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In Vitro Processing of Aleurain, a Barley Vacuolar Thiol Protease

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Aleurain, originally described from its cDNA as a thiol protease [Rogers, J.C., Dean, D., and Heck, G.R. (1985). Proc. Natl. Acad. Sci. USA 82, 6512–6516], is characterized here as a glycoprotein that is targeted to a distinct vacuolar compartment in aleurone cells. Monospecific antibodies to a bacterial *trp*E-aleurain fusion protein were used to show that aleurain is made as a 42-kilodalton (kD) proenzyme (proaleurain) that is proteolytically processed in a post-Golgi compartment in two steps to form a 32-kD protein. The first processing step is the discrete loss of 9 kD from proaleurain to yield a 33-kD intermediate that is further processed by the gradual loss of 1 kD resulting in mature 32-kD aleurain. Using proaleurain secreted from *Xenopus* oocytes as a substrate, we established an in vitro system using aleurone cell extracts that correctly processes proaleurain to a stable protein that is indistinguishable from native barley aleurain as judged by partial digestion with staphylococcal V8 protease. Proaleurain is not capable of self-cleavage in the absence of aleurone cell extracts and mature aleurain appears not to participate in processing in vitro.

INTRODUCTION

The most thoroughly characterized secretory tissue in plants is the barley aleurone. Aleurone cells form a layer that surrounds the starchy endosperm of the grain and secrete large quantities of hydrolytic enzymes during germination in response to the phytohormone gibberellic acid (GA). The secreted hydrolases, of which α-amylase is the most abundant, degrade storage products in the endosperm to supply nutrients to the rapidly growing seedling (reviewed by Yomo and Varner, 1971). Aleurone cells must also possess mechanisms that target proteins to intracellular compartments because they have protein bodies (aleurone grains) that contain storage proteins. During germination, the contents of aleurone grains are rapidly degraded and mobilized, presumably to provide amino acids for de novo protein synthesis (reviewed by Fincher, 1989). Little is known about the processes in aleurone cells that segregate proteins targeted to vacuoles from those in the pathway for secretion. We sought to identify a vacuolar protein in aleurone cells that could be used to study targeting mechanisms.

The thiol protease, aleurain, was originally described as the predicted translation product of a cDNA clone isolated from barley aleurone layers (Rogers et al., 1985; Whittier et al., 1987). The C-terminal 218 amino acids define the protease domain, which is very closely related (65% amino acid identity) to the rat lysosomal thiol protease cathepsin H. The striking similarity of these two proteins in such distantly related organisms suggests that these enzymes have an important function that will not tolerate greater sequence divergence. In addition to the protease domain of aleurain, there are 143 residues at the amino terminus, of which the first 22 to 23 residues conform well to consensus signal peptides that direct cotranslational delivery to the lumen of endoplasmic reticulum (ER). The remainder of this N-terminal domain probably corresponds to a propeptide because all thiol proteases so far described that enter the endomembrane system have amino-terminal prosequences. For example, the cDNA sequence of papain predicts that it has an N-terminal 133-residue prosegment (Cohen et al., 1986). Similarly, pulse-labeling experiments identified a 42-kD procathepsin H that was chased to a 28-kD form, suggesting that cathepsin H is initially translated with a prosegment of about 130 amino acids (Nishimura and Kato, 1987), a fact that was confirmed by comparison to the cDNA (Ishidoh et al., 1987; Fuchs et al., 1988).

Because aleurain had been defined only as a cDNA, we characterized the protein with emphasis on its proteolytic processing and final destination in aleurone cells. Using aleurain-specific antibodies raised against an aleurain-*trp*E

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fusion protein expressed in *Escherichia coli*, we now characterize aleurain as a 32-kD intracellular glycoprotein that accumulates in a specific vacuolar compartment. Aleurain is initially synthesized as a 42-kD proenzyme (proaleurain) that is proteolytically processed by two sequential steps to make the mature form. The enzymes responsible for aleurain maturation reside in a post-Golgi compartment in aleurone cells. To further characterize these processing events, we developed an in vitro assay that faithfully reproduces the proteolytic processing of proaleurain. Aleurone cell extracts contain activities that convert *Xenopus* oocyte-synthesized proaleurain into a smaller polypeptide that is indistinguishable from native mature aleurain. The in vitro processing is not autocatalytic. These experiments also show that even though aleurain is 65% identical to mammalian cathepsin H, it is not recognized by the phosphotransferase that generates a mannose 6-phosphate (M6P) group required for targeting to lysosomes in animal cells (reviewed by Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986).

RESULTS

Aleurain is a 32-kD Intracellular Glycoprotein

We characterized the protein by use of polyclonal antibodies to a fusion protein made from the aleurain protease domain and the *E. coli trp*E gene product. Antibodies for either the aleurain portion or the *trp*E portion were purified on affinity columns. The specificity of the affinity-purified antibodies is documented in Figure 1A. Preimmune serum (Figure 1A, column a) did not react with either the *trp*E protein (Figure 1A, lanes T) or the *trp*E-aleurain fusion protein (Figure 1A, lanes F). As expected, the *trp*E antibodies reacted with both the 33-kD *trp*E protein and the 65-kD *trp*E-aleurain fusion protein (Figure 1A, column b). Most importantly, the aleurain antibodies only recognized the *trp*E-aleurain fusion protein, but not *trp*E protein (Figure 1A, column c).

Figure 1B shows the results of using the aleurain antibodies on immunoblots of extracts from different tissues of barley fractionated by SDS-PAGE. Aleurain was detected as a 32-kD protein in both GA-treated aleurone layers and leaves (indicated in Figure 1B). In contrast, aleurain was not detected in media that had surrounded these aleurone layers (Figure 1B). Identical experiments using either preimmune serum or *trp*E antibodies showed no reactive proteins (data not shown). To confirm that aleurain was not secreted, the protein was immunoprecipitated from ^{35}S-labeled extracts and media of aleurone layers; aleurain, a 32-kD protein, was found only in immunoprecipitates made from aleurone layers and not the media surrounding those layers (data not shown). We did not study aleurain from barley leaves by immunoprecipi-

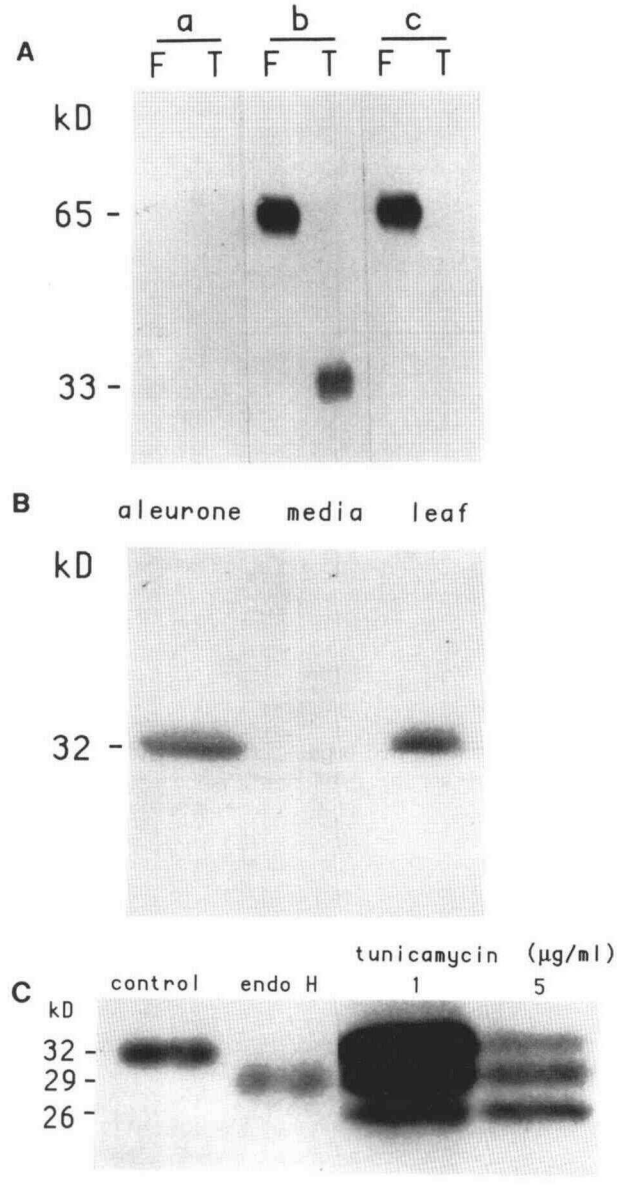

Figure 1. Identification of Aleurain as a 32-kD Intracellular Glycoprotein.

(A) Identically prepared immunoblots of 1 μg of both *trp*E-aleurain fusion protein (lanes F) and *trp*E protein (lanes T) fractionated by SDS-PAGE were treated with preimmune serum (column a), *trp*E antibody (column b), or aleurain antibody (column c).

(B) An immunoblot of 100 μg of total protein from GA-treated barley aleurone layers, media surrounding the layers, and leaf extracts was treated with aleurain antibodies. Similar membranes treated with either preimmune serum or *trp*E antibodies had no signals (not shown).

