrose gradients were analyzed. SDS-PAGE immunoblotting analysis of the fractions using anti-SH-EP antiserum was resulted in detection of the 42-kDa SH-EP near the bottom fraction (fractions 3 and 4) (Fig. 9*B*). When BiP antibody was used for SDS-PAGE immunoblot analysis, an intense band labeling BiP was observed in fractions 5, 6, and 7 (Fig. 9*A*). This indicates that the 42-kDa SH-EP localizes in a more dense cell compartment than main compartment of the ER, whereas band corresponding to the 43-kDa SH-EP was primarily detected in the same fractions with BiP, suggesting that the 43-kDa SH-EP localized in ER (Fig. 9, *A* and *B*). Although the 42-kDa SH-EP was not observed in crude extract from cotyledons (Fig. 2), the procedures for concentration of the subcellular compartment by ultracentrifugation and sucrose gradient resulted in the detection of the 42-kDa SH-EP, probably by enhancing its concentration. The low level of 42-kDa SH-EPs in cotyledon suggests that it is quickly processed to the 39- and 36-kDa SH-EP in cotyledons. This further suggests that the 42-kDa SH-EP is quite transient and may be restricted to a transport compartment bridging the ER where the 43-kDa SH-EP is accumulated and the vacuole

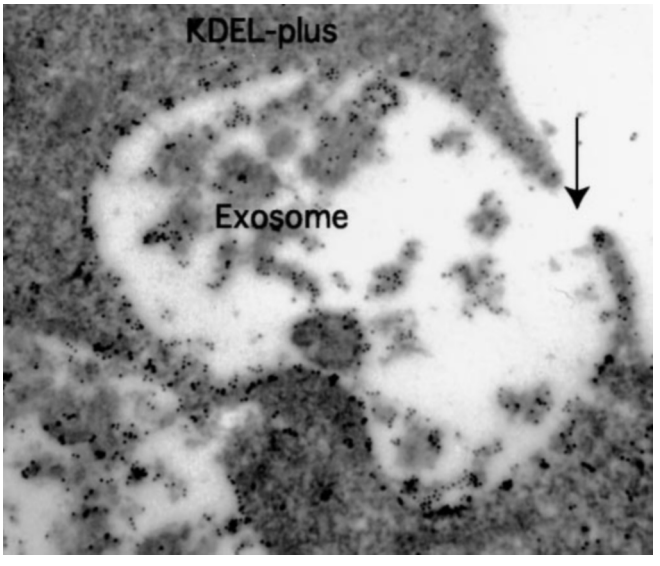

FIG. 7. **EM immunogold assay of apparent secretion of plus-KDEL SH-EP into extracellular space by expulsion of material in an exosome.** The exosomes appear to be identical to putative lysosomes shown in Figs. 5 and 6. These organelles appear to fuse to the plasma membrane to release its contents into extracellular space (*arrow*).

where the 42-kDa SH-EP is processed to the mature active 33-kDa form through two intermediates. Immunogold EM studies are in progress to identify all of the cellular compartments sequestering SH-EP forms. Through such investigations, we hope to identify and characterize the compartment containing the 42-kDa form. The presence of 42-kDa SH-EP in cotyledon cells as well as in the Sf-9 cells indicates that the initial step of processing of proSH-EP, the removal of KDEL, is not an artifact of expression on the heterologous Sf-9 cells.

Mutation of the SH-EP Active Site Cysteine Does Not Inhibit Posttranslational KDEL Removal but Does Alter the Pattern of Precursor Sequence Processing—To assess which processing steps of SH-EP maturation are the consequence of self-catalyzed events the cysteine 26 active site sulfhydryl was mutated to a glycine (C26G mutant). This produces a protein that would

