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KDEL-Containing Auxin-Binding Protein Is Secreted to the Plasma Membrane and Cell Wall[1]

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The auxin-binding protein ABP1 has been postulated to mediate auxin-induced cellular changes associated with cell expansion. This protein contains the endoplasmic reticulum (ER) retention signal, the tetrapeptide lysine-aspartic acid-glutamic acid-leucine (KDEL), at its carboxy terminus, consistent with previous subcellular fractionation data that indicated an ER location for ABP1. We used electron microscopic immunocytochemistry to identify the subcellular localization of ABP1. Using maize (*Zea mays*) coleoptile tissue and a black Mexican sweet (BMS) maize cell line, we found that ABP1 is located in the ER as expected, but is also on or closely associated with the plasma membrane and within the cell wall. Labeling of the Golgi apparatus suggests that the transport of ABP1 to the cell wall occurs via the secretory system. Inhibition of secretion of an ABP homolog into the medium of BMS cell cultures by brefeldin A, a drug that specifically blocks secretion, is consistent with this secretion pathway. The secreted protein was recognized by an anti-KDEL peptide antibody, strongly supporting the interpretation that movement of this protein out of the ER does not involve loss of the carboxy-terminal signal. Cells starved for 2,4-dichlorophenoxyacetic acid for 72 h retained less ABP in the cell and secreted more of it into the medium. The significance of our observations is 2-fold. We have identified a KDEL-containing protein that specifically escapes the ER retention system, and we provide an explanation for the apparent discrepancy that most of the ABP is located in the ER, whereas ABP and auxin act at the plasma membrane.

Several ABPs have been identified in plants (Hicks et al., 1989; Jones and Venis, 1989; Macdonald et al., 1991; Prasad and Jones, 1991), and a possible role in auxin-mediated cell expansion has been postulated for several of these (Jones and Prasad, 1992). The first ABP identified (Lobler and Klambt, 1985), now designated ABP1, has been shown to cofractionate predominantly with the ER enzyme marker Cyt *c* reductase, suggesting that at least 90% of this ABP is located in the ER lumen or on the ER membrane (Shimomura et al., 1988; Jones et al., 1989). Analysis of the cDNA encoding ABP1 (Inohara et al., 1989; Tillmann et al., 1989) revealed that the carboxy terminus of ABP1 is the tetrapeptide KDEL, an ER lumen retention signal sequence (Munro and Pelham, 1987). The data consistently indicated that ABP1 is an ER protein, making this putative growth hormone receptor unique.

There is considerable evidence that primary sites of action for auxin-induced cell expansion are located both cytoplasmically and extracytoplasmically (discussed by Jones and Prasad, 1992). Because auxin rapidly induces specific gene transcription (Key, 1989), it is reasonable to assume that the cytoplasmic site of action is within the nucleus. Auxin also rapidly induces proton secretion into the cell wall (Rayle and Cleland, 1980; Cleland et al., 1991) and causes a hyperpolarization of the plasma membrane (Ephritikhine et al., 1987); thus, a plasma membrane/cell wall site of action is also plausible. The relationship among growth, proton secretion, and membrane hyperpolarization was described by Senn and Goldsmith (1988), and a model for induction was proposed by Hager et al. (1991).

Furthermore, Barbier-Brygoo and coworkers proposed that these membrane effects are mediated by auxin and ABP1 via an H^+-ATPase at the outer face of the plasma membrane (Barbier-Brygoo et al., 1989; Barbier-Brygoo et al., 1991). This proposal is based on the observation that polyclonal antibodies directed against maize (*Zea mays*) ABP1 are able to block auxin-induced hyperpolarization of the plasma membrane of tobacco (*Nicotiana tabacum*) mesophyll protoplasts and that maize ABP1 added to the medium bathing tobacco protoplasts enhances the auxin effect. Presumably, the antibodies are binding specifically to the tobacco ABP1 homolog and only at the plasma membrane, and ABP1 is also acting at the plasma membrane. If so, several questions are raised. For example, what is the subcellular location of ABP1 in maize; does ABP1 move through the normal secretion pathway; if ABP1 is secreted, how does ABP1 escape the ER retention mechanism; and, finally, is this movement part of an auxin mechanism of action?

We report here results from electron microscopic immunocytochemistry and immunochemical assays that elucidate the subcellular distribution and secretion of ABP1 in maize coleoptiles and maize suspension-culture cells. The subcellular location of ABP1 at the plasma membrane, the pathway

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Abbreviations: ABP, auxin-binding protein; BiP, immunoglobulin-binding protein; BMS, Black Mexican Sweet; FBS/TBST, 10% fetal bovine serum in Tris-buffered saline-Tween (10 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 0.5% Tween-20); IgG, immunoglobulin G; PDI, protein disulfide isomerase.

of ABP1 trafficking, and the significance of its secretion despite the presence of an ER retention signal is discussed in the context of its possible mode of action.