(C) Aleurain immunoprecipitated from extracts of GA-treated cells was treated with endo H. Aleurain was also immunoprecipitated from extracts of layers treated with 1 μg/mL or 5 μg/mL tunicamycin. Proteins were visualized by fluorography after separation by SDS-PAGE.

tation techniques and, therefore, did not compare its size with that of aleurain immunoprecipitated from aleurone layer extracts. Additionally, in agreement with the previously reported hormonal regulation of aleurain mRNA levels (Rogers et al., 1985), GA induced the synthesis of aleurain, whereas ABA prevented its synthesis (data not shown).

The predicted amino acid sequence of aleurain indicates that there are two potential N-linked glycosylation sites (Rogers et al., 1985). We used digestion with endo-β-*N*-acetylglucosaminidase H (endo H) in addition to metabolic labeling in the presence of tunicamycin to establish that both sites are glycosylated. The results of these experiments are shown in Figure 1C. Digestion of aleurain immunoprecipitates with endo H resulted in a reduction in size from 32 kD to 29 kD, consistent with the removal of one high-mannose-type oligosaccharide (Figure 1C, compare control and endo H). Asparagine-linked oligosaccharides that had been modified to complex form would be resistant to endo H treatment. When layers were treated with 1 μg/mL or 5 μg/mL tunicamycin to prevent glycosylation of asparagine residues, two smaller forms of 29 kD and 26 kD, in addition to the 32-kD form, were identified (Figure 1C, tunicamycin). We interpret the 29-kD form to be aleurain missing one oligosaccharide and the 26-kD form to be missing two oligosaccharides. By analogy to the cathepsin H sequence, the predicted size of the mature aleurain polypeptide is about 26 kD. These results showed that both N-linked glycosylation sites are used in aleurain, and that one site has a high-mannose-type chain, whereas the other possesses a complex-type chain. Tunicamycin did not cause aleurain, in any form, to be secreted to the media (data not shown), suggesting that the N-linked oligosaccharides are not necessary for retention in aleurone cells. Furthermore, the fact that we obtained unglycosylated mature forms indicated that glycosylation was not necessary for proteolytic processing of proaleurain or delivery to the site where this occurs.

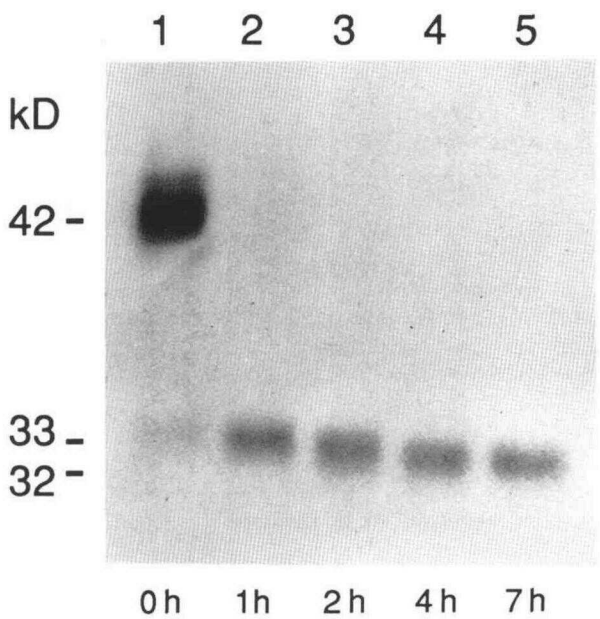

Figure 2. Proaleurain Is the 42-kD Precursor of Aleurain.

GA-treated layers were pulse labeled for 45 min and chased in media containing unlabeled amino acids for the indicated times. Immunoprecipitates of aleurain fractionated by SDS-PAGE were visualized by fluorography.

Aleurain Is Initially Made as a 42-kD Proenzyme (Proaleurain)

Because aleurain, as identified above, is only 32 kD and the translation product predicted from the cDNA is 37 kD after removal of its signal peptide, there must be proteolytic processing of a larger precursor. As illustrated in Figure 2, pulse-chase experiments using a 45-min labeling period showed that the aleurain precursor, proaleurain, is 42 kD in size (Figure 2, lane 1). The size of proaleurain is consistent with a 37-kD polypeptide plus two oligosaccharides. Proaleurain was chased to a 33-kD form within 1 hr (Figure 2, lane 2), followed by a slower trimming to a stable 32-kD form between 4 hr and 7 hr (Figure 2, lanes 3, 4, and 5). We have done a number of different pulse-chase experiments with labeling times up to 2 hr (data not shown) and found only the proteins shown in Figure 2; no other processing intermediates have been detected. These results showed that aleurain is made as a 42-kD proenzyme that undergoes two different proteolytic processing steps. The first is a discrete loss of about 9 kD, termed clipping, forming a 33-kD intermediate that is further reduced, termed trimming, to form mature 32-kD aleurain.

Proaleurain Can Be Correctly Processed to Mature Aleurain in Vitro by Barley Cell Extracts

To further characterize the activities involved in proaleurain maturation, an in vitro assay was developed. We reasoned that such an assay would require correctly folded proaleurain as a substrate. Expression of in vitro synthesized aleurain mRNA in *Xenopus* oocytes resulted in the secretion of proaleurain that is glycosylated with one endo H-sensitive, high-mannose-type oligosaccharide and one complex-type oligosaccharide (data not shown). The maturation reaction was initiated by mixing oocyte-synthesized proaleurain with extracts made from barley aleurone layers. After incubation for various time periods, the products were isolated by immunoprecipitation with antialeurain antibodies and analyzed by SDS-PAGE and fluorography.

The results of an in vitro maturation assay are shown in Figure 3. Incubation of 44-kD ^{35}S-labeled proaleurain in aleurone extracts made in buffers at pH 5 and pH 6 resulted in the time-dependent conversion of the 44-kD precursor to a stable 34-kD product that was immunoprecipitated with antibodies specific to the protease domain of aleurain (Figure 3A, indicated by arrow). In contrast, incubation with extracts prepared at pH 7 or pH 8, or in buffers alone, resulted in no detectable conversion of the 44-kD precursor to the 34-kD mature form. These results established the pH dependence and dependence of the conversion on the presence of cell extract, and ruled out the possibility that maturation was a self-catalyzed reaction (Figure 3A). Additionally, if the crude barley extract was denatured by boiling, no product was formed (Figure 3A, BOIL), showing that processing was mediated by a heat-labile factor(s) that is most likely another enzyme(s). The result from the boiled extract was identical to a zero timepoint in the assay.

Incubation of the 44-kD precursor at pH 5 or pH 6 resulted in the appearance of other degradation products at 1 hr and 4 hr (i.e., 16 kD, 17 kD, and 35 kD, see Figure 3A), but these products were unstable because they were not detected beyond 4 hr. We did not further characterize the unstable products, but speculated that they may result from proteolysis of proaleurain at alternative sites producing fragments that cannot assume a stable, tightly folded conformation. Such proteolytic fragments would be susceptible to further degradation by the abundant nonspecific proteases normally synthesized and secreted by GA-treated aleurone cells (Hammerton and Ho, 1986; Koehler and Ho, 1988). In spite of this, after in vitro processing, mature aleurain was stable in crude extracts for periods extending to 72 hr (data not shown). The fact that proaleurain was not proteolytically processed by extracts at pH 7 or pH 8 suggested that proaleurain maturation factors are localized in an acidified compartment within the aleurone cell.

To show that it was equivalent to mature aleurain, the 34-kD product was subjected to partial digestion with staphylococcal V8 protease (Cleveland et al., 1977) and compared with similar digests of native aleurain (Figure 3B). The partial digestion products of native aleurain and the 34-kD in vitro maturation product were indistinguishable with respect to size and pattern (Figure 3B; similar bands are indicated by arrowheads in lanes 3 and 6). These results indicated that the barley aleurone extracts contain the necessary factors for correct proteolytic processing of proaleurain. The slightly larger size of the in vitro product (34 kD) compared with native aleurain (32 kD) was consistent with the larger size of the substrate oocyte-proaleurain (44 kD), compared with native proaleurain (42 kD). The size differences were due to differing oligosaccharide processing in oocytes and barley cells (data not shown). The experimental strategy did not require conversion of proaleurain to mature aleurain to be quantitative; in fact, only a relatively small portion (2% to 5%) was properly processed. The important results were that processing occurred with fidelity and that the mature protein was stable.

