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Communication

Gibberellic Acid Regulates the Level of a BiP Cognate in the Endoplasmic Reticulum of Barley Aleurone Cells[1]

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ABSTRACT

The isolation of a 70-kilodalton protein from barley (*Hordeum vulgare* L.) aleurone layers that cross-reacts with an antibody against yeast binding protein (BiP) is reported. Endoplasmic reticulum isolated from aleurone layers treated with gibberellic acid contain much higher levels of the BiP cognate than do membranes isolated from layers treated with abscisic acid.

The synthesis and secretion of α-amylase and other acid hydrolases from the aleurone layer of cereals is regulated by the plant hormones GA_3 and ABA (reviewed in ref. 8). Calcium is also required for the synthesis and secretion of α-amylase by aleurone tissue (11). Like the α-amylases of bacteria and mammals, barley α-amylase is a Ca-containing metalloprotein, and the binding of one molecule of Ca^{2+} to each amylase molecule is required for enzymic activity and protein stability (6).

One of the effects of GA_3 in the barley aleurone layer is to stimulate Ca^{2+} transport into the lumen of the ER (3). GA_3-stimulated Ca^{2+} transport into aleurone ER is ATP dependent and inhibited by the vanadate anion (3), and GA_3 stimulates transport 10- to 20-fold by increasing the activity of the Ca^{2+} transporter (6). The effects of GA_3 on Ca^{2+} transport and on the synthesis and secretion of α-amylase are inhibited by ABA (5).

ER isolated from GA_3-treated barley aleurone contains at least 3 to 5 μM Ca^{2+} (3), a level at least 10 times that found in the cytosol (4). We have proposed that the high level of Ca^{2+} in the lumen of the ER is required for the synthesis of active and stable α-amylase molecules (3, 6). Our proposal is supported by experiments showing that when the Ca^{2+} concentration of isolated ER vesicles is reduced by Ca^{2+} ionophores, the activity of α-amylase within the vesicles decreases (6).

Calcium has also been implicated in the transport of secretory proteins from the ER (16). The ER of animal and yeast cells contains Ca-binding proteins that allow this compartment of the endomembrane system to accumulate as much as 3 mM Ca (17). One of the lumenal ER proteins that has been identified as a Ca-binding protein is BiP[3] (13, 17). BiP is a soluble ER protein that is homologous to heat-shock proteins in animal cells (14–16). The deduced amino acid sequence of yeast BiP consists of 682 amino acids, including 58 aspartate and 53 glutamate residues that are implicated in calcium binding (14, 16, 17). A BiP homolog with an apparent mol wt of 70,000 has recently been identified in the endosperm of the *floury-2* mutant of corn (9). The deduced amino acid sequence of this BiP homolog shows it to be 62% identical to BiP from mouse and yeast (9).

BiP has also been implicated in the folding of secretory proteins in the ER (reviewed in ref. 15). A unique feature of BiP and other resident ER proteins is the presence of the C-terminal tetrapeptide sequence KDEL or HDEL (15). This sequence has been shown to interact with a receptor in the salvage compartment such that BiP is retained in the endomembrane system (18). It has been proposed that the binding of BiP to the receptor is regulated by Ca^{2+} (14, 16).

Because barley aleurone ER is the site of synthesis and export of a Ca-containing secretory protein and accumulates high levels of Ca^{2+}, we investigated the hypothesis that BiP was present in this compartment. We show that ER membranes isolated from aleurone cells contain a BiP cognate whose synthesis is regulated by GA_3 and ABA.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* cv Himalaya, 1985 harvest, Department of Agronomy, Washington State University, Pullman, WA) grains were de-embryonated and allowed to imbibe water (11). Aleurone layers were isolated and incubated in 5 μM GA_3 or 1 μM ABA (Sigma) both with 10 mM $CaCl_2$ (20 layers/mL).