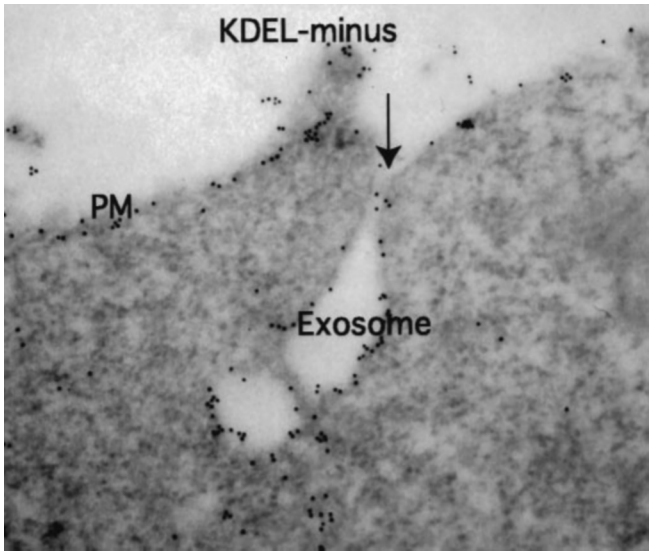

FIG. 8. **Apparent secretion of minus-KDEL SH-EP into extracellular space mediated by vesicles (exosomes) fusing to the plasma membrane (*arrow*).** A second vesicle containing SH-EP is located adjacent to the exosome that appears to be in position for a subsequent secretion event.

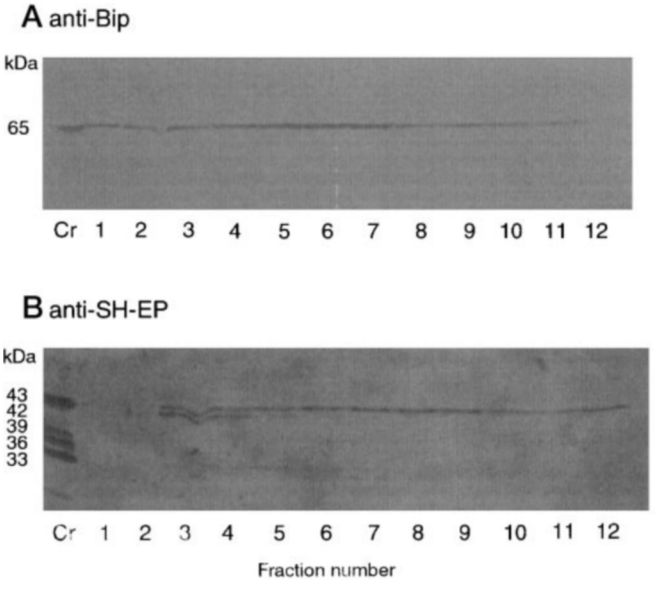

FIG. 9. **Sucrose density gradient analysis of the distribution of 43/42-kDa proSH-EP doublet in cotyledon cells.** The microsomal fraction prepared from the germinated cotyledons was separated with 11 ml 0.6–1.6 M sucrose gradient. The gradient fractions were analyzed by SDS-PAGE immunoblotting with an antiserum against maize BiP (*A*) or SH-EP (*B*). *Tubes 1* and *12* correspond to 1.6 and 0.6 M sucrose concentration, respectively. *Cr*, crude extracts prepared from germinated cotyledons.

