Mass Transport of Proform of a KDEL-tailed Cysteine Proteinase (SH-EP) to Protein Storage Vacuoles by Endoplasmic Reticulum–derived Vesicle Is Involved in Protein Mobilization in Germinating Seeds

Kiminori Toyooka, Takashi Okamoto, and Takao Minamikawa

Department of Biological Sciences, Tokyo Metropolitan University, Minami-osawa 1-1, Hachioji, Tokyo, 192-0397 Japan

Abstract. A vacuolar cysteine proteinase, designated SH-EP, is expressed in the cotyledon of germinated *Vigna mungo* seeds and is responsible for the degradation of storage proteins. SH-EP is a characteristic vacuolar proteinase possessing a COOH-terminal endoplasmic reticulum (ER) retention sequence, KDEL. In this work, immunocytochemical analysis of the cotyledon cells of germinated *V. mungo* seeds was performed using seven kinds of antibodies to identify the intracellular transport pathway of SH-EP from ER to protein storage vacuoles. A proform of SH-EP synthesized in ER accumulated at the edge or middle region of ER where the transport vesicle was formed. The vesicle containing a large amount of proSH-EP, termed KV, budded off from ER, bypassed the Golgi complex, and was sorted to protein storage vacuoles. This massive transport of SH-EP via KV was thought to mediate dynamic protein mobilization in the cotyledon cells of germinated seeds. We discuss the possibilities that the KDEL sequence of KDEL-tailed vacuolar cysteine proteinases function as an accumulation signal at ER, and that the mass transport of the proteinases by ER-derived KV-like vesicle is involved in the protein mobilization of plants.

Key words: cysteine proteinase • endoplasmic reticulum • intracellular transport • KDEL sequence • protein storage vacuole

Introduction

In higher plants, dynamic protein mobilization occurs in germinated seeds for supplying amino acids to grow seedlings. Seeds accumulate storage proteins in protein storage vacuoles of the embryo or endosperm cell during seed maturation (Bewley and Black, 1994). In exalbuminous dicot seeds, such as legume seeds, globulin, the major storage protein, is stored in the protein storage vacuole of cotyledon cells. The storage globulin is synthesized, folded, and oligomized in the ER (Pedrazzini et al., 1997; Galili et al., 1998; Vitale and Denecke, 1999). In general, storage globulins are transported to the vacuole via the Golgi-mediated pathway (Greenwood and Chrispeels, 1985; Shotwell and Larkins, 1988). Recently, Hara-Nishimura et al. (1998) reported that a proform of 11S globulin of castor bean is aggregated and packed in vesicles in ER, termed precursor-accumulating (PAC)[1] vesicle, and that PAC vesicles are directly sorted to vacuoles through the Golgi-independent pathway. In the case of cereal grains, both Golgi-mediated and ER-derived vesicle-mediated sorting pathways of storage proteins have been reported as well (Levanony et al., 1992; Herman and Larkins, 1999). After storage proteins accumulated in seeds, the desiccation of seeds proceeds, and the moisture content of quiescent seeds decreases to 5–15% with the metabolic activity almost at a standstill (Bewley and Black, 1994).

Rehydration and suitable temperature are generally needed for seed germination, although priming treatments such as light stimulus and a period at low temperature are essential for some kinds of seeds to break seed dormancy. Germinating seeds synthesize de novo papain family cysteine proteinases that rapidly mobilize storage proteins during seeds germination and early seedling growth (Müntz, 1996). In contrast to the accumulation of proteins during seed maturation, the mechanism of the rapid breakdown of proteins is not well understood, although a number of the enzymes responsible for the breakdown of the proteins have been identified.

Papain-type proteinases function as major enzymes involved in the degradation of seed storage proteins (Baumgartner and Chrispeels, 1977; Mitsuhashi et al., 1986; Boy-

Address correspondence to Dr. Takashi Okamoto, Department of Biological Sciences, Tokyo Metropolitan University, Minami-osawa, Hachioji, Tokyo, 192-0397 Japan. Tel.: +81 426-77-2559. Fax: +81 426-77-2559. E-mail: okamoto-takashi@c.metro-u.ac.jp

[1]*Abbreviations used in this paper:* KV, KDEL-tailed cysteine proteinase-accumulating vesicle; PAC, precursor accumulating; PSV, protein storage vacuole; TB, toluidine blue.

© The Rockefeller University Press, 0021-9525/2000/02/453/11 $5.00

lan and Sussex, 1987; Holwerda et al., 1990; Kato and Minamikawa, 1996; Müntz, 1996). In the cotyledons of germinated *Vigna mungo* seeds, a cysteine proteinase, designated SH-EP, has a major role in the breakdown of seed globulin (Okamoto and Minamikawa, 1998). SH-EP is synthesized in ER as a proform of 43 kD through cleavage of the signal sequence. The 43-kD SH-EP (proSH-EP) is further processed to the enzymatically active 33-kD mature enzyme via 39- and 36-kD intermediates during or after transport to vacuoles (Mitsuhashi and Minamikawa, 1989). In addition, 43-kD proSH-EP is known to be converted into the mature enzyme by autocatalytic and asparaginyl endopeptidase (VmPE-1)–mediated fashions (Okamoto et al., 1999a). SH-EP is a unique vacuolar proteinase, since it has a COOH-terminal KDEL sequence (Akasofu et al., 1989) that is known as the ER retention sequence (Munro and Pelham, 1987; Pelham, 1989; Denecke et al., 1992; Napier et al., 1992; Lee et al., 1993). The function of the KDEL sequence of SH-EP is supposed to store SH-EP as a transient zymogen in ER (Okamoto et al., 1999b).

In this study, the intracellular sorting pathway of SH-EP was intensively studied by an immunocytochemical technique using specific antibodies raised to 43-kD SH-EP, 33-kD mature SH-EP, storage globulin, VmPE-1, complex glycan, and KDEL peptide. The results obtained show that a unique vesicle (200–500 nm in diameter) containing a large amount of proSH-EP buds off from ER, and the vesicle, tentatively designated KDEL-tailed cysteine proteinase-accumulating vesicle (KV), is transported to protein storage vacuoles by the Golgi-independent pathway. The function of the mass transport of proSH-EP by KV will be discussed.

Materials and Methods

Plant Materials

V. mungo seeds were germinated on layers of wet filter paper at 27°C in darkness, and cotyledons were collected on days 1 to 3 post-imbibition.

Gel Electrophoresis and Immunoblotting

SDS-PAGE and immunoblotting were performed as described previously (Mitsuhashi and Minamikawa, 1989).

Preparation of Antibodies

The recombinant proform of SH-EP (43-kD SH-EP) was produced as described (Okamoto and Minamikawa, 1999), and antiserum to the recombinant proenzyme was prepared according to Mitsuhashi and Minamikawa (1989). To amplify the DNA sequence of SH-EP cDNA encoding a partial sequence of the NH_2-terminal prosequence (Phe-23 to Tyr-80), primers for T7 promoter (ATTAATACGACTCACTATAG) and SH-EP cDNA (TTATCCATCTAGTTAGTGTT) were set to a pET17b vector (Novagen) harboring signal sequence–deleted SH-EP cDNA (Okamoto and Minamikawa, 1999). The PCR was performed in 100 μl for 35 cycles (94°C 1 min, 55°C 2 min, 72°C 2 min), and the amplified fragment was subcloned into a TA vector (Invitrogen). The insert in the vector was cut by NdeI and BamHI, and the excised fragment was subcloned to the pET17b vector cut by the same enzymes. The expression of a partial peptide of the NH_2-terminal propeptide (Phe-23 to Tyr-80) consisting of 57–amino acid residues in *E. coli* and the isolation of inclusion bodies accumulating the peptide were performed as described (Okamoto and Minamikawa, 1999). The recombinant peptide (0.6 mg) was immobilized to 3 ml of ECH-Sepharose 4B (Pharmacia) according to the manufacturer's instruction, and the partial propeptide-immobilized Sepharose was packed into a column and used for isolation of the antibody to 43-kD SH-EP from the antiserum to 43-kD SH-EP. 25 ml of antiserum to 43-kD SH-EP was precipitated by the addition of 12.5 ml of saturated ammonium sulfate solution, and the precipitate was dialyzed against PBS. After centrifugation of the dialyzed solution, the supernatant was applied to the column of the partial propeptide-immobilized Sepharose that had been equilibrated with PBS. The column was washed first with PBS and further with 0.5 M NaCl in PBS. The antibody bound to the column was eluted by 0.1 M glycine-HCl (pH 2.5) containing 0.5 M NaCl, and the eluate was immediately neutralized with 1 M Tris-Cl (pH 8.0). The antibody obtained from the column was dialyzed against PBS containing 0.1% sodium azide and used as anti–43-kD SH-EP antibody. Recombinant 33-kD mature SH-EP was prepared as described previously (Okamoto et al., 1999a), and the proteins were immobilized to ECH-Sepharose 4B. Isolation of antibody to 33-kD SH-EP by the column of 33-kD SH-EP–immobilized Sepharose from antiserum to 43-kD SH-EP was carried out as described above.