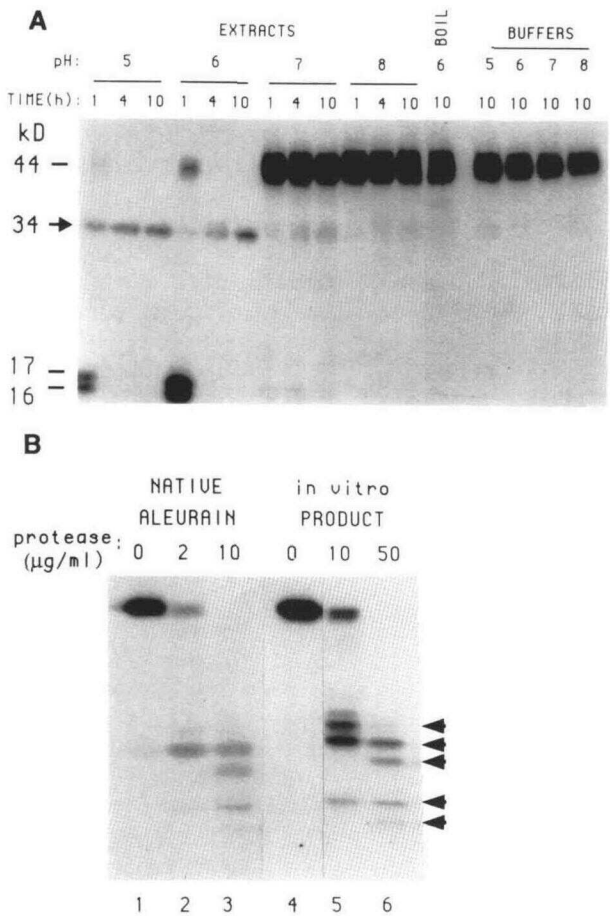

Figure 3. In Vitro Proaleurain Maturation Assay.

(A) Radioactively labeled *Xenopus* oocyte-synthesized proaleurain was added either to barley aleurone extracts made in buffers of the indicated pH or to corresponding buffers at the indicated pH. A pH 6 extract incubated at 100°C before addition of proaleurain is indicated by BOIL and is identical to a zero timepoint. After incubation for the times indicated, reaction products immunoprecipitated with antialeurain antibodies were analyzed by SDS-PAGE and fluorography. The arrow (→) indicates the 34-kD mature aleurain.

(B) Native aleurain immunoprecipitated from radioactively labeled aleurone layers and the product recovered from a 10-hr reaction of oocyte-synthesized proaleurain at pH 6 were incubated with staphylococcal V8 protease at the indicated concentrations for 1 hr at 37°C. The reaction products were analyzed as described in **(A)**. Arrowheads (◄) indicate partial digestion products that are shared by the two proteins.

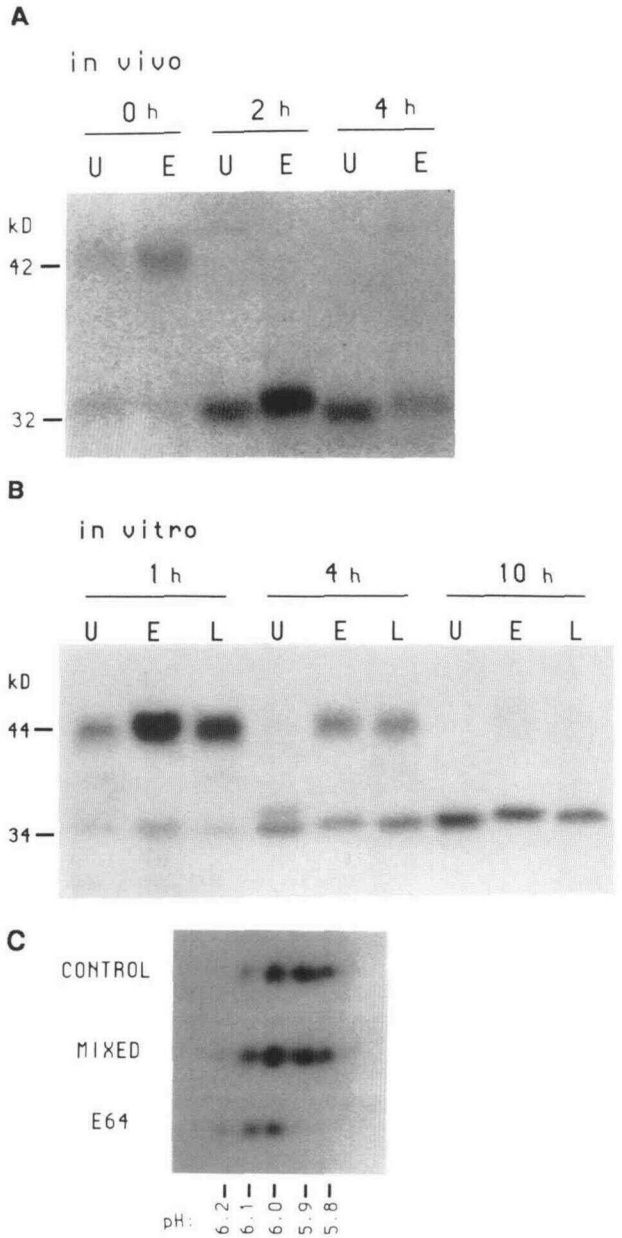

Figure 4. Inhibition of Trimming by the Thiol Protease Inhibitor E64.

(A) Pulse-chase experiment on aleurone layers treated with E64. Aleurone layers were preincubated, pulse labeled, and chased in the presence (E) or absence (U) of 200 μg/mL E64. At the times indicated, immunoprecipitates from extracts of washed layers were made using antialeurain antibodies and analyzed by SDS-PAGE and fluorography.

(B) In vitro maturation assay in the presence of 200 μg/mL E64 (E) and 10 μg/mL leupeptin (L). Aleurone extracts made in pH 6 buffer were preincubated with the respective thiol protease inhibitors at 20°C for 30 min before addition of oocyte-synthesized proaleurain. Untreated control reactions are indicated by U. After incubation for the indicated times, the reaction products were isolated with antialeurain antibodies and analyzed as described in **(A)**.

(C) Two-dimensional gel electrophoretic analysis (O'Farrell, 1975) of in vitro maturation reaction products with untreated and E64-treated extracts. Immunoprecipitates from a 12-hr reaction described in **(B)** were analyzed either separately or mixed as indicated. The separate gels were aligned by reference to nonradioactive standards and the locations of radiolabeled proteins were visualized by fluorography.

The Proaleurain Trimming and Clipping Maturases Are Separate Enzymes

Proaleurain maturation in vivo occurs in two steps identified as clipping (i.e., 42 kD → 33 kD), followed by trimming (i.e., 33 kD → 32 kD) to form mature aleurain. As shown in Figure 4, we found that E64, a thiol protease inhibitor, inhibited the trimming step of maturation both in vivo (Figure 4A) and in vitro (Figure 4B). In a pulse-chase experiment, E64 had no effect upon formation of the 33-kD intermediate (clipping: 42 kD → 33 kD), but further processing of the 33-kD intermediate (trimming: 33 kD → 32 kD) was blocked, as evidenced by the accumulation of this slightly larger product by cells treated with E64 [Figure 4A, compare lanes U (untreated controls) with lanes E (E64-treated cells)]. Similarly, in vitro, E64 had no effect on clipping but caused the formation of a slightly larger product relative to the untreated control (Figure 4B, compare lanes U with lanes E). Two-dimensional gel electrophoresis was used to show clearly the inhibitory effect of E64 in vitro (Figure 4C). A mixture of control and E64-treated reaction products resolved on the same gel showed that the respective reactions yielded different products (Figure 4C, compare MIXED with CONTROL and E64). Untreated control reactions produced three major charge forms at pH 6.0, pH 5.9, and pH 5.8 and a minor one at pH 6.1 (Figure 4C, CONTROL), whereas E64-treated reactions yielded only three products at pH 6.2, pH 6.1, and pH 6.0 (Figure 4C, E64). Although both control and E64-treated reactions produced two products with the same charge (at pH 6.0 and pH 6.1), the E64-treated extract did not produce any products at pH 5.9 or pH 5.8 that were seen in untreated controls. The inhibition of the trimming maturase was specific for E64 because leupeptin, another thiol protease inhibitor, had no effect [Figure 4B, compare lanes L (leupeptin treated) with lanes U (untreated controls)]. The inhibition by E64 suggested that trimming of the 33-kD proaleurain intermediate was mediated by a thiol protease.

Leupeptin and E64 both appeared to slow the loss of proaleurain compared with controls (Figure 4B, at 4 hr compare lanes L and E with lanes U at 44 kD). As shown in Figure 5, the accumulation of clipped product was not affected. If the amount of radioactivity in the 34-kD region of each lane of the gel shown in Figure 4B is plotted

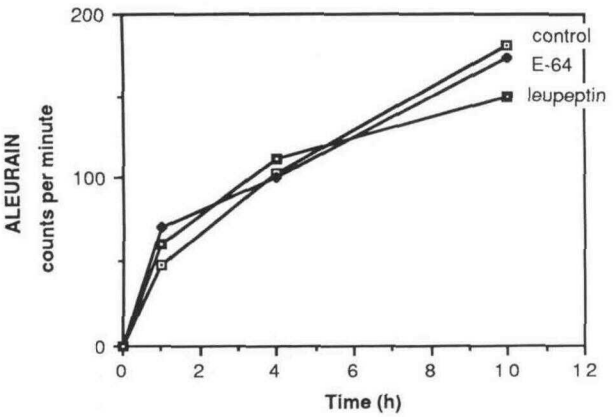

Figure 5. Time Course of Aleurain Accumulation in an in Vitro Maturation Reaction.