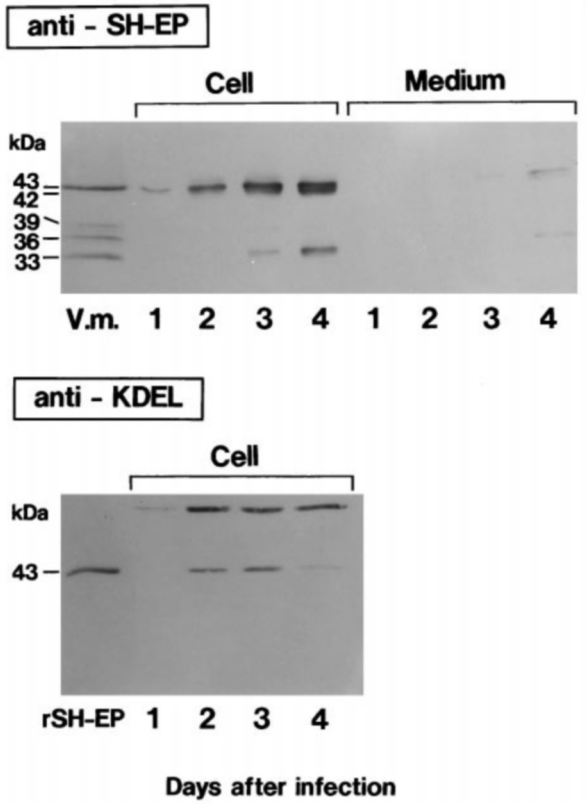

FIG. 10. **SDS-PAGE-immunoblot analysis of the expression of C26G mutant SH-EP that removes the catalytic amino acid.** A time course of 1–4 days postinfection of the mutant of the cellular and secreted proteins. The mutant SH-EP is initially synthesized as a full-length 43-kDa protein that accumulated throughout the time course and is secreted in small quantities by day 4. Posttranslational processing of the 43-kDa to the 42-kDa polypeptide is observed by day 3 and continues to accumulate through day 4. Mature 33-kDa SH-EP also accumulates during days 3 and 4 in the cells and on day 4 in the medium without the parallel accumulation of the 39- and 37-kDa processing intermediates. Parallel SDS-PAGE immunoblots assayed with anti-KDEL monoclonal antibody 1D3 demonstrate that the initial 43-kDa polypeptide possesses KDEL and that the KDEL signal is gradually lost during days 2–4 as the 42-kDa polypeptide accumulates. An additional polypeptide that is probably a reticuloplasmin is observed and functions as an internal control for the anti-KDEL immunoreactivity. *V.m.* is lysate from 3 day *V. mungo*, which provides control polypeptides for SH-EP and its processing intermediates. *rSH-EP* is *E. coli*-produced proSH-EP to provide a control.

presumably be proteolytically inactive and similar to a cysteine protease-related protein found in soybean seeds (P34) (31). A time course of C26G mutant expression in Sf-9 cells was analyzed by SDS-PAGE immunoblots (Fig. 10). The processing of proSH-EP-C26G to mature 33-kDa protein proceeds much more slowly than either plus- or minus-KDEL SH-EP in parallel expression experiments. On day 3 postinfection, the 43/42-kDa doublet is observed in the C26G form, indicating that the processing of the 43-kDa proSH-EP possessing KDEL to the 42-kDa minus-KDEL form can occur in the absence of the catalytic cysteine. Using the anti-KDEL monoclonal antibody 1D3, we confirmed the presence of KDEL in the 43-kDa form, and the absence of the KDEL in the 42-kDa form. Intermediate 39- and 37-kDa processing steps of SH-EP were not observed in the C26G mutant at any time point postinfection, suggesting that these intermediate processing products are only produced when the catalytic cysteine is available.

DISCUSSION

The removal of KDEL from SH-EP in homologous *V. mungo* cells and in heterologous Sf-9 cells is so far unique among the retention sequence-bearing proteins that have been studied. There have been a number of studies that have added K/HDEL sequences to proteins and expressed these in transgenic plants (35, 38, 56–58). For the most part, these studies have shown partial or enhanced retention within the ER/nuclear envelope lumen with eventual progression to distal sites in the endomembrane system. These results would appear to indicate that the retention sequence of itself is insufficient to introduce complete retention of these proteins. However, in none of these studies was the fate of the retention signal examined, and the possibility remains that posttranslational processing of the retention sequence might explain their progression through the endomembrane system. In contrast, the KDEL-bearing protein