MATERIALS AND METHODS

Plant Materials and Chemicals

Maize (*Zea mays*) seedlings (B73XMO17) were harvested after 3½ d of growth in complete darkness at 25°C. BMS cells were obtained from Dr. R. Boston, North Carolina State University, and cultured in modified Murashige-Skoog medium as described by Green (1977). BMS cells doubled every week after a 5-d lag and grew in clusters of about 20 to 200 cells. In the first 72 h after transfer, the time of harvest for most of the experiments described here, cultures increased in fresh weight approximately 10 to 20%. Brefeldin A was purchased from Epicentre Technologies (Madison, WI).[2] EM grade glutaraldehyde was purchased from Ted Pella, Inc. (Redding, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. ABP1 was purified from maize seedlings as described by Napier et al. (1988).

Fixation and Microscopy

Immunocytochemical analysis was performed as described by Herman and Melroy (1990). Specifically, BMS cells and coleoptiles of 4-d-old dark-grown maize (cultivar) seedlings to be used for immunolocalization experiments were fixed in 4% formaldehyde, 2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4) at 7°C. Parallel aldehyde-fixed coleoptile samples were postfixed in 1% OsO_4 for 2 h at room temperature to provide samples for structural analysis. The cells and tissue were dehydrated in a graded ethanol series and embedded in hard-grade L.R. White resin. Ultrathin sections mounted on nickel grids were blocked with FBS/TBST for 10 min at room temperature. The grids were incubated in either monospecific anti-ABP antibodies (1:20 dilution) or control IgG diluted in FBS/TBST. The grids were then washed in TBST and indirectly labeled with 10 nm of colloidal gold goat anti-rabbit IgG (BioCell; Ted Pella, Inc.) or protein A-colloidal gold (12 nm) diluted 1:1 in FBS/TBST for 10 min at room temperature. The grids were washed in TBST and distilled water and then stained in 5% aqueous uranyl acetate for 30 min. The grids were examined and photographed with Hitachi H300 and H500 electron microscopes.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE, immunoblot analyses, and antibody production were performed as described by Jones et al. (1991). Anti-ABP1 antibodies (NCO4) were antigen purified using pure maize ABP1 by the method of Ausubel et al. (1990). Oxidation of glycans on the immunoblots and grids was performed by the method of Woodward et al. (1985) with slight modifications. Briefly, the immunoblots and grids previously blocked with milk buffer or FBS/TBST were exposed to 20 mM sodium periodate, 50 mM sodium acetate (pH 4.5) for up to 30 min, followed by exposure to 10 mM sodium borohydrate for 10 min. Rabbit anti-BiP antibodies were described by Fontes et al. (1991) and were provided by Dr. Rebecca Boston, North Carolina State University. Rabbit anti-KDEL peptide antibodies were made using the peptide KETEKESTEKDEL as described by Vaux et al. (1990) and were provided by Dr. S. Fuller (European Molecular Biology Laboratory, Heidelberg, Germany). Rabbit anti-PDI antibodies were described by Shorrosh and Dixon (1991) and were provided by Dr. Richard Dixon, S.R. Noble Foundation.

Secretion Experiments

In experiments on secretion, cells were grown to a density of about 50 to 200 mg fresh weight mL^{-1} medium. Cells were washed with medium three times by allowing the cells to settle and then aspirating the medium. Cells in fresh medium were then harvested, typically 72 h later, by filtration or at the indicated times (see Fig. 14). Proteins in the medium were precipitated by 12% TCA and then washed in acetone. Cells were washed with 5 mL of water. SDS-PAGE sample buffer was added to precipitated protein (20 μL/mL equivalent of medium) and to collected BMS cells (equal volume). Brefeldin A (0.2 μM) or monensin at the indicated concentration was added to washed cells. Experiments on the effect of 2,4-D in the medium were performed with the following modification. Cells were washed and then cultured for 24 h in the appropriate medium (with or without 10 μM 2,4-D) before transfer to fresh medium (with or without 10 μM 2,4-D) and cultured for 72 h before harvesting. Quantitation of ABP in cells and medium was made by scanning the blots and integrating the volume of the band using a Molecular Dynamics Image Analyzer.

Auxin Uptake Experiments

Cells were cultured in the presence or absence of 2,4-D for 72 h as described above. The medium was then replaced with fresh medium containing 0.5 μM 7-[^{3}H]5-azidoindole-3-acetic acid or [^{3}H]benzoic acid, and 1 mL of cell culture was collected by filtration over time. The cells were washed, weighed, and counted for ^{3}H. Ethanol was the carrier solvent in all cases and never exceeded 1%. Accumulation averaged approximately 20-fold over the medium.