Aleurain production was estimated by excising the 34-kD areas of the gel used to make the fluorogram shown in Figure 2B and quantitating the radioactivity by liquid scintillation counting.

relative to the time of incubation, the resulting curves are indistinguishable. Both leupeptin and E64 inhibit the GA-induced secreted thiol proteases (Koehler and Ho, 1988), and the protective effect toward the 44-kD proaleurain is explained by slowing nonspecific degradation. Interestingly, we have not been able to detect inhibition of the in vitro maturation reactions with the serine protease inhibitor phenylmethylsulfonyl fluoride, the aspartic protease inhibitor pepstatin, or the metalloprotease inhibitors EDTA and 1,10-phenanthroline (data not shown).

Mature Aleurain Is not Involved in the Processing of Its Own Precursor

It was possible that aleurain itself could participate in the maturation of proaleurain. To assess this, we took advantage of the fact that aleurain synthesis is induced by GA and repressed by ABA in aleurone tissue. Figure 6 shows the results of a direct comparison of in vitro maturation reactions using extracts that had either high or very low concentrations of mature aleurain. Although extracts from GA-treated cells had much more aleurain than extracts from ABA-treated cells, as shown in immunoblots [Figure 6, compare lanes G (GA-treated cells) with lanes A (ABA-treated cells)], both reactions produced similar amounts of the mature 34-kD product (Figure 6A, compare lanes G with lanes A). This strongly suggested that mature aleurain is not involved in the maturation of its own precursor. Because the extracts are complex mixtures that have many differences (e.g., the GA-treated extract would have additional unlabeled proaleurain), it is not possible to compare processing rates between the two (GA versus ABA). These differences would explain why, for example, more 44-kD proaleurain remained in reactions containing GA-treated extracts than those containing ABA-treated ex-

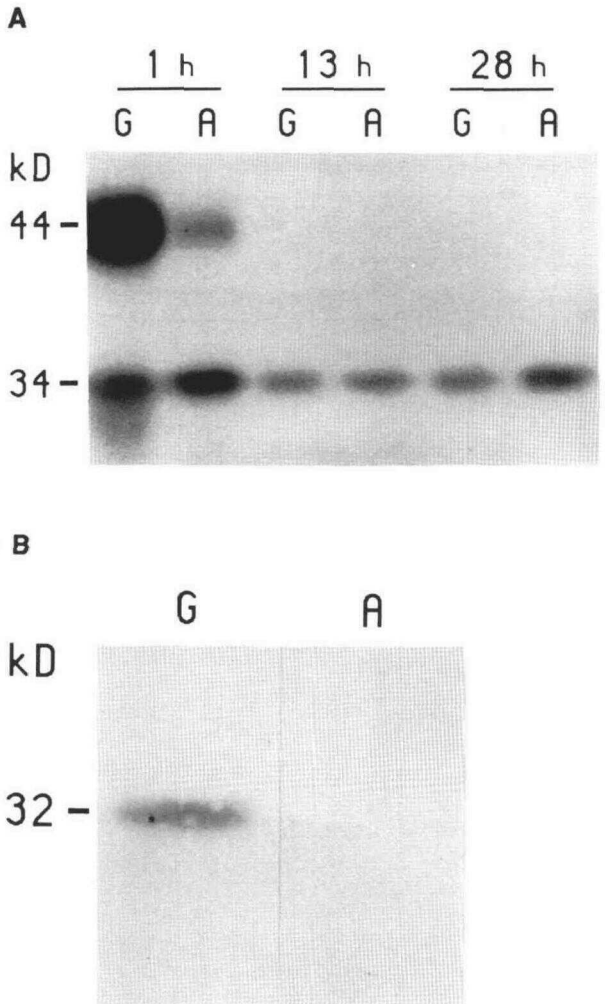

Figure 6. Effect of Mature Aleurain on the Maturation of Proaleurain.

(A) In vitro maturation reactions were done using extracts made at pH 6 from aleurone layers treated either with GA (G) or ABA (A). For the latter, the aleurone layers were prepared from deembryonated seeds that had also been imbibed in the presence of ABA. Immunoprecipitates were made at the indicated times and analyzed by SDS-PAGE and fluorography.

(B) Immunoblot analysis of extracts used in **(A)**. Extracts (50 μg of total protein) were fractionated by SDS-PAGE, blotted onto nitrocellulose, and treated with antialeurain antibodies. After incubation with a secondary antibody conjugated to alkaline phosphatase, the location of aleurain was visualized by staining for alkaline phosphatase activity.

tracts after 1 hr (Figure 6A, at 1 hr compare lanes G with lanes A).

Proteolytic Processing of Proaleurain Occurs at a Post-Golgi Location

To characterize the subcellular location of the aleurain maturation events, we did a pulse-chase experiment in the presence of monensin. Monensin blocks secretory traffic at the medial Golgi (Tartakoff, 1983). As shown in Figure 7, monensin completely blocked processing of the 42-kD precursor [Figure 7, compare lanes without (−) and with (+) monensin]; this indicated that maturation occurs in a post-Golgi compartment. To confirm the effect of monensin on secretion, we also measured the amount of α-amylase secreted into the incubation media. It can be seen in Figure 7 (lower panel) that monensin treatment of aleurone layers (Figure 7, lanes +) caused a drastic inhibition of α-amylase secretion. Consistent with these results, brefeldin A, a drug that disrupts secretory traffic between ER and Golgi (see Lippincott-Schwartz et al., 1989), also caused inhibition of proaleurain processing similar to that described above for monensin (data not shown).

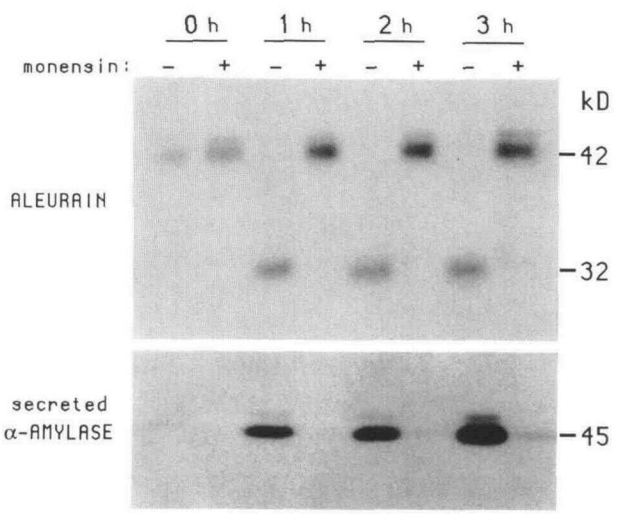

Figure 7. Proteolytic Processing of Proaleurain Occurs in a Post-Golgi Compartment.

GA-treated aleurone layers were preincubated, pulse labeled, and chased in media containing 10 μM monensin (+). Untreated controls are indicated by a minus sign (−). At the times indicated, samples were taken and immunoprecipitates made either from cell extracts using antialeurain antibodies (upper panel) or media using anti-α-amylase antiserum (lower panel). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Proaleurain, a Plant Vacuolar Protein, Is not Recognized by the M6P Receptor, an Animal Lysosomal Targeting Receptor

Because aleurain is targeted to vacuoles in plant (barley) cells and is also closely related to cathepsin H (65% identical residues; Rogers et al., 1985), which is targeted to lysosomes in animal cells, it was of interest to determine whether aleurain would be recognized as a protein to be targeted to lysosomes in an animal cell. This was tested by expressing aleurain in *Xenopus* oocytes because these cells readily express foreign proteins (Coleman, 1984) and correctly target mammalian lysosomal enzymes (see Faust et al., 1987). Figure 8 shows the results of these experiments. Aleurain expressed by microinjection of in vitro synthesized mRNA caused the oocytes to secrete a 44-kD protein that resembled native proaleurain. The proaleurain secreted by oocytes was used as substrate for the above described maturation assays. Figure 8A shows that the increasing amount of proaleurain in the media was associated with a loss from the oocytes [Figure 8A, compare lanes O (oocytes) with lanes M (media) over 24 hr to 72 hr]. There was no evidence that the oocytes produced any mature aleurain even on extended exposure of fluorograms.