Membrane Isolation

Microsomal and ER membranes were isolated using methods described previously (3). Crude microsomal membranes were prepared by discontinuous density gradient centrifuga-

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[3] Abbreviations: BiP, binding protein; CCR, Cyt *c* reductase; PM, plasma membrane; TP tonoplast or vacuolar membrane.

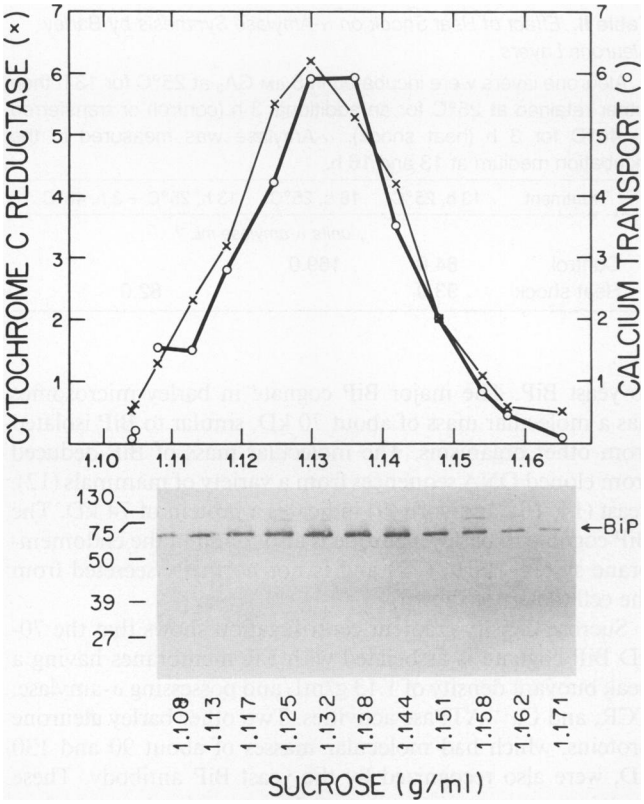

Figure 1. Localization of BiP, the ER marker enzyme CCR, and Ca^{2+} transport activity in membranes isolated from GA_3-treated barley aleurone layers. Upper panel, distribution of CCR and Ca^{2+} transport activities in density gradient fractions. Lower panel, protein blot of density gradient fractions probed with yeast anti-BiP. Molecular mass is indicated in kD.

tion, and ER membranes were purified from this fraction by isopycnic gradient centrifugation (3). Sucrose concentrations were measured refractometrically. ER was identified by the presence of α-amylase and CCR (EC 1.6.2.1) (11), and the TP by the TP-specific antibody TP-25 (10). The PM was localized by its buoyant density at 1.17 g/mL (3).

Calcium Transport

Net Ca^{2+} uptake into purified microsomal membrane fractions was monitored using the method of Bush *et al.* (3). Membranes (100 μL, approximately 100 μg protein/mL) were mixed with 400 μL of medium containing 25 mM Hepes adjusted to pH 7.4 with bistris{[bis(2-hydroxyethyl)imino]-trishydroxymethyl-methane}, 10 mM potassium oxalate, 3 mM $MgSO_4$, 10 μM $CaCl_2$, 100 μM sodium azide with or without 1 mM ATP, and $^{45}CaCl_2$ (3.7×10^4 Bq/mL, specific activity 5.6×10^6 Bq/mmol; Amersham). Calcium uptake was continued for 20 min, and duplicate 200-μL samples of the reaction mixture were withdrawn and filtered onto 0.45-μm filter disks (type HA, Millipore) under vacuum. Radioactivity was measured by scintillation counting. Ca^{2+} transport was calculated as the mean difference between $^{45}Ca^{2+}$ accumulation in membranes in the presence and absence of ATP (3).