To prepare the monoclonal antibody to 33-kD SH-EP, 0.2 ml of recombinant 43-kD SH-EP (0.4 mg) was emulsified with an equal volume of Freund's complete adjuvant (Wako Pure Chemical Co.) and BALB/c female mice were injected with the antigen. The mice were boosted three times every 2 wk. The spleens were removed from the mice, and spleenoocytes were fused with log-phase NS-1 myeloma cells by the methods of Galfre and Milstein (1981). Cells were plated into five 96-well plates, and hybridomas were selected on a hypoxanthine, aminopterin, and thymidine (HAT) medium. After 10 d on the HAT medium, cells were refed with a hypoxanthine-thymidine (HT) medium. When the cells increased to ~50% confluence, the culture medium of the well was used for screening. Screening was carried out by SDS-PAGE/immunoblotting of the crude extract from day-3 cotyledons of germinated *V. mungo* seeds. Positive cells were diluted 1,000-fold and plated into 24-well plates. The medium of the wells in which a single colony of hybridomas grew was used for the second screening. Cells of positive clones were further diluted and plated again. The third screening was carried out with the culture medium from a single colony. Positive cells were plated into 12-well plates to prepare the stock medium, which was used as the monoclonal antibody to 33-kD SH-EP.

To prepare the recombinant proform of VmPE-1, a pET17b vector harboring full-length VmPE-1 cDNA was cut by NheI and HindIII, and both sides of the vector were blunted by a DNA-Blunting kit (Takara). The blunted vector having cDNA encoding the signal-sequence–deleted VmPE-1 was ligated and transformed to *E. coli* BL21(DE3). The expression and isolation of recombinant proVmPE-1 were conducted according to Okamoto and Minamikawa (1999). Antibody to proVmPE-1 was affinity-purified from antiserum to proVmPE-1 by proVmPE-1–immobilized Sepharose 4B prepared as above. 7S globulin, the major storage protein of *V. mungo* seeds, was isolated from dry seeds according to Basha and Beevers (1975), and, after SDS-PAGE of the 7S globulin, the 54-kD polypeptide of 7S globulin was cut out from the gel and used as antigen. Antiserum to the 54-kD polypeptide of 7S globulin and 7S globulin-immobilized column was used for purification of anti-7S globulin antibody. Antiserum raised against β-xylosidase of sycamore (*Acer pseudoplatanus* L.; Tezuka et al., 1993) was provided from Dr. Ikuko Hara-Nishimura (National Institute of Basic Bioloby, Okazaki, Japan), and antibody to complex glycan was isolated from the antiserum according to Hara-Nishimura et al. (1998). Monoclonal antibody, 1D3, recognizing the COOH-terminal KDEL sequence was purchased from Stressgene.

Immunocytochemistry and Ultrastructural Analysis

Day-3 cotyledons of *V. mungo* seeds were cut into ~0.5-mm^3 cubes and fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.4) for 4 h at 4°C. After dehydration of the tissue pieces in a graded methanol series, the pieces were further dehydrated in acetone/methanol (1:1), 100% acetone, acetone/methanol (1:1), and 100% methanol. Procedures for ultrastructural analysis were conducted as described (Hara-Nishimura et al., 1993). For immunocytochemical analysis, the dehydrated pieces were embedded in the hard formulation of LR White resin. Ultrathin sections mounted on nickel grids (600 mesh; Electron Microscopy Sciences) were blocked with 10% fetal bovine serum in TBS (25 mM Tris-Cl, pH 7.4, 150 mM NaCl) for 10 min at room temperature. The sections were then labeled with affinity-purified polyclonal antibody to 43-kD SH-EP (diluted 1:1), 33-kD SH-EP (1:1), 7S globulin (1:5), proform of VmPE-1 (1:1) or complex glycan (1:10) in TBS, or monoclonal antibody to 33-kD SH-EP (1:1) or KDEL sequence (1:100) in TBS. After being washed with TBS, sections were indirectly labeled with colloidal gold particles coupled to goat anti–rabbit IgG or colloidal

gold particles coupled to goat anti–mouse IgG. Gold-labeled sections were then washed with TBS, rinsed in water, and stained with 5% aqueous uranyl acetate. The grids were examined and photographed with a transmission electron microscope (model 1010EX; JEOL) at 80 kV.

Results

Preparation of Antibodies

When crude extracts from day-3 cotyledons of germinated *V. mungo* seeds were analyzed by SDS-PAGE/immunoblotting with anti–43-kD SH-EP antiserum, 43-kD proform, 39- and 36-kD intermediate forms of SH-EP, as well as 33-kD mature SH-EP were detected (Fig. 1 A, lane 1). Affinity purification of the antiserum by the mature SH-EP–immobilized column and by the NH_2-terminal prosequence-immobilized column resulted in the isolation of antibodies recognizing all four SH-EP–related polypeptides (Fig. 1 A, lane 2) and only 43-kD SH-EP (Fig. 1 A, lane 3), respectively. The monoclonal antibody to 33-kD SH-EP recognized no polypeptides other than four SH-EP–related polypeptides (Fig. 1 A, lane 4). Affinity-purified anti-proVmPE-1 antibody recognized only mature VmPE-1 of 33 kD in extracts from day-3 cotyledons (Fig. 1 B; Okamoto and Minamikawa, 1995). When extracts from dry seeds of *V. mungo* were analyzed by SDS-PAGE/immunoblotting with purified antibody to the 54-kD polypeptide of 7S globulin, 56-kD polypeptide was detected in addition to the 54-kD polypeptide (Fig. 1 C). 7S globulin of *V. mungo* seeds is mainly composed of 64-, 56-, 54-, and 51-kD polypeptides (Okamoto and Minamikawa, 1998). The 56-kD polypeptide detected in Fig. 1 C would be due to contamination of the 56-kD polypeptide in preparations of antigen 54-kD polypeptide, from purified 7S

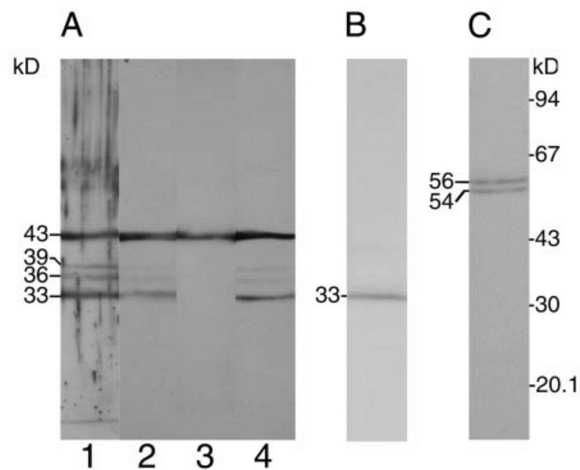

Figure 1. SDS-PAGE/immunoblotting of extracts from the cotyledon of *V. mungo* seeds. (A) Protein extracts (0.1 mg protein) from day-3 cotyledons were separated by SDS-PAGE and the proteins in the gel were blotted to a membrane. The membrane was cut into four strips and probed with antiserum to 43-kD SH-EP (lane 1), polyclonal antibody to 33-kD SH-EP (lane 2), polyclonal antibody to 43-kD SH-EP (lane 3), or monoclonal antibody to 33-kD SH-EP (lane 4). (B) Protein extracts (0.1 mg protein) from day-3 cotyledons were separated by SDS-PAGE and the proteins in the gel were blotted to a membrane. The membrane was probed with polyclonal antibody to proVmPE-1. (C) Protein extracts (0.1 mg protein) from dry seeds of *V. mungo* were separated by SDS-PAGE and the proteins in the gel were blotted to a membrane. The membrane was probed with polyclonal antibody to 54-kD polypeptide of 7S globulin.