In animal cells, soluble lysosomal enzymes acquire M6P residues that are recognized by an M6P receptor that subsequently targets the enzyme to lysosomes. The acquisition of M6P groups requires the presence of specific determinants on lysosomal enzymes that are recognized by a phosphotransferase (UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosaminyl phosphotransferase) that transfers *N*-acetylglucosamine 1-phosphate to selected mannose residues (Hasilik et al., 1981; Reitman and Kornfeld, 1981a, 1981b). The resulting phosphodiester intermediate is then further processed by removal of the terminal *N*-acetylglucosamine (by *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase), exposing an M6P group that is recognized by the M6P receptor (reviewed by Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986). Whether or not oocyte-proaleurain had acquired M6P residues was assessed by testing its ability to bind to an M6P receptor affinity column (Figure 8B). Both intracellular and media forms of oocyte-proaleurain were quantitatively recovered in the flow-through fraction from the M6P receptor column (Figure 8B, lanes F), indicating that neither form of proaleurain had acquired recognizable M6P groups. This assay is sensitive enough to detect M6P residues on as little as 0.5% of the total protein; otherwise, the column will bind greater than 95% of a lysosomal enzyme, cathepsin D, similarly expressed in oocytes (Faust et al., 1987). Additionally, other work has shown that aleurain expressed in COS-1 cells (African green monkey kidney cells) is secreted and does not incorporate detectable ^{32}P, as would be expected had it acquired M6P groups (G. Sahagian, B. Holwerda, and J. Rogers, unpublished data).

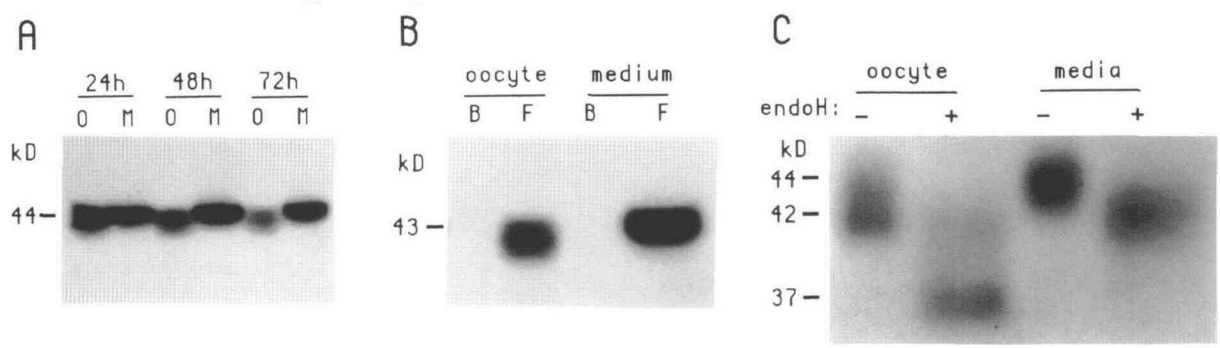

Figure 8. Aleurain Expressed in *Xenopus* Oocytes Is Secreted and Does not Bind to the Mannose 6-Phosphate Receptor.

(A) After microinjection of in vitro synthesized aleurain mRNA into *X. laevis* oocytes, aleurain was immunoprecipitated from either the oocytes (O) or incubation media (M). The indicated timepoints start from the addition of radioactive amino acids. Each lane contains immunoprecipitable material from the equivalent of 0.5 oocyte. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

(B) Oocyte extracts and media from 72 hr in **(A)** were passed through an M6P receptor affinity column and immunoprecipitates made using antialeurain antibodies from material that bound to the column (B) and unbound flow-through material (F). Immunoprecipitates were analyzed as described in **(A)**.

(C) Proaleurain immunoprecipitated from oocytes and media was incubated with endo H (+) and compared with untreated control immunoprecipitates (–). The reaction products were analyzed as described in **(A)**.

The lack of oligosaccharide phosphorylation was not caused by an absence of high-mannose-type oligosaccharides because both forms of proaleurain were susceptible to digestion with endo H [Figure 8C, compare lanes with (+) and without (–) endo H]. These results demonstrated that proaleurain is not recognized by the enzymes that specifically phosphorylate high-mannose-type oligosaccharides on lysosomal enzymes in animal cells.

Aleurain Is Targeted to a Vacuolar Compartment in Aleurone Cells

Immunogold labeling with the antialeurain antibodies was used to show the location of aleurain in aleurone tissue from germinating barley seeds. As illustrated in Figure 9, light microscopy of sections treated with aleurain antibodies showed many evenly distributed, discrete sites that contained aleurain, as shown by the deposition of silver in aleurone (al) cells. These were not present in either pericarp (pc) or endosperm (en) tissues. The specificity of this result is shown by the lack of staining in control sections treated with the anti-*trp*E antibodies (Figure 9A).

Immunogold localization using antibodies labeled with 10-nm colloidal gold was performed at the ultrastructural level to obtain a more precise identification of the aleurain-containing structures. As shown in Figure 10, the antialeurain antibody is specifically localized to structures we have termed aleurain-containing vacuoles (acv). Three additional examples of these structures are shown in Figure 10 (panels D, E, and F). The presence of the antialeurain antibodies is shown by the 10-nm gold particles (appearing as strong black dots) that overlay the aleurain-containing vacuoles in large numbers. This immunogold electron microscopy has been repeated using secondary antibodies labeled with 5-nm gold (which gives smaller particles) alone, and after silver enhancement of 1-nm gold particles; in each case, identical results were obtained (data not shown). Moreover, the size and distribution of aleurain-containing vacuoles are consistent between light and electron micrographs. The specificity of our immunogold technique is shown by the fact that similar structures did not bind any gold particles on grids treated with control *trp*E antibodies (Figure 10, panels A, B, and C). Antibody localization to aleurain-containing vacuoles was emphasized by the fact that much lower densities of gold particles were associated with other structures. Quantitation of several grids yielded a density of 166 (±18) gold particles μm^{-2} (mean ± SE) for aleurain-containing vacuoles, whereas aleurone grains had 9.2 (±2.7) μm^{-2}, cell walls had 1.3 (±0.1) μm^{-2}, and lipid bodies had 4.4 (±0.4) μm^{-2}. The aleurain-containing vacuoles were slightly more electron opaque than surrounding cytoplasm and were not circumscribed with lipid bodies. They varied in shape from circular to elliptical and kidney-shaped with diameters ranging from 0.4 μm to 1.5 μm, but irregular-shaped structures were seen (e.g., Figure 10, panel H). Interestingly, we found 36.5 (±8.2) gold particles μm^{-2} over areas that were dense in ribosomes and rough ER. This lower density of labeling may represent aleurain on its way through the secretory apparatus to its final vacuolar destination. Optimal preservation of aleurain antigenicity required a mild fixation protocol without the use of osmium. For that reason, membranous structures are not visualized in these sec-

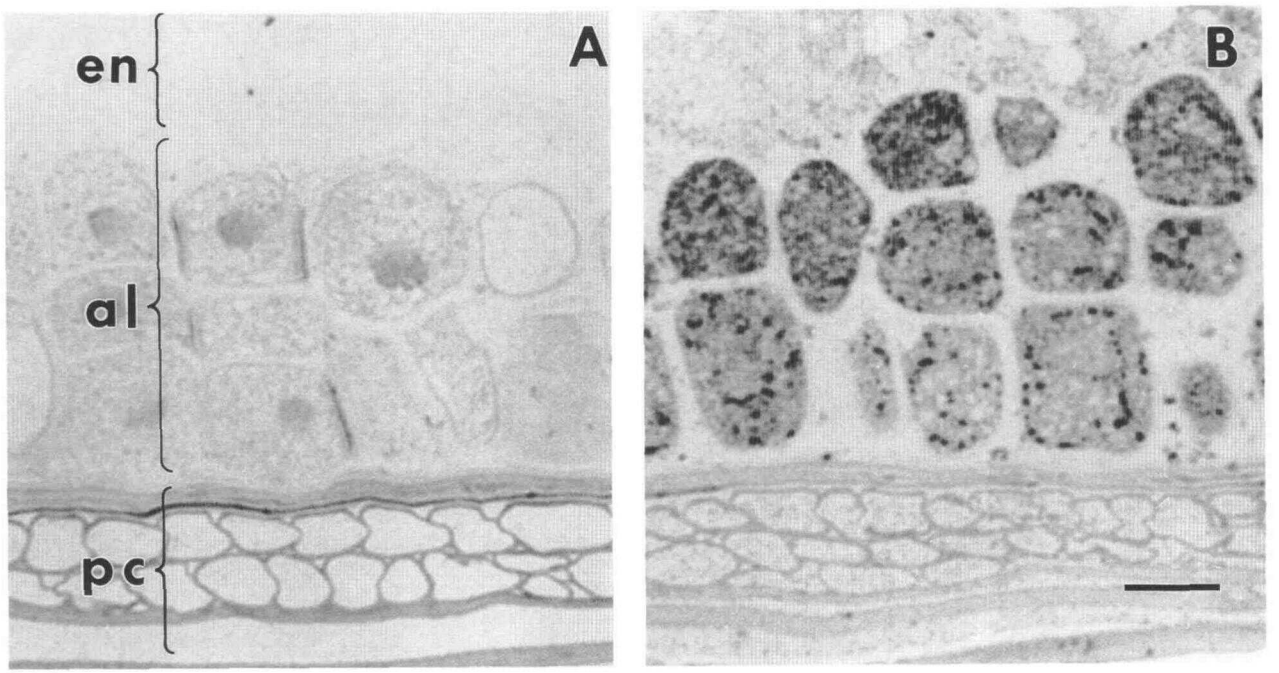

Figure 9. Aleurain Is Located in Discrete Locations in Aleurone Cells.