Electrophoresis and Protein Blotting

SDS-PAGE was performed in a minigel system (6 × 9 × 0.8 cm) using 12% acrylamide. Protein blots were probed with bean anti-TP-25, yeast anti-BiP, and barley anti-α-amylase, all raised in rabbits, followed by goat anti-rabbit serum coupled to peroxidase (Cappel Laboratories). Peroxidase activity was visualized with 4-chloro-1-naphthol (Sigma) and H_2O_2.

RESULTS

Purified microsomal membranes isolated from GA_3-treated barley aleurone layers contained three proteins recognized by an antibody raised to yeast BiP (Fig. 1). The major protein had an apparent molecular mass of about 70 kD, and two more weakly cross-reacting proteins had molecular masses of about 90 and 130 kD. The 70-kD protein was broadly distributed across the gradient and peaked at 1.13 g/mL. The 90- and 130-kD proteins exhibited peaks of antigenicity at 1.12 to 1.13 g/mL and 1.16 g/mL (Fig. 1).

The distribution of the 70-kD BiP cognate on an isopycnic gradient matched that of the ER marker enzyme CCR and ATP-dependent Ca^{2+} transport activity, *i.e.* it was broadly distributed between 1.10 and 1.17 g/mL with a peak at 1.13 g/mL (Fig. 1). The ER and TP were also localized by probing protein blots of SDS-PAGE gels of the gradient fractions with barley anti-α-amylase and bean anti-TP-25, respectively. Whereas the fractions with the highest antigenicity to the α-amylase antibody had densities between 1.12 and 1.13 g/mL, the fractions showing the highest cross-reactivity to TP-25 were at 1.14 to 1.15 g/mL (data not shown).

To establish whether GA_3 and ABA affect the levels of the BiP cognate, we isolated microsomal membranes from barley aleurone layers incubated in GA_3 or ABA for 16 h. Mem-

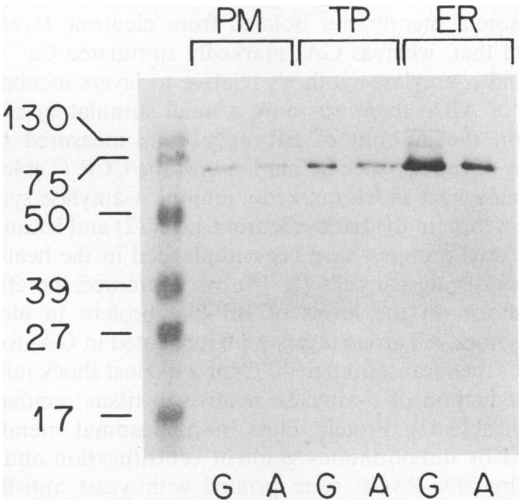

Figure 2. Effects of GA_3 and ABA on the BiP cognate in PM, TP, and ER fractions of density gradients of membranes isolated from barley aleurone layers incubated in GA_3 (G) or ABA (A) for 16 h. Fractions enriched in PM, TP, and ER from gradients of membranes from GA_3- or ABA-treated layers were analyzed by SDS-PAGE, and protein blots were probed with yeast anti-BiP. Left hand lane shows position of prestained molecular mass markers (kD).

Table I. *Effect of Incubation in GA_3 or ABA on α-Amylase Synthesis by Aleurone Layers and on Ca^{2+} Transport and ER Marker Enzyme Activity in Isolated Microsomal Membranes*

Aleurone layers (50) were incubated in 10 mM $CaCl_2$ in the presence or absence of 5 μM GA_3 or 1 μM ABA for 16 h. α-Amylase secreted into the incubation medium was measured and microsomal membranes were isolated. CCR and Ca^{2+} transport activity were measured in the microsomal membrane fraction.