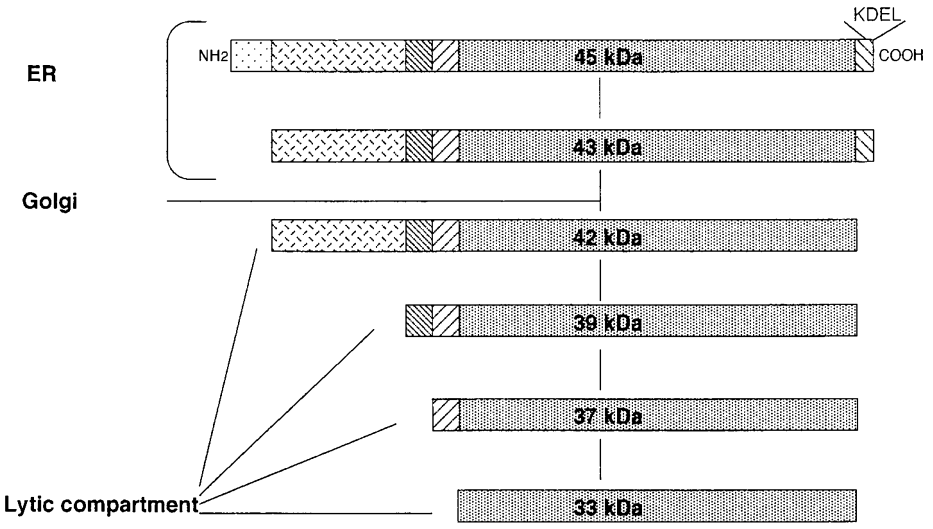

FIG. 11. **A model for posttranslational processing and intracellular transport of SH-EP and its intermediates in Sf-9 cells.** The SH-EP mRNA coding 45-kDa polypeptide is translated on membrane-bound polysomes to a 43-kDa intermediate through co-translational cleavage of a signal sequence. A carboxyl-terminal propeptide of 1 kDa containing KDEL sequence is processed from the 43-kDa intermediate in the ER lumen or immediately after exit from the ER. The 42-kDa intermediate is autocatalytically converted to the 33-kDa mature enzyme via 39- and 37-kDa intermediates.

auxin-binding protein was examined by Jones and Herman (59), who found that the secreted protein retained its KDEL sequence assayed by immunoreactivity with the same monoclonal antibody 1D3 used in the present study.

The ER-resident lumen proteins or reticuloplasmins with their carboxyl-terminal KDEL and HDEL retention sequences are conserved among eukaryotic cells. Each type of reticuloplasmin is highly similar among plant, animal, and fungal cells. Examples of these proteins include well characterized molecular chaperones BiP and GRP94 and protein disulfide isomerase (25, 26). The reticuloplasmins are primarily localized within the ER/nuclear envelope lumen, although there are studies that have indicated that at least under some circumstances these proteins may escape the ER-retention system and are secreted (60–63).

Plant cells possess two proteins, SH-EP and auxin-binding protein, that are not obvious members of the class of reticuloplasm proteins but do possess carboxyl-terminal KDEL sequences. The function of auxin-binding protein remains unknown, although it is widely assumed that the protein may be involved in signal transduction of plant hormone auxin. Auxin-binding protein is localized primarily in the ER as if it were a reticuloplasmin (64, 65), although a small fraction of the auxin-binding protein is secreted to the cell surface (59, 66, 67) with its KDEL sequence intact (59). The binding of ligand, auxin, may alter the exposure of the KDEL sequence, and this could change the trafficking of the protein (68) so that its removal is not necessary to allow its exit from the ER. It is possible that auxin binding shuttles reversibly between the cell surface and the endomembrane system by exploiting the ER-retention system for targeting and transport.

The crystal structure of cathepsin B (69), papain (70), actinidin (71), and procathepsin L (72) have been elucidated. The approximately 120-amino acid prosegment functions to occlude the active site with an antiparallel peptide chain (72) that is an inhibitor of the enzyme (73). The position of the prosegment on the papain precursor is folded so it is oriented close to the carboxyl terminus. Assuming that proSH-EP with its high sequence homology to papain is structurally homologous to the propapain crystal structure, the only way to determine whether the carboxyl-terminal KDEL sequence of SH-EP is exposed to be functionally presented is to test the proSH-EP protein's retention in an eukaryotic heterologous expression system. We found that plus-KDEL proSH-EP is processed more slowly than the minus-KDEL proSH-EP, indicating that the KDEL sequence of the proSH-EP is properly displayed to be recognized by the ER-retention system. That is because processing of the SH-EP is a post-Golgi event, retarding the protein in the pre-Golgi endomembrane system will retard the rate at which it is processed to the mature enzyme. This interpretation may be supported by the sucrose gradient fractionation experiment of the plus-KDEL SH-EP expressing Sf-9 cells, which shows that the 43-kDa proSH-EP that possesses the KDEL is in a distinct membrane compartment compared with the 42-kDa partially processed minus-KDEL proSH-EP. In cotyledon cells of *V. mungo* seedlings, the 42-kDa SH-EP was present in cell compartment distinct from primary fraction of ER. This result may support our proposal that the KDEL sequence of the SH-EP functions to retard the exit from ER in cotyledon cells.