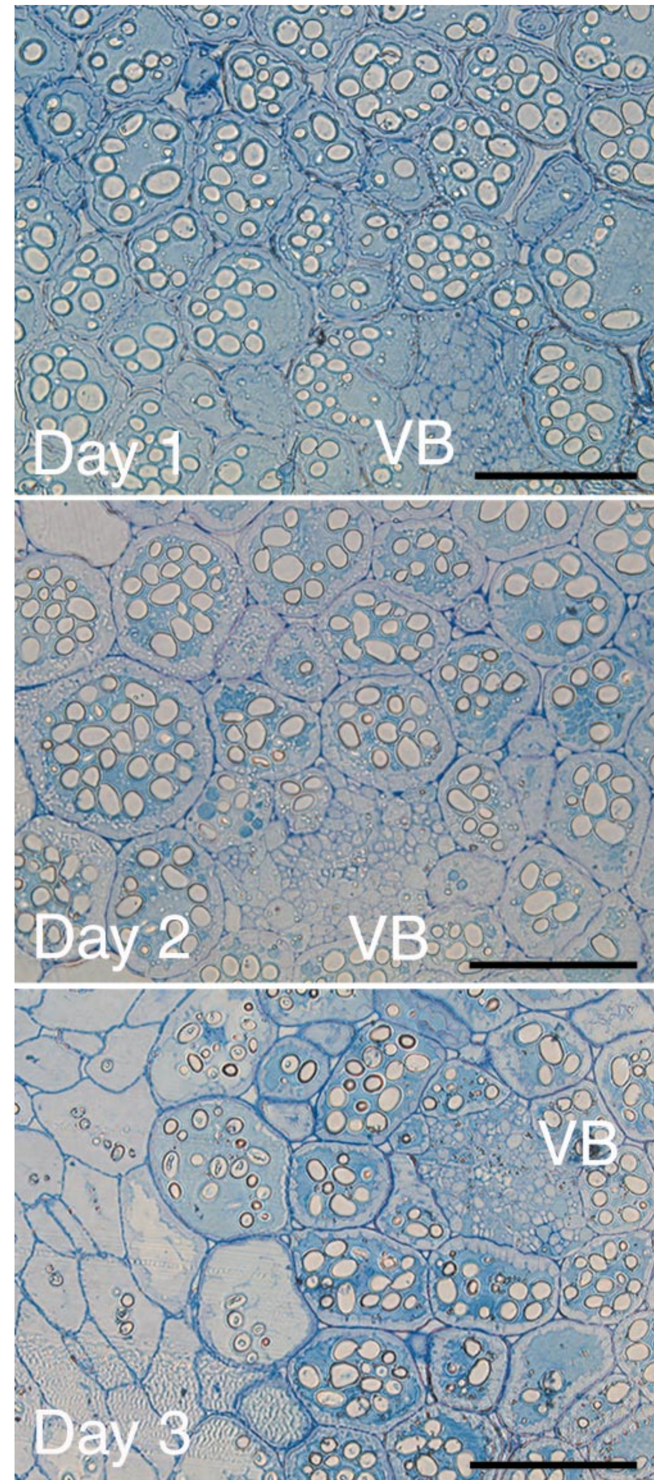

Figure 2. Toluidine blue staining of sections from cotyledons of germinated *V. mungo* seeds. Cotyledons were collected on days 1, 2, and 3 post-imbibition, and sections were prepared as described in Materials and Methods. VB, vascular bundles. Bars, 100 μm.

globulin, and both 54- and 56-kD polypeptides might be recognized by the anti-7S globulin antibody.

Mobilization Pattern of Storage Proteins in Cotyledon Cells of Germinated V. mungo Seeds

To observe the mobilization pattern of protein reserves in the cotyledons of germinated *V. mungo* seeds, transverse sections were prepared from the organs on days 1, 2, and 3 post-imbibition and stained with toluidine blue (TB; Fig. 2). On day 1 of seed germination, most cells were stained with toluidine blue, indicating that little mobilization of seed proteins occurred in the cotyledons. The white large organelles in the cells represent starch granules, which were stained with iodine-potassium iodide (data not shown). On day 2, TB-stainless cell, a cell that did not contain seed proteins, was observed in the tissue area farthest from the vascular bundle. The degradation of storage proteins proceeded on day 3. This indicates that the mobilization of storage materials in the cotyledons of *V. mungo* seeds starts at the farthest region from the vascular bundle. This result is consistent with histochemical and biochemical observations of storage protein mobilization in cotyledons of *Vigna radiata* seeds (Harris and Chrispeels, 1975).

The TB-stainless cells, in which the protein degradation was completed, existed just adjacent to the TB-stained cells which were filled with storage proteins. This suggests that TB-stained cells are converted to TB-stainless cells very quickly in the cotyledon. We hypothesized that the dynamic mobilization of storage proteins occurs in TB-stained cells or slightly TB-stained cells that were adjacent to the TB-stainless cells. Immunocytochemical analysis with seven kinds of antibodies was thus performed to observe the transport pathway of SH-EP to protein storage vacuoles of such cotyledon cells.

SH-EP Accumulated in Large Vesicles (KV) which Were Distinct from Protein Storage Vacuoles (PSV)

By immunogold labeling cotyledon cells with anti–33-kD SH-EP polyclonal antibody, it was seen that a large amount of SH-EP was localized in a vesicle of 300 nm in a diameter (Fig. 3 A). In contrast, only a small number of particles were present in protein storage vacuoles (PSVs). The diameters of the vesicles containing SH-EP were in a range of 200–500 nm (Figs. 3 and 4). Ultrastructural analysis of the cotyledon cells indicated that a vesicle with a diameter and electron density similar to the vesicle accumulating SH-EP was surrounded by a single membrane (Fig. 3 B).

Double immunogold staining with anti-7S globulin polyclonal antibody and anti–33-kD SH-EP monoclonal antibody was carried out to distinguish the vesicle from PSV. 7S globulin (10-nm particles) was localized only in PSV, and SH-EP (15-nm particles) was observed in the vesicle (Fig. 3 C). This indicates that the vesicle is distinct from the PSV, and we tentatively termed the vesicle KV. When

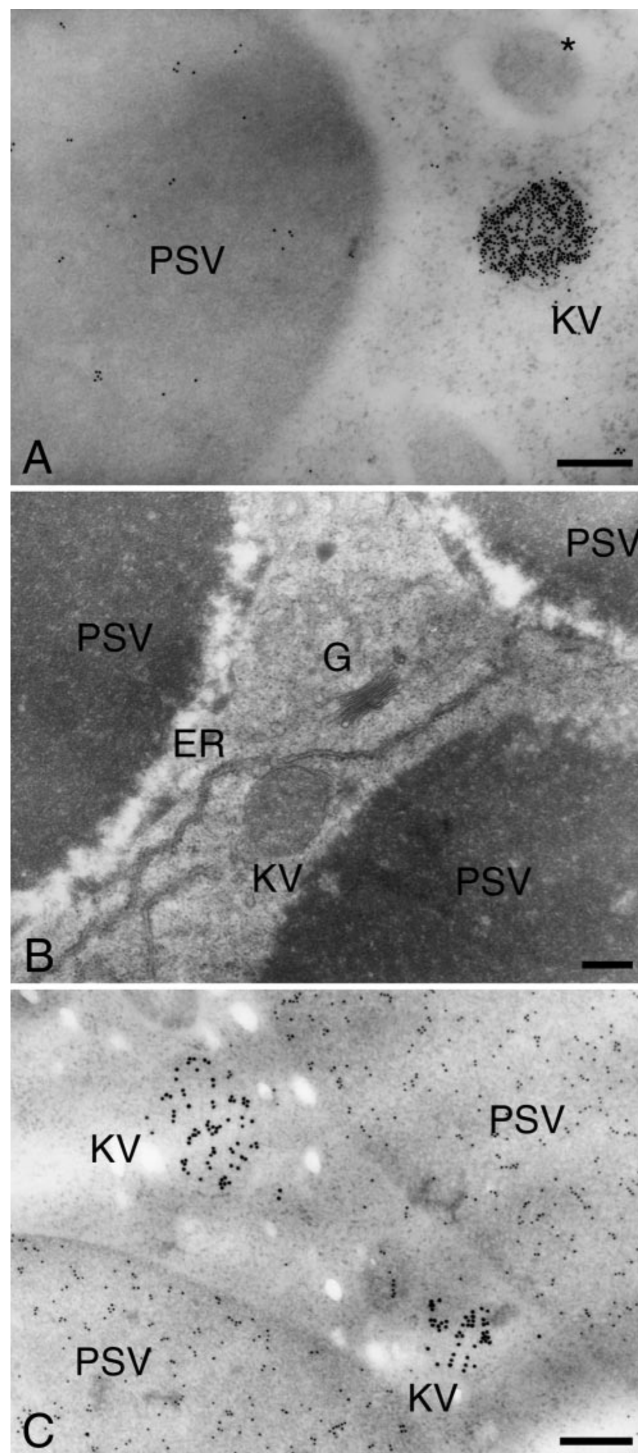

Figure 3. Electron photographs showing vesicles immunogold labeled with anti–33-kD SH-EP antibody (A and C), and ultrastructural analysis of cotyledon cells (B). (A) Immunogold localization of SH-EP. The vesicle (KV) with a diameter of 300 nm was densely labeled by gold particles. A slight of gold particles labeled the protein storage vacuole (PSV). The asterisk indicates an unidentified cell compartment. (B) Ultrastructure of cotyledon cells. The vesicle having similar electron density and diameter to KV in A and C was surrounded by single membrane. (C) Immunogold localizations of SH-EP (15-nm particles) and 7S storage globulin (10-nm particles). The vesicles accumulating SH-EP (KV) with diameters of 200 and 300 nm were labeled by antibody to 33-kD SH-EP, and the protein storage vacuole (PSV) by antibody to 7S storage globulin. ER, endoplasmic reticulum; G, Golgi complex; PSV, protein storage vacuole. Bars, 200 nm.