Treatment	α-Amylase	CCR Activity	Net $^{45}Ca^{2+}$ Transport
	units mL^{-1}	*pmol min^{-1} mL^{-1}*	
Ca^{2+}	7.8	415	1.44
$GA_3 + Ca^{2+}$	66.0	520	9.03
$ABA + Ca^{2+}$	3.2	473	0.76

branes were resolved by isopycnic centrifugation, and the fractions corresponding to the positions of the ER, TP, and PM were analyzed by SDS-PAGE. Protein blots of these gels were probed with anti-BiP (Fig. 2). One major protein at 70 kD was found in ER, TP, and PM fractions of membranes isolated from GA_3- and ABA-treated tissue. Two minor bands at about 90 and 130 kD were also seen in the ER fraction of membranes isolated from GA_3-treated aleurone layers (Fig. 2). The distribution of BiP-like protein among ER, TP, and PM fractions was similar to that shown in Figure 1, the highest level of BiP cognate occurring in the ER and lower levels in the TP and PM fractions (Fig. 2).

GA increased the level of the BiP cognate in ER, TP, and PM fractions relative to layers incubated in ABA (Fig. 2). Antibody staining of the 70-kD band showed that GA_3 caused an increase in the amount of the protein that cross-reacts with the BiP antibody (Fig. 2). In this same experiment, aleurone layers incubated in GA_3 secreted 77 units of α-amylase, whereas layers incubated in ABA produced only 3 units of α-amylase.

GA_3 and ABA also regulated the levels of Ca^{2+} transport in microsomal membranes isolated from aleurone layers. We showed that, whereas GA_3 markedly stimulated Ca^{2+} transport and α-amylase synthesis relative to layers incubated in $CaCl_2$ or ABA, there was only a small stimulatory effect of GA_3 on the amount of ER membrane measured by the activity of the ER-specific marker enzyme CCR (Table I).

Because heat shock markedly inhibits α-amylase synthesis and secretion in the barley aleurone layer (2) and because BiP and related proteins have been implicated in the heat-shock response in animal cells (1, 15), we examined the effect of heat shock on the levels of BiP-like protein in aleurone microsomes. Aleurone layers were incubated in GA_3 for 13 h at 25°C, then transferred to 40°C for 3 h. Heat shock inhibited the production of α-amylase relative to tissue incubated at 25°C (Table II). Protein blots of microsomal membranes isolated by discontinuous gradient centrifugation and separated by SDS-PAGE were probed with yeast anti-BiP. A prominent band of 70 kD that cross-reacted with yeast anti-BiP was present in approximately equal amounts in microsomal membranes isolated from control and heat-shocked tissue (Fig. 3).

DISCUSSION

Microsomal membranes isolated from the aleurone layer of barley contain proteins recognized by an antibody raised

Table II. *Effect of Heat Shock on α-Amylase Synthesis by Barley Aleurone Layers*

Aleurone layers were incubated in 5 μM GA_3 at 25°C for 13 h then either retained at 25°C for an additional 3 h (control) or transferred to 40°C for 3 h (heat shock). α-Amylase was measured in the incubation medium at 13 and 16 h.

Treatment	13 h, 25°C	16 h, 25°C	13 h, 25°C + 3 h, 40°C
	units α-amylase mL^{-1}		
Control	84.0	169.0	
Heat shock	93.0		82.0

to yeast BiP. The major BiP cognate in barley microsomes has a molecular mass of about 70 kD, similar to BiP isolated from other organisms. The molecular mass of BiP deduced from cloned DNA sequences from a variety of mammals (12), yeast (14, 16), and corn (9) indicates a protein of 74 kD. The BiP cognate in barley aleurone is also found in the endomembrane system (Figs. 1, 2) and is not normally secreted from the cell (data not shown).

Sucrose density gradient centrifugation shows that the 70-kD BiP cognate is associated with ER membranes having a peak buoyant density of 1.13 g/mL and possessing α-amylase, CCR, and Ca^{2+}-ATPase activities. Two other barley aleurone proteins, which had molecular masses of about 90 and 130 kD, were also recognized by the yeast BiP antibody. These proteins were prominent in membranes with a buoyant density of 1.16 g/mL as well as in the ER fraction (Fig. 1). Although the identity of these membranes is not known, they are intermediate in density between the TP (1.15 g/mL) and the PM (1.17 g/mL) of the aleurone layer (3). It is interesting to note that protein disulfide isomerase, which also has the C-terminal tetrapeptide KDEL, is associated with the PM and the membranes of the Golgi apparatus of rat exocrine pancreas cells (19).