Thick sections (0.5 μm) of aleurone tissue from 3-day germinating barley seeds were treated with the indicated antibodies. Binding of the primary antibodies was visualized by silver enhancement of colloidal gold-conjugated goat anti-rabbit IgG secondary antibody. en, starchy endosperm; al, aleurone layer; pc, pericarp. Bar is 20 μm.
(A) Anti-*trp*E antibodies.
(B) Antialeurain antibodies.

tions. Otherwise, the morphology of our sections was very consistent with results obtained by other investigators (Jones, 1969; Vigil and Ruddat, 1973). Current efforts are being directed at preserving both aleurain antigenicity and membranes to clearly identify aleurain (or proaleurain) associated with ER and/or Golgi.

DISCUSSION

Our overall goal is to understand the cell biology of protein processing and targeting in aleurone cells. As one effort toward this goal, we developed an antibody specific to the previously uncharacterized thiol protease aleurain. Although aleurone cells secrete thiol proteases in response to treatment with GA (Hammerton and Ho, 1986; Koehler and Ho, 1988), we could only detect aleurain in cell extracts and not in media containing other secreted proteins. Although we cannot exclude the possibility that some aleurain is secreted from cells and rapidly degraded, our data from immunoblotting, immunoprecipitates, and immunogold microscopy showed that aleurain is primarily targeted to a specific vacuolar compartment in aleurone cells.

Barley aleurone cells have multiple vacuoles and abundant lipid bodies and aleurone grains (protein bodies) (Jones, 1969; Yomo and Varner, 1971; Vigil and Ruddat, 1973). During seed germination, the contents of the aleurone grains are rapidly mobilized, presumably by proteases contained within the same protein body (reviewed by Fincher, 1989). However, we found that aleurain does not localize directly within aleurone grains, but is associated with other vacuoles that are clearly different. Aleurone-containing vacuoles may represent a different type of protein body in aleurone cells. It is possible, however, that aleurain-containing vacuoles or smaller vesicles originating from these vacuoles fuse with aleurone grains to degrade their contents. A study of reserve protein metabolism in germinating mung beans suggested that newly synthesized endopeptidase activity became associated with protein bodies by fusion with smaller vesicles budding from the ER (Chrispeels et al., 1976). The nature of the aleurain-containing vacuole is not yet clear, but our ability to tag it with a biochemical marker will permit a more detailed functional characterization of this organelle.

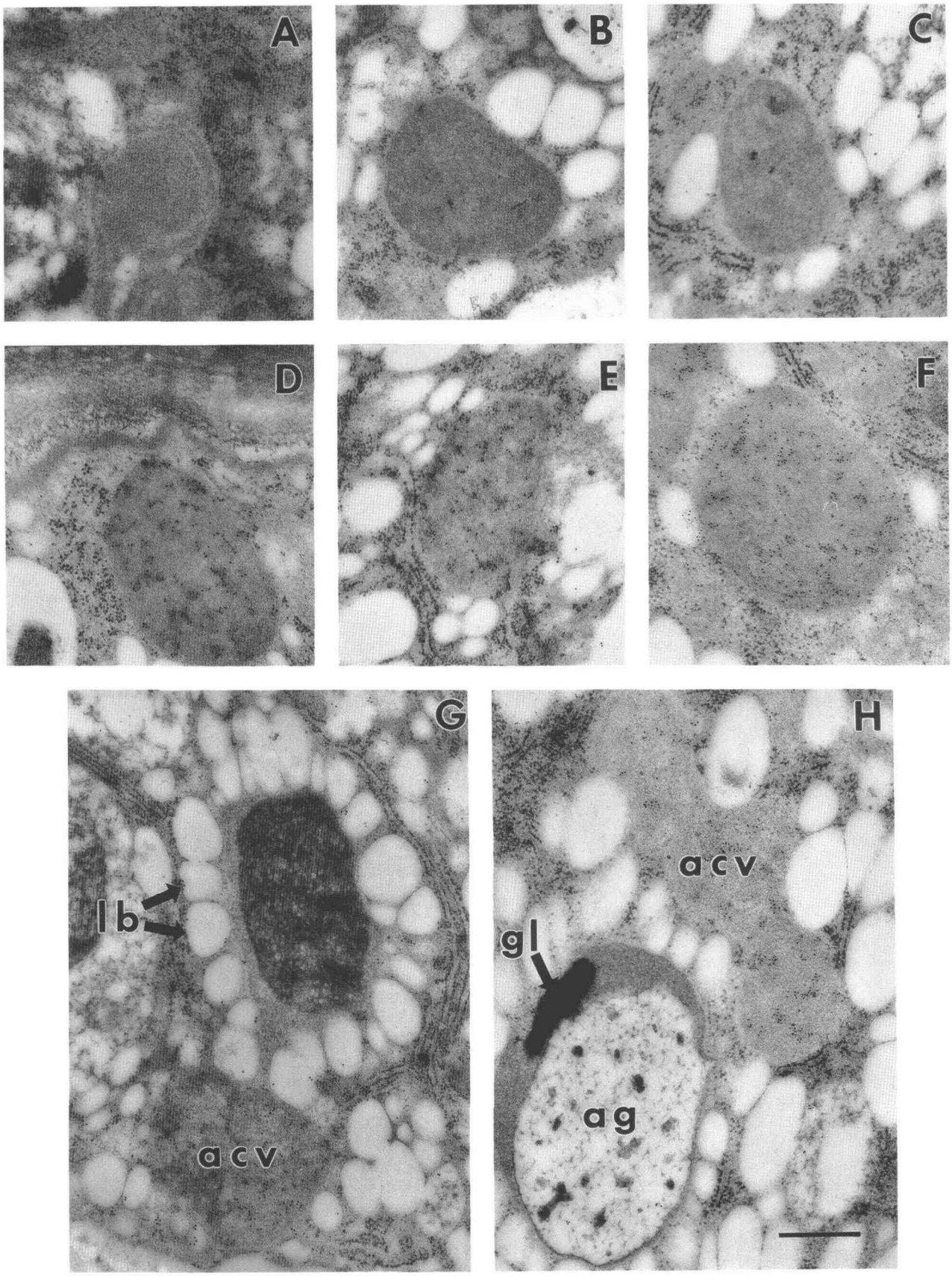

Figure 10. Examples of Aleurain-Containing Vacuoles.

Aleurain was also found in mature leaf tissue at levels similar to that in GA-stimulated aleurone layers. Rogers et al. (1985) showed that aleurain mRNA was also found in root cells in addition to leaves and aleurone layers. The wide tissue distribution of aleurain suggests that it may function in general protein turnover in plant cells, similar to the role proposed for closely related cathepsin H in mammalian cells (Barrett and Kirschke, 1981).

Aleurain is synthesized as a 42-kD proenzyme before being processed to a final 32-kD mature form. The size of proaleurain is consistent with predictions made from the cDNA. Cotranslational removal of a signal peptide from aleurain should yield a 37-kD polypeptide backbone that upon addition of two N-linked oligosaccharides would result in a proenzyme of about 42 kD. Alignment of the predicted aleurain amino acid sequence with the mature sequences of cathepsin H and papain (see Rogers et al., 1985) suggests that mature aleurain results from the removal of an amino-terminal prosegment. We have not yet established the amino-terminal sequence of mature aleurain, but the unglycosylated 26-kD form made by tunicamycin-treated cells is similar in size to the predicted 25-kD protein if cleavage were to occur at a site homologous to cathepsin H and papain (see Rogers et al., 1985). Furthermore, the alignment of predicted amino acid sequences from the cDNAs of cathepsin H (Fuchs et al., 1988) and papain (Cohen et al., 1986) to the respective mature polypeptides indicates that both undergo removal of only N-terminal residues during processing. We cannot, however, rule out the possibility that some proteolytic processing occurs at the carboxyl terminus of aleurain because another related thiol protease, cathepsin B, is known to undergo removal of a 6-amino-acid C-terminal extension during its maturation (Chan et al., 1986).

We characterized the proteolytic processing of aleurain further by developing an in vitro maturation assay. The use of correctly folded *Xenopus* oocyte-synthesized proaleurain as a substrate was crucial to developing a faithful in vitro maturation assay. The results showed that proteolytic processing of proaleurain is mediated by two different factors that are active in aleurone cell extracts. The maturase activities catalyze both the clipping (42 kD → 33 kD) and the trimming (33 kD → 32 kD) reactions. We were able to show that two different reactions were occurring because trimming alone was inhibited by the thiol protease inhibitor E64. Because monensin blocked proaleurain maturation, these reactions occur either within the aleurain-containing vacuole or at a site between the Golgi and this vacuole (i.e., the trans-Golgi network). In view of the fact that several different plant storage proteins are known to be proteolytically processed at their site of deposition in vacuoles or protein bodies (Chrispeels, 1984), it is a reasonable possibility that proaleurain maturation occurs in the aleurain-containing vacuole. This conjecture is supported by the fact that in vitro processing worked only in an acidic environment (pH 5 to pH 6) similar to one that is characteristic of plant vacuoles. This assay will enable us to purify the proaleurain maturation proteases and thereby establish their localization in barley cells. Whatever the post-Golgi processing site may be, however, the asparagine-linked oligosaccharides on proaleurain have no role in targeting proaleurain to it. These results are consistent with previous work that has definitively shown oligosaccharides have no role in storage protein targeting in plant cells (Voelker et al., 1989; Sonnewald et al., 1990; Wilkins et al., 1990).