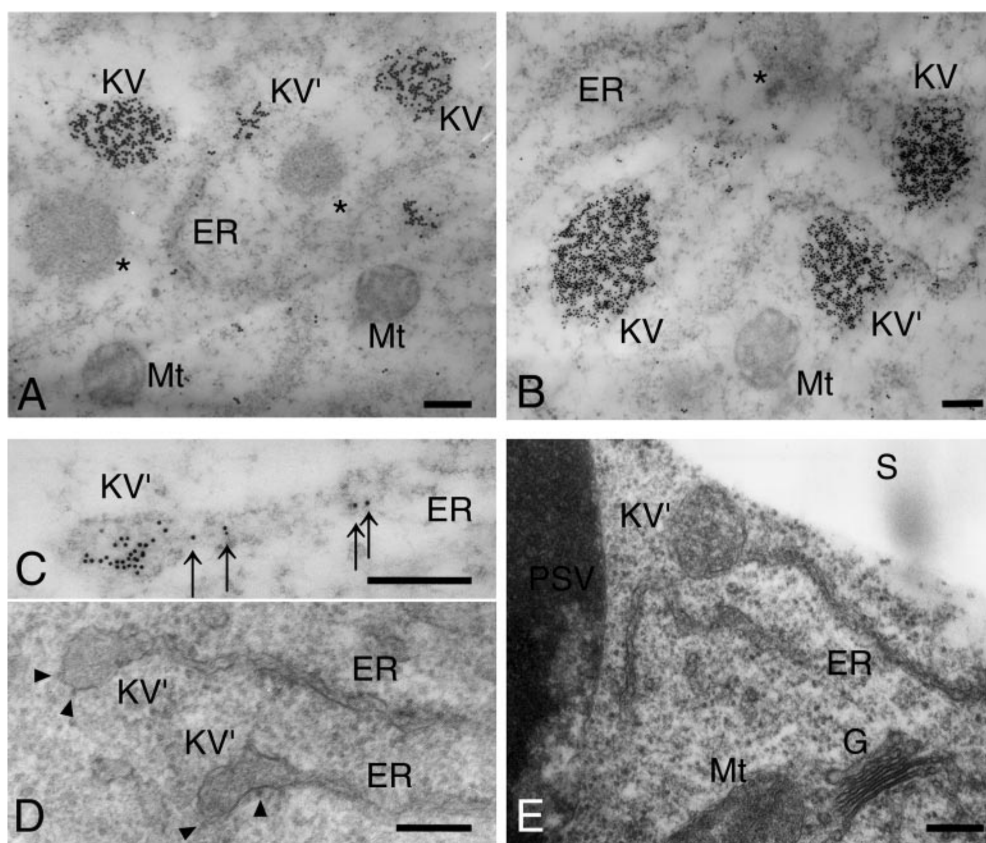

Figure 4. Electron photographs showing the budding of KV from ER (A, B, and C) and ultrastructural analysis of cotyledon cells (D and E). (A) Accumulation of SH-EP at the edge of ER (KV′). The area where SH-EP localized was swollen. Two KVs existed close to ER. (B) Accumulation of SH-EP at the middle region of ER (KV′). Two KVs existed close to ER. (C) Magnified image of the accumulation of SH-EP at the edge of ER (KV′). SH-EP in the lumen of ER (arrows) seemed to be moving toward the edge of ER where a large amount of SH-EP accumulated (KV′). (D) Ultrastructure of cotyledon cells, showing ER terminates in a small vesicle (KV′). The size and shape of the vesicle correspond to those of the swollen vesicle in C. Arrowheads indicate ribosomes. (E) Ultrastructure of cotyledon cells, showing the existence of KV-like vesicles (KV′) adjacent to ER. ER, endoplasmic reticulum; PSV, protein storage vacuole; S, starch granule; Mt, mitochondrion; Asterisk, unidentified cell compartment. Bars, 200 nm.

we examined maturing cotyledons of *V. mungo* seeds by immunoelectron microscopy with anti-7S globulin antibody, the proteins were transported to PSV via Golgi-derived dense vesicles having a 50–80-nm diameter, but no vesicles of similar electron density or size to KV were observed (data not shown). KV will be formed de novo in the cotyledon cell of germinated *V. mungo* seeds.

KV Was a ER-derived Vesicle and Sorted to PSV by the Golgi-independent Pathway

ER is the port of entry of proteins into the endomembrane system. When anti–33-kD SH-EP polyclonal antibody was used for immunoelectron microscopy of the cotyledon cells of germinated *V. mungo* seeds, a large amount of SH-EP accumulated at the edge or middle region of ER, and the area looked swollen (Fig. 4, A–C). In addition, KVs were frequently close to ER (Fig. 4, A and B). This strongly suggests that SH-EP synthesized in ER was packed into KV that is formed at the edge or middle area of ER, and that the budding of KV from ER is the first step of transport of SH-EP from ER to PSV. Under a conventional electron microscopy, it was observed that ER often terminated in a small vesicle surrounded by a ribosome-attached membrane (Fig. 4 D), and the size and shape of the vesicle are closely similar to those of the swollen vesicle observed in Fig. 4 C. In addition, a KV-like vesicle often existed adjacent to ER (Fig. 4 E). ER was well immunogold labeled with the anti–33-kD SH-EP polyclonal antibody. However, the Golgi complex was never labeled with the anti–33-kD SH-EP monoclonal antibody (Fig. 5 A) or the anti–33-kD SH-EP polyclonal antibody (Fig. 5 B), indicating that KV is transported to PSV via the Golgi-independent pathway. Although normal ER cisternae was either unlabeled with anti–33-kD SH-EP monoclonal antibody (Fig. 5 A) or poorly labeled with anti–33-kD SH-EP polyclonal antibody (Fig. 4, A and B), some labeling of the Golgi complex would be expected if SH-EP localized in the Golgi complex because proteins should be more concentrated in the Golgi stacks. Moreover, the diameter of KVs (200–500 nm) will probably be too large to discharge their content into the Golgi complex without destroying it.

Next, to investigate the contribution of proteins from the Golgi complex to KV, antibody to the complex glycan was used to immunogold electron microscopy of cotyledon cells, since the modification of Asn-linked glycan occurs in the Golgi complex. The antibody clearly stained the Golgi complex, while KV was not stained with the antibody (Fig. 5 C). PSV was also immunogold labeled with anti-complex

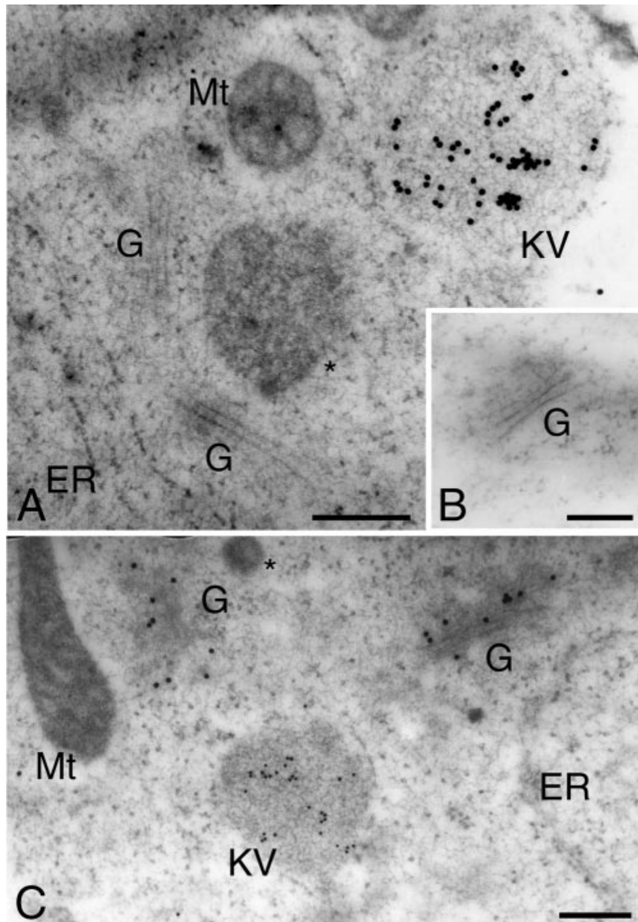

Figure 5. Electron photographs showing the transport of SH-EP by KV bypassing the Golgi complex. (A) KV was immunogold labeled (15-nm particles) with monoclonal antibody to 33-kD SH-EP. (B) The anti–33-kD SH-EP polyclonal antibody never immunogold labeled the Golgi complex. (C) The anti-complex glycan polyclonal antibody (15-nm particles) and anti–33-kD SH-EP monoclonal antibody (10-nm particles) specifically immunogold labeled the Golgi complex and KV, respectively. ER, endoplasmic reticulum; G, Golgi complex; Mt, mitochondrion; Asterisk, unidentified cell compartment. Bars, 200 nm.

glycan antibody (data not shown). These strongly indicate that proteins from the Golgi complex do not contribute to content or formation of KV.