The 43-kDa C26G SH-EP mutant expressed in Sf-9 cells is processed to remove the carboxyl-terminal KDEL sequence, producing the 42-kDa proSH-EP. Thus, the autocatalytic activity of SH-EP is not required for removal of the KDEL sequence. Whether the KDEL sequence removal is the consequence of an alternate self-catalyzed reaction or the exogenous enzyme activity of another protein remains to be determined. Our experiments with the SH-EP mutant lacking the catalytic cysteine 26 residue indicates that the processing of the 42-kDa proSH-EP to 39- and 37-kDa intermediate precursors and mature 33-kDa is the result of autocatalytic self-processing. The C26G mutant is processed to a mature form in the insect cells without the accumulation of the 39- and 37-kDa intermediate precursors and at a much slower rate. This processing is likely to be the consequence of an endogenous insect or viral cysteine protease that processes the SH-EP protein in a single step. Experiments on *in vitro* processing of the recombinant proSH-EP mutant lacking the catalytic cysteine by mature active wild-type SH-EP resulted in conversion of the proprotein to a mature molecular mass without the accumulation of intermediate processing products.[2] These findings indicate that exogenous thiol protease activity including those thiol proteases found in Sf-9 cells will process the proSH-EP to its mature molecular mass but that only self-processing will yield intermediate processing products.

That a lytic protease possesses an ER retention sequence and is retained within the ER lumen is curious. Such potentially destructive proteases would not seem to be candidates to be ER lumen resident proteins. The ER lumen is well established as a compartment involved in folding and processing newly synthesized proteins for export into the endomembrane system (74,

[2] T. Okomoto, A. Yuki, N. Mitsuhashi, and T. Minamikawa, submitted for publication.

75). However, our results clearly demonstrate that only the inactive proSH-EP possesses the KDEL retention sequence. The KDEL sequence on proSH-EP is removed either in the ER or shortly after the protein's exit from the ER. The removal of the retention sequence in the ER is an obvious mechanism by which the proprotein could be changed from an ER-retained (or ER-retarded form) to a secretion-competent form that would pass by the *cis*-Golgi ERD-2 retention sequence receptor in the bulk secretory flow to its final destination. Plant cells have been shown to possess an ERD-2-type K/HDEL receptor protein that is highly similar to forms characterized in yeast and animal cells (76–80). We suggest that proSH-EP with its KDEL sequence intact constitutes an inactive form of the protein that is functionally stored by continuous retrieval within the ER. If SH-EP was processed in bulk removing the retention sequence that would permit efficient and pulse-like progression to its destination and functional activation in the vacuole. The proSH-EP with its KDEL by recycling within the pre-Golgi endomembrane lumen is then functionally equivalent to a zymogen (81) of proteases and is perhaps best described as a "transient zymogen."

The posttranslational processing of carboxyl-terminal KDEL sequence in SH-EP expressed in the homologous plant and heterologous Sf-9 cells suggests a previously unrecognized means of utilizing the information content of the ER retention sequence. An outline of the proposed sequence of processing events is shown in Fig. 11. The regulated removal of the KDEL sequence offers the opportunity to permit a protein to progress through the endomembrane system to either the lytic compartment or secretion. This could allow a protein to serve dual roles within the ER and elsewhere in the cell. The removal of the ER retention sequence could allow the protein to be transported to the lytic compartment for disposal, or in the case of SH-EP for activation as an acid protease. We speculate that the selective removal of KDEL sequences will not be restricted to SH-EP but might also include reticuloplasmins to permit efficient turnover of the proteins during normal growth and during recovery from stress overexpression. Experiments are currently in progress in our laboratories to examine whether retention sequence processing and removal is a characteristic of reticuloplasmins.

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