Our results also show that proaleurain does not have the structural features necessary for oligosaccharide phosphorylation and subsequent targeting to lysosomes when it is expressed in an animal cell. The phosphorylation of lysosomal enzymes requires a restricted three-dimensional arrangement of amino acid residues to form a determinant on one side of the molecule that is recognized by a specific phosphotransferase (Baranski et al., 1990). Apparently, aleurain cannot form this recognition patch although it is 65% identical with the lysosomal enzyme cathepsin H.

Because aleurain is probably a thiol protease itself (Rogers et al., 1985), it was important to consider the possibility that maturation of proaleurain was either an intramolecular (i.e., self-catalyzed) reaction and/or mediated by mature aleurain in an intermolecular manner. Intramolecular and intermolecular reactions are both involved in the conversion of the aspartic protease precursor pepsinogen into pepsin (Al-Janabi et al., 1972; Sanny et al., 1975; Marciniszyn et al., 1976) and, furthermore, Smith and Gottesman (1989) have shown that recombinant procathepsin L, a thiol protease precursor, can self-catalyze its own maturation. Our results indicated that neither intramolecular nor intermolecular reactions were responsible for the maturation of proaleurain, but instead, other protease activities were involved.

The proteolytic processing of yeast vacuolar proteases provides one of the better defined systems for comparison to our results. Proteinase A, an aspartic protease, is responsible for the processing of several hydrolases targeted to the yeast vacuole. Soluble enzymes such as carboxypeptidase Y, proteinase B, and membrane-bound vacuolar alkaline phosphatase are dependent upon proteinase A,

Figure 10. (continued).

The binding of antialeurain or control anti-*trp*E antibodies to thin sections of aleurone cells was visualized with a secondary antibody conjugated to 10-nm gold particles. ag, aleurone grain; acv, aleurain-containing vacuole; gl, phytic acid globoid; lb, lipid body. Bar is 0.5 μm.

(A) through **(C)** Anti-*trp*E antibodies.

(D) through **(H)** Antialeurain antibodies.

the *PEP4* gene product, for maturation (Ammerer et al., 1986; Mechler et al., 1987, 1988; Klionsky and Emr, 1989). Proteinase A appears to be activated in the vacuole by a self-catalyzed mechanism (Woolford et al., 1986). The proaleurain clipping maturase activity, however, was not inhibited by an aspartic protease inhibitor, and the trimming maturase appears to be a thiol protease. There are two broad categories of other well-characterized processing proteases. One category includes endopeptidases that cleave substrates on the carboxyl-terminal side of pairs of basic amino acids, e.g., the yeast *KEX2* gene product (Fuller et al., 1989). The other category of processing enzymes includes dipeptidyl aminopeptidases that cleave either -X-Ala- or -X-Pro- dipeptides from the amino terminus of polypeptides, as occurs in processing of *Yarrowia lipolytica* alkaline extracellular protease (Matoba and Ogrydziak, 1989). Similar activities have not yet been identified in plants. In fact, in the examples from well-studied storage proteins, different cleavage specificities are seen. For example, cleavage of the 15-residue carboxyl-terminal propeptide from barley lectin occurs between glycine and valine residues (Wilkins et al., 1990).

In vitro processing of the precursors of seed storage proteins has been shown in two other systems. One report (Harley and Lord, 1985) showed processing of castor bean lectins by extracts of protein bodies and another (Hara-Nishimura and Nishimura, 1987) showed that pumpkin cotyledon proglobulin was correctly processed by a vacuolar extract. It is not known whether these processing events are mediated by maturases related to those that process proaleurain. Pumpkin proglobulin and castor bean lectin processing enzymes showed tissue-specific expression, being restricted to seeds, whereas proaleurain processing occurs not only in barley grains but in other tissues such as leaves. The proaleurain processing activities may be members of a class of maturases that process other plant thiol proteases such as papain (see Cohen et al., 1986), barley EP-B (Koehler and Ho, 1990), and a sulfhydryl endoprotease from mung beans (Mitsuhashi and Minamikawa, 1989). Each of these enzymes is made as a proenzyme of about 43 kD in size that is eventually processed to a 32-kD to 33-kD mature enzyme, but little is known about the enzymology involved in these processes. In vitro processing systems, such as that used here, should ultimately permit purification of the maturases and give a better understanding of their tissue distribution and substrate specificities.

Finally, the availability of cDNAs for two homologous thiol proteases, aleurain and EP-B (Koehler and Ho, 1990), that are simultaneously expressed in aleurone cells but have different destinations (vacuolar versus secreted, respectively) should allow identification of the protein sequences responsible for targeting aleurain. As an initial strategy, experiments are underway to switch prosequences from the two proteins and to express the chimeric proteins in plant cells to determine their intracellular destinations.

METHODS

Production of Antibodies to Aleurain Fusion Proteins

A fusion protein with an *Escherichia coli trp*E segment fused to the aleurain protease domain was used to make antialeurain antibodies. The protease domain is defined by alignment of the predicted translation product of the aleurain cDNA (nucleotides 466 through 1119) with the amino acid sequence of rat liver cathepsin H (Rogers et al., 1985). An aleurain cDNA fragment bounded by an NciI site at 468 bp and a BspMI site at 1093 bp was made blunt ended, and BamHI linkers were attached at both ends. This was ligated into the BamHI site of pATH2 (Dieckmann and Tzagoloff, 1985) to make pJR135. The DNA sequence through the ligation site was determined to assure the correct reading frame was maintained. In *E. coli* strain HB101, pJR135 produced an indole acrylic acid-inducible fusion protein where aleurain was carboxyl-terminal to a portion of the *E. coli trp*E gene product. The fusion protein was purified by preparative SDS-PAGE as described by Hoffman et al. (1987). Immunization by multiple intradermal injection (Hurn and Chantler, 1980) was done with 100 μg of protein in Freund's complete adjuvant with boosts of 50 μg given intramuscularly at 4-week to 8-week intervals using Freund's incomplete adjuvant. The *trp*E-aleurain fusion protein was soluble only in buffers containing a minimum of 0.1% SDS and was quantitated using bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL) with BSA as the standard.

Polyclonal antibody was purified from an immune serum IgG fraction by first removing *trp*E-specific antibodies by repeated passage through a *trp*E-Sepharose column before isolation on aleurain-*trp*E-Sepharose. The affinity supports were made by coupling either 10 mg of crude *trp*E protein (Hoffman et al., 1987) or 10 mg of SDS-PAGE-purified *trp*E-aleurain fusion protein to 1 g of cyanogen bromide-activated Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) in 0.1 M sodium borate, pH 9.0, containing 0.5 M NaCl and 0.1% SDS. After extensive washing with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.9, and 150 mM NaCl), the antibodies were removed from their respective affinity columns by elution with 0.2 M glycine, pH 2.0, at room temperature. Eluted fractions were neutralized, followed by concentration and dialysis against TBS containing 0.1% sodium azide. Antibodies eluted from *trp*E-Sepharose were shown to be specific to *trp*E protein on immunoblots and were used as a control in immunogold microscopy of aleurone tissue.

Preparation of Tissue Extracts and Media

Barley seeds (*Hordeum vulgare* cv Himalaya) were surface sterilized, de-embryonated, and imbibed under sterile conditions for 72 hr to 96 hr as previously described (Rogers and Milliman, 1983). Aleurone layers were incubated in 20 mM sodium succinate, pH 5.2, containing 10 mM $CaCl_2$, 50 μg/mL carbenicillin, and 0.125 μg/mL amphotericin B (Sigma). All incubations were done at room temperature with constant gentle agitation. ^{35}S-labeled

amino acids were supplied by including TranS^{35}-label (70% ^{35}S-methionine and 15% ^{35}S-cysteine; >1000 Ci/mmol; ICN Biomedicals) at approximately 0.2 mCi/mL. Gibberellic acid (Sigma) or abscisic acid (Sigma) were added to the incubation medium at final concentrations of 2×10^{-6} M and 1×10^{-5} M, respectively. In some experiments, tunicamycin (Sigma) was added 1.5 hr before addition of TranS^{35}-label and included throughout the remainder of the incubation period. For pulse-chase experiments, incubation with isotope was for 45 min, followed by three washes and further incubation in similar medium without TranS^{35}-label but supplemented with 10 mM methionine and 5 mM cysteine. In some experiments, 10 μM monensin (Sigma), 200 μg/mL E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Sigma] or 10 μg/mL leupeptin (Sigma) were included in a 45-min preincubation before labeling and included at the same concentration in labeling media and chase media. Medium was removed from the layers and adjusted to pH 7.5 by addition of 1 M Tris-HCl, pH 9, and brought to final concentrations of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/mL leupeptin. Layers were thoroughly washed before homogenizing in TBS containing 10 mM EDTA, 0.1 mM PMSF, and 5 μg/mL leupeptin using a Polytron homogenizer (Brinkman Instruments). After centrifugation at 10,000*g* for 10 min, the clarified homogenates and media were stored at −80°C until use.