Fig. 6 A represents the fusion of KV with PSV and possible release of SH-EP from KV into PSV. Observation of the cotyledon cells with conventional microscopy suggested that the KV-like vesicle appeared to be merging with the membrane of PSV (Fig. 6 B). The KV-like vesicle fusing with PSV had a diameter of ~700 nm, which is larger than that of other KVs observed in this study. Fusion of the KV vesicle might result in enlargement of diameter of the vesicle. As shown in Fig. 6 A, the electron density of PSV fused with KV was lower than that of PSV filled with storage proteins (Fig. 3, A and C). Both vacuolized and unvacuolized PSVs were observed under a conventional electron microscopy (Fig. 6 C). Mobilization of storage proteins proceeded in vacuolized PSV, but not in unvacuolized PSV, suggesting that the degra-

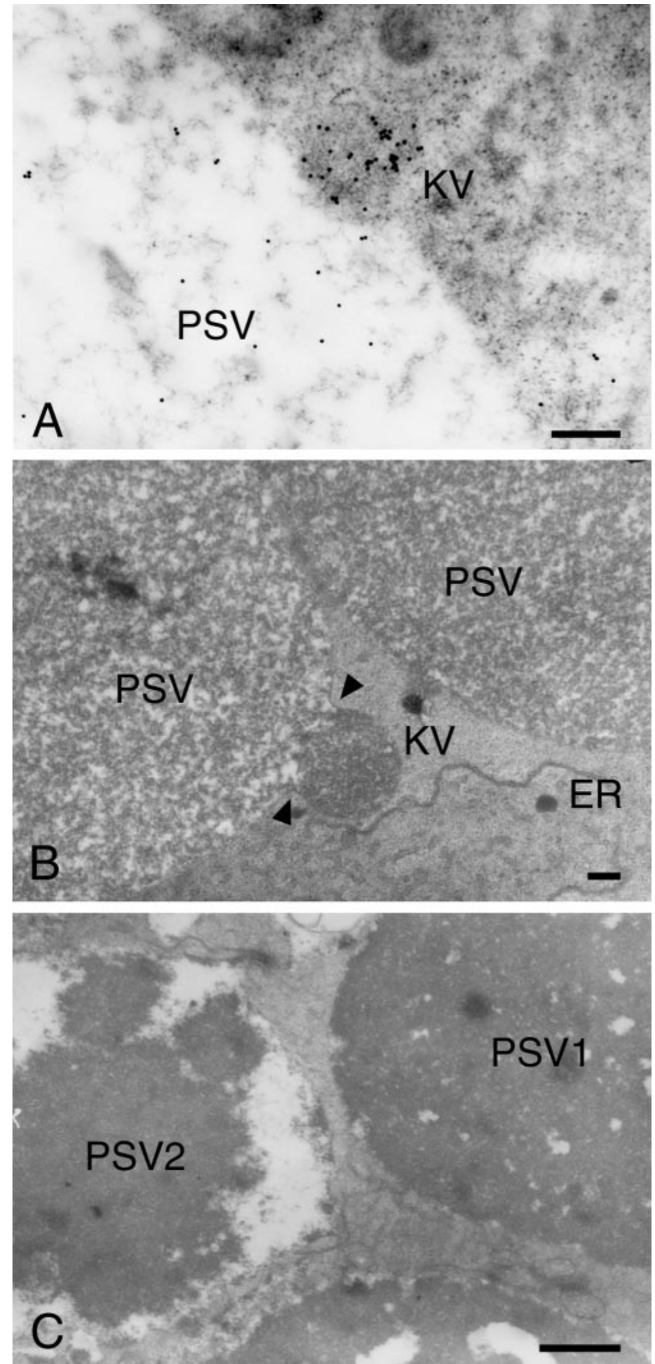

Figure 6. Electron photographs showing the fusion of KV with a protein storage vacuole (A) and ultrastructural analysis of cotyledon cells (B and C). (A) KV immunogold labeled with the anti–33-kD SH-EP polyclonal antibody fused with a protein storage vacuole (PSV). Gold particles are localized in PSV and KV. (B) Ultrastructural photograph showing that a KV-like vesicle fuses to PSV. Arrowheads indicate the region where KV is merging with membrane of PSV. (C) Ultrastructural photograph of two kinds of protein storage vacuole. One protein storage vacuole (PSV2), in which the degradation of storage protein proceeded, is vacuolized. In the other protein storage vacuole (PSV1), little vacuolization took place. Bars: (A and B) 200 nm; (C) 1 μm.

dation of storage proteins takes place in PSV with which KV fuses (Fig. 6 A).

SH-EP Existed as Its Proform in KV

The polyclonal antibody to 43-kD SH-EP and the monoclonal antibody to 33-kD SH-EP were used in immunogold labeling of cotyledon cells to see whether SH-EP exists as the proform or mature form in KV. KV was densely labeled with the anti–43-kD SH-EP antibody (Fig. 7 A) or with the polyclonal antibody to 43-kD SH-EP and the monoclonal antibody to 33-kD SH-EP (Fig. 7 B). The results indicate that SH-EP accumulated in KV as the enzymatically inactive proenzyme. The possibility that the NH_2-terminal prosequence removed from the proenzyme was recognized by the antibody to 43-kD SH-EP could not be ruled out, but the prosequence removed from 43-kD SH-EP during enzymatic activation is unstable and is rapidly degraded by the active mature SH-EP (Okamoto et al., 1999a). This evidence supports the possibility that the gold particles from anti–43-kD SH-EP antibody indicate the presence of 43-kD SH-EP in KV.

To observe whether or not the COOH-terminal KDEL sequence of SH-EP remains attached in KV, the monoclonal antibody to the KDEL sequence was used for immunogold labeling of cotyledon cells, since the monoclonal antibody has been shown to recognize the COOH-terminal KDEL sequence of proSH-EP (Okamoto et al., 1999b). The gold particles labeled the KV-like vesicle (Fig. 8 A). Anti–33-kD SH-EP polyclonal antibody was used to ascertain whether the KV-like vesicle labeled with the anti-KDEL antibody contains SH-EP. Both antibodies labeled KV (Fig. 8 B), but the results cannot indicate that the KDEL sequence is not removed during transport of proSH-EP from ER to KV, since the anti-KDEL antibody would recognize reticuloplasmins as well as proSH-EP.

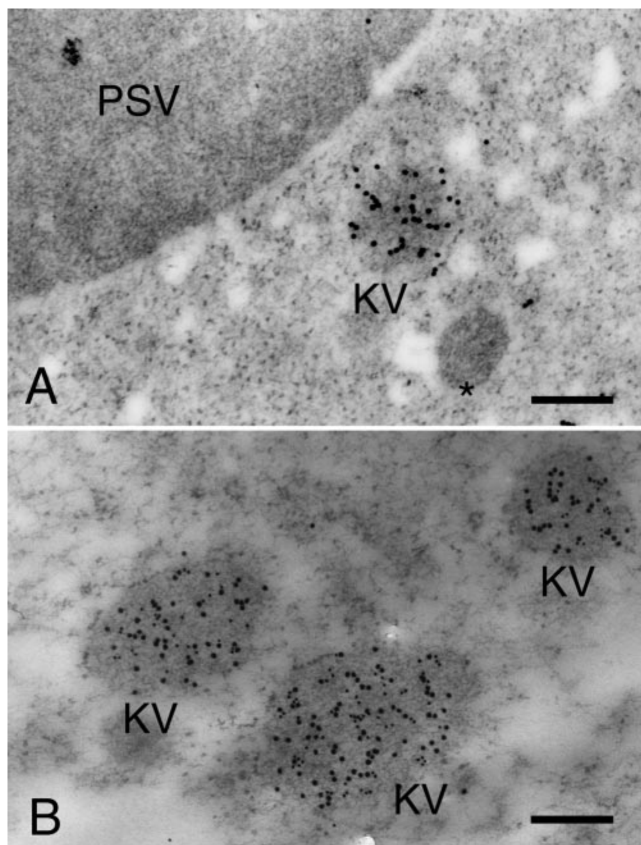

Figure 7. Electron photographs showing the localization of the proform of SH-EP in KV. (A) KV was immunogold labeled with anti–43-kD SH-EP polyclonal antibody. The asterisk indicates an unidentified cell compartment. (B) Three KVs having diameters of 200, 300, and 500 nm were immunogold labeled with anti–43-kD SH-EP polyclonal antibody (15-nm particles) and anti–33-kD SH-EP monoclonal antibody (10-nm particles). PSV, protein storage vacuole. Bars, 200 nm.