The cross-reactivity between yeast anti-BiP and the 90- and 130-kD proteins in aleurone cells may not be specific. It should be noted, however, that a large number of proteins

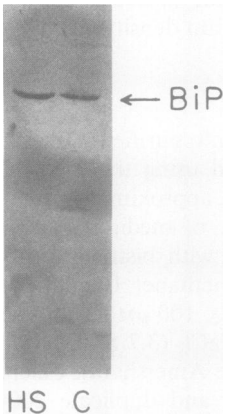

Figure 3. Effect of heat shock on the levels of BiP cognate in microsomal membranes isolated from barley aleurone layers incubated in GA_3 for 13 h at 25°C then transferred to 40°C for 3 h (heat shock, HS) or incubated at 25°C for 16 h (control, C).

having C-terminal KDEL/HDEL sequences have now been isolated (15). For example, an abundant ER protein, GRP94, having a C-terminal KDEL sequence and a molecular mass of about 94 kD, has been isolated from mammalian cells and is thought to be involved in the folding of secretory proteins (15).

The level of the BiP cognate in barley aleurone layers is dramatically altered by incubation in GA_3 and ABA (Fig. 2). GA_3 increases the level of BiP cognate relative to layers incubated in ABA (Fig. 2) or in the absence of hormone (data not shown). GA_3 also increases the rate of Ca^{2+} transport and stimulates α-amylase synthesis in aleurone ER (Table I). The levels of BiP, α-amylase, and Ca^{2+} transport activity are lower in layers incubated in ABA (Fig. 2, Table I), but the levels of ER membrane are about the same (Table I), indicating that GA_3 and ABA do not bring about changes in ER-associated proteins by altering the amount of ER.

BiP is structurally and functionally related to the heat shock protein hsp70 (15). Treatments, such as heat shock, that affect the synthesis of secretory proteins also increase the level of BiP/hsp70 in animal cells (7). In barley aleurone, however, heat shock inhibits α-amylase synthesis and secretion but does not affect the level of BiP cognate.

BiP could play two important roles in regulating the synthesis and secretion of α-amylase in the aleurone cell. It could catalyze the folding of the α-amylase molecule and it could serve to maintain high lumenal ER Ca^{2+} levels (17). Evidence that the folding of barley α-amylase is catalyzed and not spontaneous comes from experiments showing that amylase inactivated by Ca^{2+} removal cannot be reactivated *in vitro* by the addition of Ca^{2+} (6). Because of its ability to bind Ca^{2+} with low affinity (17), BiP may be particularly important in the folding of Ca-containing metalloenzymes such as α-amylase. Besides the 70-kD BiP cognate described in this paper, we have found several other Ca^{2+}-binding proteins in barley microsomes whose levels are increased by GA_3 and decreased by ABA (D. S. Bush and R. L. Jones, unpublished observations).

Our data on the BiP cognate in the endomembrane system of barley aleurone are consistent with a role for this protein in α-amylase synthesis and secretion. The synthesis and secretion of α-amylase requires the coordination of translational and posttranslational events in the ER, and we propose that GA_3 and ABA play key roles in these processes. GA_3 and ABA affect α-amylase synthesis by regulating the transcription of the α-amylase genes (8). GA_3 and ABA could also affect both the synthesis and the secretion of amylase by regulating Ca^{2+} transport and BiP synthesis. We propose that BiP regulates α-amylase synthesis by catalyzing the folding of the amylase molecule, and it regulates secretion of α-amylase by buffering Ca^{2+} in the lumen of the ER.

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