Leaf extracts were made by homogenizing barley leaves in 50 mL of 200 mM Mes, pH 6.0, 200 mM NaCl, 10 mM EDTA, 5 mM sodium diethyldithiocarbamate, 10 mM 2-mercaptoethanol, and 2% insoluble polyvinylpolypyrrolidone (Sigma) with a Polytron, clarified, and stored as described above for aleurone layer extracts.

Immunoblotting

Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose (BA85; Schleicher & Schuell) in 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol. The membranes were blocked with TBS containing 5% skim milk powder before incubation with primary antibody in TBS containing 3% BSA, 1% goat serum (Sigma), and 0.01% gelatin (Sigma) at room temperature for 2 hr. After thorough washing in TBS containing 0.1% Nonidet P-40, membranes were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1:5000 in TBS containing 0.1% Nonidet P-40 for 2 hr. Membranes were washed and stained for alkaline phosphatase in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl, 5 mM $MgCl_2$, 50 μg/mL nitro blue tetrazolium (Sigma), and 50 μg/mL 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Immunoprecipitation

Aleurain was immunoprecipitated from ^{35}S-labeled extracts and media by incubation with the aleurain antibodies, followed by isolation of the immune complexes with protein A-Sepharose as described by Anderson and Blobel (1983). SDS was added to 1% and the samples were then incubated at 100°C for 5 min. After dilution with TBS to yield 0.2% SDS and addition of Nonidet P-40 to 1%, samples were chilled on ice, antialeurain antibodies added (0.5 μg), and mixtures incubated overnight at 4°C. Protein A-Sepharose (Sigma) was added and the mixtures were incubated at room temperature for 2 hr with continuous mixing. The protein A-Sepharose beads were washed four times with TBS containing 0.2% SDS and 1.0% Nonidet P-40 and finally with 0.1 M Tris-HCl, pH 8.0, before releasing the bound proteins by incubation at 100°C for 4 min in SDS sample buffer (Laemmli, 1970).

Analysis of Immunoprecipitated Proteins

SDS-PAGE was done as described by Laemmli (1970) using 12% acrylamide in the separation gel. Molecular weights were determined by comparison to the migration of a mixture of standard proteins purchased from Bethesda Research Laboratories and included myosin heavy-chain, M_r 200,000; phosphorylase *b*, M_r 97,400; bovine serum albumin, M_r 68,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 29,000; β-lactoglobulin, M_r 18,400; and lysozyme, M_r 14,300. Two-dimensional gel electrophoresis was done as described (O'Farrell, 1975), and the pH gradient of the isoelectric focusing gels was determined directly by equilibrating gel slices in degassed 9 M urea and measuring the pH of this solution. Gels containing radiolabeled proteins were prepared for fluorography by treatment with *EN^{3}HANCE* (DuPont). Endo-β-*N*-acetylglucosaminidase H (endo H; Boehringer Mannheim) digestions were done on immunoprecipitates released from protein A-Sepharose in 100 mM sodium citrate, pH 6.0, containing 0.075% SDS and 0.2% 2-mercaptoethanol. Reaction mixtures contained 4 milliunits of endo H and were incubated at 37°C for 18 hr. For analysis of in vitro matured aleurain by partial digestion with staphylococcal V8 protease (Sigma), immunoprecipitates were released from protein A-Sepharose with 0.2% SDS. These were adjusted to a final concentration of 40 mM sodium phosphate, 0.16% SDS, and 0 μg/mL to 50 μg/mL V8 protease and incubated at 37°C for 1 hr (see Cleveland et al., 1977). The digestions were stopped by adding SDS to 2% and immediately boiling before analysis by SDS-PAGE.

In Vitro Proaleurain Maturation Assay

The in vitro proaleurain maturation assay was initiated by mixing proaleurain secreted by *Xenopus laevis* with an extract of barley aleurone layers. In the standard assay, aleurone layers isolated from de-embryonated and imbibed barley seeds were incubated 16 hr to 20 hr in media containing either GA or ABA as described above. The layers were washed thoroughly before chilling and homogenizing in 75 mM Mes, pH 6.0, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $ZnSO_4$ and 5 mM 2-mercaptoethanol for 30 sec with a Polytron (150 layers per 15 mL of buffer). The homogenate was sonicated for 30 sec at 150 W to ensure disruption of membranes before clarifying by centrifugation at 15,000*g* for 15 min. The resulting supernatant was used as the enzyme source for the maturation assay. The effect of pH was tested by using aleurone extracts made as described above, but the Mes buffer was replaced with another buffer of different pH. These were 75 mM sodium acetate, pH 5.0, 75 mM sodium phosphate, pH 7.0, or 75 mM Tris-HCl, pH 8.0. The maturation reaction was initiated by the addition of 20 μL to 30 μL of media from metabolically labeled oocytes expressing the aleurain mRNA to 1.0 mL of aleurone extract and incubating at 20°C. Aliquots (190 μL) were removed at various timepoints and immunoprecipitates were made using antialeurain antibodies.

Expression and Analysis of Aleurain in *Xenopus* Oocytes

The full-length aleurain cDNA (Rogers et al., 1985) was subcloned into pSP65 (Melton et al., 1984) to make in vitro RNA transcripts for microinjection into *X. laevis* oocytes. A BamHI linker was attached to the NaeI site just preceding the ATG translation initiation codon in the aleurain cDNA and this was ligated between the BamHI and HindIII sites of pSP65. RNA transcripts were made from the resulting construct, pJR215, after it was linearized as described by Melton et al. (1984) with the modifications of Faust et al. (1987). Manipulations, injection of RNA into oocytes, and metabolic labeling with TranS^{35}-label (>1000 Ci/mmol) were exactly as described by Faust et al. (1987). At the end of incubations, oocytes and media were separated and stored at −70°C. Oocyte extracts were made by homogenizing oocytes in TBS containing 0.1 mM PMSF, 5 μg/mL leupeptin, and 5 mM EDTA by sonication (25 μL/oocyte) and clarified by centrifugation. Oocyte extracts and media were first denatured with SDS before aleurain was immunoprecipitated.

Media and oocyte extracts were passed through a bovine liver 215-kD M6P receptor affinity column that was prepared and run as described previously (Faust et al., 1987). Flow-through and eluted (bound) fractions were denatured with SDS before making immunoprecipitates using antialeurain antibodies.

Immunogold Microscopy

Barley seeds germinated in a moist chamber for 3 days were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, overnight at 4°C, dehydrated through a graded series of ethanol, and infiltrated with LR White embedding medium (Polysciences, Inc., Warrington, PA). Embedded tissue slices were polymerized overnight in an oven at 50°C to 55°C. Semi-thin sections (0.5 μm to 1 μm thick) were mounted on glass slides for light microscopic immunolocalization. Sections were rehydrated by 30-sec treatments in 50% ethanol, 25% ethanol, and then TBS before incubation in blocking solution (TBS containing 3% BSA, 2% goat serum, 0.1% gelatin, and 0.05% sodium azide) at room temperature for 30 min. After rinsing with wash solution (20 mM Tris-HCl, pH 8.2, containing 0.9% NaCl, 0.1% BSA, and 20 mM sodium azide), sections were incubated in 5 μg/mL antialeurain antibody (anti-*trp*E antibody for controls) in blocking solution at room temperature for 90 min. These were washed thoroughly by three cycles of 10 min each in fresh changes of wash solution before incubation for 60 min at room temperature in goat anti-rabbit IgG conjugated to 1-nm colloidal gold (Janssen Life Science Products, Piscataway, NJ) diluted 1:50 in blocking solution. The sections were washed again as described above and additionally for 10 min in TBS at room temperature. After a brief wash in water, bound gold particles were visualized by silver enhancement as described by the manufacturer (Janssen). The sections were counterstained with 1% neutral red in water.

Ultrathin sections (80 nm thick) were mounted on Formvar-coated nickel grids for immunogold electron microscopy. Nonspecific binding was blocked by a 30-min incubation in EM blocking solution [PBS containing 0.8% BSA, 5 μg/mL normal goat IgG (Sigma) and 0.1% gelatin] before overnight incubation at 4°C in 5 μg/mL antialeurain antibodies (anti-*trp*E antibodies for controls) in EM blocking buffer. The grids were washed extensively with EM wash solution (PBS containing 0.05% Tween 20 and 0.5% NaCl) before incubation in 10-nm colloidal gold-labeled goat anti-rabbit IgG (Janssen) diluted 1:100 in EM blocking solution for 4 hr at 4°C. After extensive washing with EM wash solution, the grids were fixed briefly in 2% glutaraldehyde before counterstaining with uranyl acetate and lead citrate. Sections were viewed in a JEOL 100cx transmission electron microscope.

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