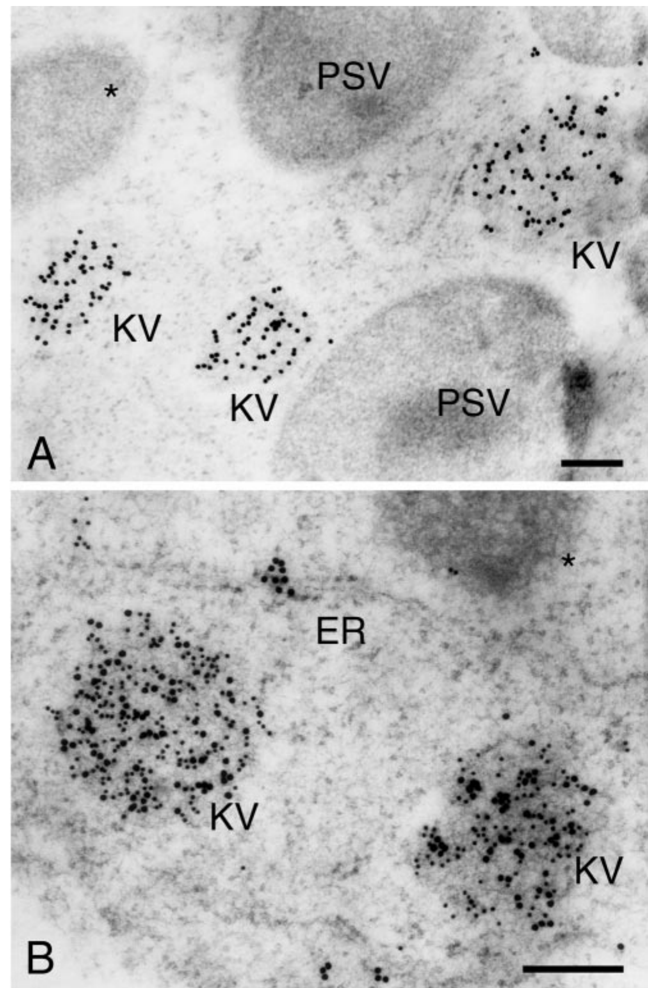

Figure 8. Electron photographs showing that KV was immunogold labeled with antibody to KDEL sequence. (A) KV was immunogold labeled by anti-KDEL monoclonal antibody. Three KVs having diameters of 200 nm to 400 nm were observed. (B) KV was immunogold labeled with anti-KDEL monoclonal antibody (15-nm particles) and anti–33-kD SH-EP polyclonal antibody (10-nm particles). Two KVs having a diameter of 300 nm were observed. ER, endoplasmic reticulum; PSV, protein storage vacuoles; Asterisk, unidentified cell compartment. Bars, 200 nm.

VmPE-1, a Processing Enzyme of SH-EP Proform, Was Transported to PSV via the Golgi-dependent Endomembrane System

An asparaginyl endopeptidase, termed VmPE-1, is in-

volved in the processing of proSH-EP (Okamoto and Minamikawa, 1995; Okamoto et al., 1999a). Immunogold labeling of cotyledon cells with the antibody to the proform of VmPE-1 was examined to see whether VmPE-1 is sorted to PSV by KV or by the Golgi-mediated endomembrane system. The Golgi complex and PSV were immunogold labeled by the anti-proVmPE-1 antibody (Fig. 9, A and B), but KV was immunogold labeled only by the anti–33-kD SH-EP monoclonal antibody (Fig. 9, A and B). This indicates that VmPE-1 is transported to PSV via the Golgi complex, and that VmPE-1 is separated from SH-EP until both enzymes are transported to PSV. Moreover, these results revealed that cotyledon cells use two sorting pathways to transport proteolytic enzymes from ER to PSV, the Golgi-mediated route for VmPE-1 and the KV-mediated route for SH-EP.

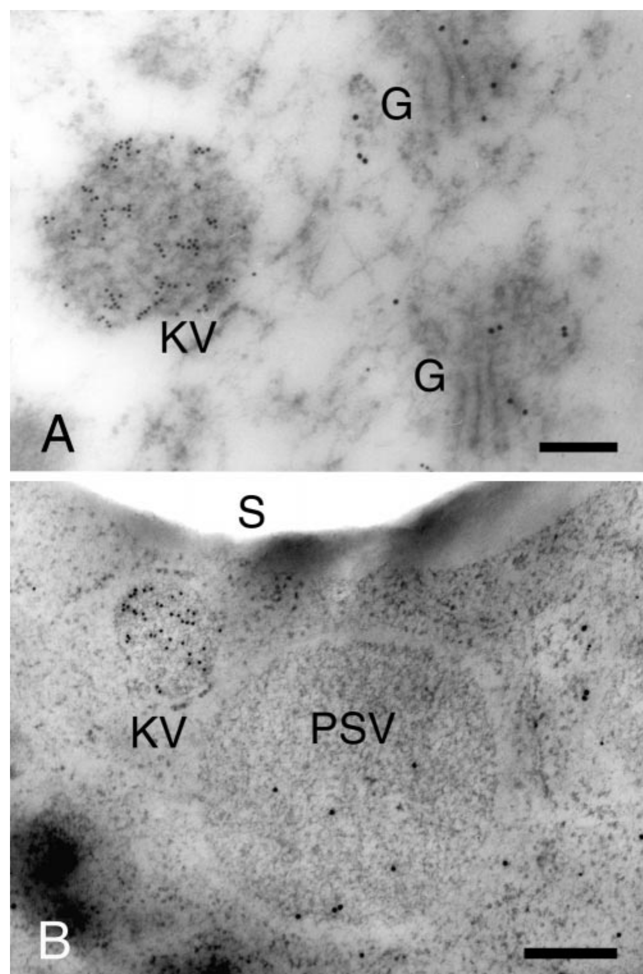

Figure 9. Electron photographs showing the transport pathway of VmPE-1 to the protein storage vacuole via the Golgi complex. (A) The Golgi complex was immunogold labeled only with anti-proVmPE-1 antibody (15-nm particles) and KV was immunogold-labeled only with anti–33-kD SH-EP monoclonal antibody (10-nm particles). (B) Immunogold-labeled PSV (15-nm particles) with anti-proVmPE-1 antibody. KV was immunogold labeled (10-nm particles) only with anti-33 kD SH-EP monoclonal antibody. G, Golgi complex; PSV, protein storage vacuole; S, starch granule. Bars, 200 nm.

Discussion

Molecular Mechanism of Rapid Mobilization of Storage Proteins in Cotyledons of Germinated V. mungo Seeds

Results of present immunocytochemical analysis indicated that SH-EP synthesized in ER is packed into a KV at the edge or middle region of ER, and KV (200–500 nm in diameter) filled with proSH-EP buds off from ER, bypasses the Golgi complex and fuses with PSV, resulting in the release of proSH-EP into the inside of PSV. In PSV, proSH-EP will be effectively activated, since in vitro experiments for the processing of proSH-EP have shown that the proenzyme is autocatalytically processed to the mature enzyme within 1 h at pH 5.8, which is equivalent to in vivo vacuolar pH measured by ^{31}P nuclear magnetic resonance (NMR) of intact cotyledons (Okamoto et al., 1999a). In addition, the localization of VmPE-1, the processing enzyme of proSH-EP, in PSV gave additional evidence for the efficient activation of SH-EP in PSV. Fully activated SH-EP (mature SH-EP) in PSV has potential to degrade the storage globulin rapidly. This putative rapid degradation is supported by the fact that 1 μg of purified mature SH-EP completely cleaved 100 μg of the isolated 7S globulin into smaller peptides within 12 h in vitro (Okamoto and Minamikawa, 1998). Together with these biochemical data and the results of present immunocytochemical analysis, a mobilization mechanism of storage proteins stored in PSV of the cotyledon cells of *V. mungo* seeds is proposed in Fig. 10. A large amount of proSH-EP sorted to PSV via ER-derived KV is converted to the mature enzyme by autocatalytical and/or VmPE-1–mediated fashions, and activated mature SH-EP massively degrades the storage globulin. These processes mediate the immediate change of the cotyledon cells filled with storage proteins into vacuolized cells.

KDEL-tailed Cysteine Proteinase-accumulating Vesicle: A Cell Compartment Distinct from Lytic Vacuole or Protein Body

It has been known that two types of vacuoles exist in plant cells. One is a protein storage vacuole and the other is called a primary lysosome or lytic/second vacuole (Chrispeels et al., 1976; Paris et al., 1996; Swanson et al., 1998). The lytic/second vacuole is an acidic compartment (Paris et al., 1996; Swanson et al., 1998). Aleurain, a papain-like cysteine protease, is localized in the lytic vacuole of root tip cells of barley (Paris et al., 1996) and cysteine protease(s) in the second vacuole of barley aleurone cells (Swanson et al., 1998). Paris et al. (1997) reported that the lytic vacuole is built up by a clathrin-coated vesicle derived from the Golgi complex, and that the diameter of the vacuole being ∼10 μm (Swanson et al., 1998). On the other hand, KV is derived from ER and has a diameter of 200–500 nm, and it is strongly suggested that proteins from the Golgi complex does not contribute to KV (Figs. 5, A–C, and 9 A). The occurrence of SH-EP as the proform in KV suggests that pH of the inside of KV should be neutral, since proSH-EP has potential to be autoactivated into the mature enzyme at acidic pH (Okamoto et al., 1999a). In addition, VmPE-1 was not found in KV although lytic vacuoles generally contain many kinds of hydrolases. These

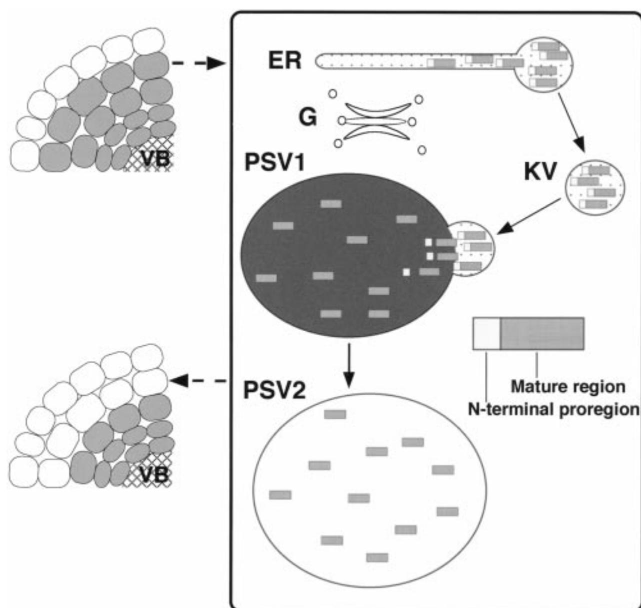

Figure 10. A model of protein mobilization in the cotyledon cell of germinated *V. mungo* seeds. The digestion of storage proteins in germinated cotyledons occurs in cells farthest from the vascular bundle (VB). The proform of SH-EP synthesized in the lumen of ER is packed into the KDEL-tailed cysteine proteinase-accumulating vesicle (KV) at the edge or the middle region of ER, and KV (200–500 nm in diameter) filled with proSH-EP, buds off from ER, bypasses the Golgi complex and fuses with PSV, resulting in the release of proSH-EP into the inside of PSV. A large amount of proSH-EP sorted to PSV is converted to the mature enzyme by autocatalytic and/or VmPE-1–mediated fashions, and the activated mature SH-EP massively degrades the storage globulin. This process mediates the change of the cotyledon cells filled with storage proteins (shaded cells) to vacuolized cells (non-shaded cells). ER, endoplasmic reticulum; G, Golgi complex; KV, KDEL-tailed cysteine proteinase-accumulating vesicle; PSV, protein storage vacuole; VB, vascular bundle; PSV1, protein storage vacuole filled with proteins; PSV2, vacuole in which storage proteins were degraded.

suggest that KV has distinct characters and is a different cell compartment from the lytic/second vacuole detected in barley cells. Chrispeels et al. (1976) reported that, in germinated cotyledons of *V. radiata* seeds, a primary lysosome-like compartment buds off from ER and that the compartment fuses with PSV. A proteinase, named vicilin peptidehydrolase, possibly localizing in the compartment has been isolated (Baumgartner and Chrispeels, 1977). In addition, by means of immunofluorescent technique, the proteinase was found to be localized in the small foci of the cytoplasm of cotyledon cells of germinated *V. radiata* seeds (Baumgartner et al., 1978). Moreover, Schmid et al. (1998) recently reported that a proform of a cysteine protease which possesses a KDEL tail accumulated in an organelle, termed ricinosome, in the cotyledon of germinated castor bean seeds, although its origin and function are still unclear. KV will be a cell compartment analogous to the primary lysosome of *V. radiata* seeds and to the ricinosome of castor bean seeds with respect to its similarity in size and localization of the KDEL-tailed cysteine protease proform in the vesicle/organelle.

Accumulation of proSH-EP occurs in the lumen of ER. The aggregation of seed proteins in ER to form the protein body was well characterized in the prolamins of cereal grains (reviewed by Galili et al., 1998) and in 11S globulin of castor bean (Hara-Nishimura et al., 1998). However, the aggregation of SH-EP will not occur in ER, since the electron contrast of KV was much lower than that of the protein body (Fig. 4) when KV was compared with those of protein bodies (Levanony et al., 1992; Hara-Nishimura et al., 1998) and since SH-EP must be soluble to function in PSV. In contrast to alcohol-soluble prolamins and salt-soluble globulins, SH-EP is a water-soluble protein (Mitsuhashi et al., 1986). There will be a different mechanism for the accumulation of proSH-EP in ER from that of storage proteins. The KDEL sequence of SH-EP is a candidate for the signal for its accumulation in ER.

KDEL Sequence of SH-EP: Putative Accumulation Signal in ER

SH-EP is known to have the KDEL ER retention sequence at the COOH terminus. Our previous report of a heterologous expression of SH-EP and its KDEL deletion mutant in insect Sf-9 cells and subcellular fractionation of cotyledons of germinated *V. mungo* seeds showed that the KDEL sequence of SH-EP delayed the transport of proSH-EP from ER along the endomembrane system and that the removal of KDEL from SH-EP occurred within ER or immediately after exit from ER (Okamoto et al., 1999b). From these observations, we proposed that the KDEL tail of proSH-EP temporarily stores proSH-EP as transient zymogen within ER and the removal of the KDEL permits the proenzyme to enter the endomembrane system. One of these two proposals, the temporary retention of proSH-EP in ER by the KDEL sequence, is consistent with our results, which clearly showed the accumulation of proSH-EP in a part of ER of cotyledon cells. These results with both cotyledon cells and Sf-9 cells strongly indicate that the KDEL sequence plays a role in the accumulation of proSH-EP in the edge or middle region of ER at which the formation of KV proceeds. For the KDEL sequence of proSH-EP, the phrase "accumulation signal at ER" is preferred to "retention signal to ER." The other proposal, removal of KDEL tail in ER, still remains open. Although the KDEL antibody labeled KV, it does not imply that the gold particles in KV represent the KDEL tail of proSH-EP since the antibody recognizes reticuloplasmins as well as proSH-EP. By subcellular fractionation of the cotyledon of germinated *V. mungo*, SH-EP precursor was resolved into two bands of 43- and 42-kD SH-EP, the smaller one lacking the KDEL tail and being enriched in a dense subcellular fraction, and the larger one possessing KDEL and being present mainly in the ER (Okamoto et al. 1999b). The dense subcellular fraction may be most likely a candidate for KV. ProSH-EP in KV may be the mixture of processed (KDEL minus) and unprocessed SH-EP. Isolation of KV from cotyledon cells is needed to determine whether the KDEL tail of proSH-EP is removed or attached in KV. The molecular mechanism of the formation of KV at ER is an interesting problem and remains open to research. Experiments are currently in progress in our laboratory to examine whether

the KDEL sequence of SH-EP is essential for the formation of KV.

KDEL-tailed Proteinase: Possible Key Enzyme for Protein Mobilization in Plants

SH-EP was the cysteine proteinase which was first found to have a KDEL tail (Akasofu et al., 1989) in spite of the fact that the proteinase localizes in the vacuole (Okamoto et al., 1994). Recently, at least seven KDEL-tailed vacuolar cysteine proteases have been identified in other plants. Interestingly, they are expressed in germinated seeds (Becker et al., 1997; Lee et al., 1997; Schmid et al., 1998; Cercos et al., 1999) or in senescing organs, such as senescing pods and fruits (Tanaka et al., 1993; Valpuesta et al., 1995; Guerrero et al., 1998). It should be noted that all KDEL-tailed cysteine proteinases are expressed in organs in which dynamic protein mobilization occurs, and that two enzymes from the eight KDEL-tailed cysteine proteinases, SH-EP and the castor bean enzyme (Schmid et al., 1998), were found to be packed as proenzymes in a similar vesicle. Moreover, our work suggested that the KV-dependent mass transport of proSH-EP to PSV mediates rapid protein mobilization. It also should be noted that KDEL-tailed proteases have been identified only in higher plants, but not in animals or prokaryotes. Higher plants may have evolved to accumulate a large amount of KDEL-tailed proteinases and transport the proenzymes by a specific vesicle, since the massive protein mobilization, such as from cotyledon to hypocotyl and from senescing pod and leaf to maturing seed, is essential for plant organs. The KV-dependent sorting mechanism of proSH-EP presented in this study will provide a clue to explaining the mechanism of dynamic protein mobilization in plants.

We thank Dr. Isao Uemura (Tokyo Metropolitan University) for his kind and useful advice on experiments with electron microscopy, and Dr. Ikuko Yazaki (Tokyo Metropolitan University) for her instructing us on the preparation of monoclonal antibody. We also thank Dr. Eliot Herman (USDA/ARS at Beltsville) for his critical reading of the manuscript and suggestions, and Drs. Ikuko Hara-Nishimura and Maki Kondo (National Institute for Basic Biology) for their useful discussions and technical advice in experiments with electron microscopy. We are also grateful to Dr. I. Hara-Nishimura for her generous gift of antiserum against sycamore β-xylosidase.

This work was supported in part by Grants-in-Aid for Scientific Research (10740374 and 09640776) from the Ministry of Education, Science and Culture of Japan.

Submitted: 24 August 1999
Revised: 15 December 1999
Accepted: 4 January 2000

References

Akasofu, H., D. Yamauchi, W. Mitsuhashi, and T. Minamikawa. 1989. Nucleotide sequence of cDNA for sulfhydryl-endopeptidase (SH-EP) from cotyledons of germinating *Vigna mungo* seeds. *Nucleic Acids Res.* 17:6733.

Baumgartner, B., and M.J. Chrispeels. 1977. Purification and characterization of vicilin peptidehydrolase, the major endopeptidase in the cotyledons of mung bean seedlings. *Eur. J. Biochem.* 77:223–233.

Basha, S.M., and L. Beevers. 1975. The development of proteolytic activity and protein degradation during the germination of *Pisum sativum* L. *Planta.* 124: 77–87.

Baumgartner, B., K.T. Tokuyasu, and M.J. Chrispeels. 1978. Localization of vicilin peptidehydrolase in the cotyledons of mung bean seedlings by immunofluorescent microscopy. *J. Cell Biol.* 79:10–19.

Becker, C., V. Senyuk, A.D. Shutov, V.H. Nong, J. Fischer, C. Horstmann, and K. Müntz. 1997. Proteinase A, a storage-globulin-degrading endopeptidase of vetch (*Vicia sativa* L.) seeds, is not involved in early steps of storage protein mobilization. *Eur. J. Biochem.* 248:304–312.

Bewley, J.D., and M. Black. 1994. Seeds: Physiology of Development and Germination. 2nd edition. Plenum Press, New York. 1–31.

Boylan, M.T., and I.M. Sussex. 1987. Purification of an endopeptidase involved with storage-protein degradation in *Phaseolus vulgaris* L. cotyledons. *Planta.* 170:343–352.

Cercos, M., S. Santamaria, and J. Carbonell. 1999. Cloning and characterization of TPE4A, a thiol-protease gene induced during ovary senescing and seed germination in pea. *Plant Physiol.* 119:1341–1348.

Chrispeels, M.J., B. Baumgartner, and N. Harris. 1976. Regulation of reserve protein metabolism in the cotyledons of mung bean seedlings. *Proc. Natl. Acad. Sci. USA.* 73:3168–3172.

Denecke, J., R. Derycke, and J. Botterman. 1992. Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2345–2355.

Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73:3–47.

Galili, G., C. Sengupta-Gopalan, and A. Ceriotti. 1998. The endoplasmic reticulum of plant cells and its role in protein maturation and biogenesis of oil bodies. *Plant Mol. Biol.* 38:1–29.

Greenwood, J.S., and M.J. Chrispeels. 1985. Immunocyochemical localization of phaseolin and phytohemagglutinin in the endoplasmic reticulum and Golgi complex of developing bean cotyledons. *Planta.* 164:295–302.

Guerrero, C., M. Calle, M.S. Reid, and V. Valpuesta. 1998. Analysis of the expression of two thioprotease genes from daylily (*Hemerocallis* spp.) during flower senescence. *Plant Mol. Biol.* 36:565–571.

Hara-Nishimura, I., I. Takeuchi, K. Inoue, and M. Nishimura. 1993. Vesicle transport and processing of the precursor to 2S albumin in pumpkin. *Plant J.* 4:793–800.

Hara-Nishimura, I., T. Shimada, K. Hatano, and M. Nishimura. 1998. Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. *Plant Cell.* 10:825–836.

Harris, N., and M.J. Chrispeels. 1975. Histochemical and biochemical observations on storage protein metabolism and protein body autolysis in cotyledons of germinating mung beans. *Plant Physiol.* 56:292–299.

Herman, E.M., and B.A. Larkins. 1999. Protein storage bodies and vacuoles. *Plant Cell.* 11:601–613.

Holwerda, B.C., N.J. Galvin, T.J. Baranski, and J.C. Rogers. 1990. In vitro processing of aleurain, a barley vacuolar thiol protease. *Plant Cell.* 2:1091–1106.

Kato, H., and T. Minamikawa. 1996. Identification and characterization of a rice cysteine endopeptidase that digests glutelin. *Eur. J. Biochem.* 239:310–316.

Lee, H.I., S. Gal, T.C. Newman, and N.V. Raikhel. 1993. The Arabidopsis endoplasmic reticulum retention receptor functions in yeast. *Proc. Natl. Acad. Sci. USA.* 90:11433–11437.

Lee, K.M., Z.W. Liu, A.L. Tan-Wilson, and W.A. Wilson. 1997. Transcript levels for a mung bean cysteine protease during early seedling growth. *Seed Sci. Res.* 7:359–372.

Levanony, H., R. Rubin, Y. Altschuler, and G. Galili. 1992. Evidence for a novel route of wheat storage proteins to vacuoles. *J. Cell Biol.* 119:1117–1128.

Mitsuhashi, W., T. Koshiba, and T. Minamikawa. 1986. Separation and characterization of two endopeptidases from cotyledons of germinating *Vigna mungo* seeds. *Plant Physiol.* 80:628–634.

Mitsuhashi, W., and T. Minamikawa. 1989. Synthesis and posttranslational activation of sulfhydryl-endopeptidase in cotyledons of germinating *Vigna mungo* seeds. *Plant Physiol.* 89:274–279.

Munro, S., and H.R.B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.

Müntz, K. 1996. Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. *J. Exp. Bot.* 298:605–622.

Napier, R.M., L.C. Fowke, C.R. Hawes, M. Lewis, and H.R.B. Pelham. 1992. Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum. *J. Cell Sci.* 102:261–271.

Okamoto, T., H. Nakayama, K. Seta, T. Isobe, and T. Minamikawa. 1994. Posttranslational processing of a carboxy-terminal propeptide containing a KDEL sequence of plant vacuolar cysteine endopeptidase (SH-EP). *FEBS Lett.* 351:31–34.

Okamoto, T., and T. Minamikawa. 1995. Purification of a processing enzyme (VmPE-1) that is involved in post-translational processing of a plant cysteine endopeptidase (SH-EP). *Eur. J. Biochem.* 231:300–305.

Okamoto, T., and T. Minamikawa. 1998. A vacuolar cysteine endopeptidase (SH-EP) that digests seed storage globulin: characterization, regulation of gene expression, and posttranslational processing. *J. Plant Physiol.* 152:675–682.

Okamoto, T., and T. Minamikawa. 1999. Molecular cloning and characterization of *Vigna mungo* processing enzyme 1 (VmPE-1), an asparaginyl endopeptidase possibly involved in posttranslational processing of a vacuolar cysteine endopeptidase (SH-EP). *Plant Mol. Biol.* 39:63–73.

Okamoto, T., A. Yuki., N. Mitsuhashi, and T. Minamikawa. 1999a. Asparaginyl endopeptidase (VmPE-1) and autocatalytic processing synergistically activate the vacuolar cysteine proteinase (SH-EP). *Eur. J. Biochem.* 264:223–232.

Okamoto, T., T. Minamikawa, C. Edward, V. Vakharia, and E. Herman. 1999b. Posttranslational removal of the carboxy-terminal KDEL of the cysteine protease SH-EP occurs prior to maturation of the enzyme. *J. Biol. Chem.* 274:11390–11398.

Paris, N., C.M. Stanley, R.L. Jones, and J.C. Rogers. 1996. Plant cells contain two functionally distinct vacuolar compartments. *Cell.* 85:563–572.
Paris, N., S.W. Rogers, L. Jiang, T. Kirsch, L. Beevers, T.E. Philips, and J.C. Rogers. 1997. Molecular cloning and further characterization of a probable plant vacuolar sorting receptor. *Plant Physiol.* 115:29–39.
Pedrazzini, E., G. Giovinazzo, A. Bielli, M. de Virgilio, L. Frigerio, M. Pesca, F. Faoro, R. Bollini, A. Ceriotti, and A. Vitale. 1997. Protein control along the route to the plant vacuole. *Plant Cell.* 9:1869–1880.
Pelham, H.R.B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1–23.
Schmid, M., D. Simpson, F. Kalousek, and C. Gietl. 1998. A cysteine endopeptidase with a C-terminal KDEL motif isolated from castor bean endosperm is a marker enzyme for the ricinosome, a putative lytic compartment. *Planta.* 206:466–475.
Shotwell, M.A., and B.A. Larkins. 1988. The biochemistry and molecular biology of seed storage proteins. *In* The Biochemistry of Plants. B.J. Miflin, editor. Academic Press, New York. 295–345.
Swanson, S.J., P.C. Bethke, and R.L. Jones. 1998. Barley aleurone cells contain two types of vacuoles: characterization of lytic organelles by use of fluorescent probes. *Plant Cell.* 10:685–698.
Tanaka, T., T. Minamikawa, D. Yamauchi, and Y. Ogushi. 1993. Expression of an endopeptidase (EP-C1) in *Phaseolus vulgalis* plants. *Plant Physiol.* 101: 421–428.
Tezuka, K., M. Hayashi, H. Ishihara, M. Nishimura, K. Onozaki, and N. Takahashi. 1993. Purification and substrate specificity of β-xylosidase from sycamore cell (*Acer pseudoplatanus* L.): application for structural analysis of xylose-containing N-linked oligosaccharides. *Anal. Biochem.* 211:205–209.
Valpuesta, V., N.E. Lange, C. Guerrero, and M.S. Reid. 1995. Up-regulation of a cysteine protease accompanies the ethylene-insensitive senescence of daylily (*Hemerocallis* spp.) flowers. *Plant Mol. Biol.* 28:575–582.
Vitale, A., and J. Denecke. 1999. The endoplasmic reticulum—gateway of the secretory pathway. *Plant Cell.* 11:615–628.