RESULTS

Validation of Anti-ABP1 Antibodies as Immunocytochemical Reagents

The affinity-purified antibodies used in this study were shown previously to recognize a single 22-kD polypeptide on an immunoblot overloaded with crude maize shoot tissue (see figure 3 of Jones et al., 1991). This result was repeated here in Figure 1A, lanes 1 and 2. In addition, monospecificity was demonstrated for the affinity-purified antibodies using crude extracts of BMS maize cells (Fig. 1A, lane 3). Glutaraldehyde fixation of the blot had no effect on antibody recog-

[2] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

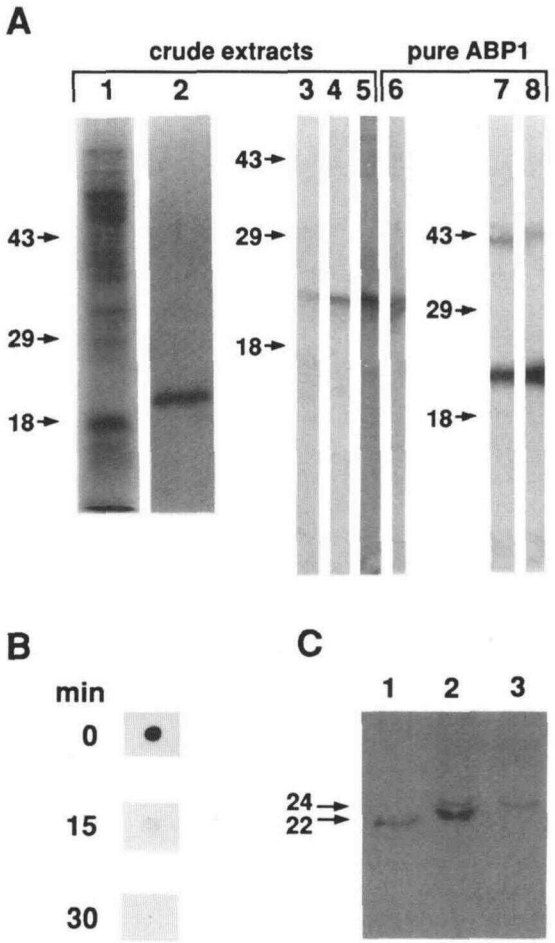


Figure 1. Affinity-purified anti-ABP1 antibodies specifically recognize ABP1 and an ABP homolog in BMS cells. The antibodies were affinity purified by the method of Ausubel et al. (1990). A, Crude extracts of maize coleoptile tissue (10 μg of protein) were subjected to SDS-PAGE (15%) and stained with Coomassie blue. The corresponding blot was probed with affinity-purified antibodies against maize ABP1 (lane 2). Crude cell extracts of BMS cells (approximately 50 μg of protein) were subjected to SDS-PAGE (12%) and immunoblot analysis using affinity-purified anti-ABP antibodies (lanes 3–5). Lane 4 is a strip of the immunoblot that was treated with 1% glutaraldehyde for 30 min. Lane 5 is a strip that was treated with periodate for 30 min as described in B. Shown for comparison in lane 6 is a strip containing untreated ABP1 standard from the blot used to make strips 3 to 5. Lanes 7 and 8 are, respectively, untreated and periodate-treated ABP1 purified from maize. Molecular mass in kD is indicated (arrows). B, Thyrogobulin (2 μg) spotted on nitrocellulose and treated for the indicated time with 20 mM sodium periodate in 50 mM sodium acetate, pH 4.5, followed by 10 mM sodium borohydrate. The control treatment (0 min) was incubated for 30 min in 50 mM sodium acetate, pH 4.5, followed by the sodium borohydride treatment. Strips were blocked in 5% milk buffer and probed with Con A coupled to horseradish peroxidase. Development was with 4-chloronaphthol. This method was more fully described by Woodward et al. (1985). C, BMS cell extracts and purified ABP1 from maize were subjected to SDS-PAGE (14%) and immunoblot analysis and probed with polyclonal serum directed against maize ABP1. Lane 1, Maize ABP1 alone; lane 2, maize ABP1 and BMS cell crude extract; lane 3, BMS cell crude extract alone. Molecular mass in kD is indicated.

nition (Fig. 1A, lane 4), demonstrating that under the mild conditions we used to fix cells for microscopy, the antibodies should retain immunoreactivity. Because ABP1 is a glycoprotein containing a single high-mannose complex (Hesse et al., 1989), it was necessary to demonstrate that the affinity-purified antibody does not contain a significant subpopulation of antibodies directed against sugar epitopes. This was done by oxidizing the sugar residues using sodium periodate and then testing for immunoreactivity. The efficiency of this method was demonstrated in Figure 1B using the test glycoprotein thyroglobulin. After periodate treatment, the anti-ABP1 antibody had not lost immunoreactivity to ABP in BMS cell extracts (Fig. 1A, lanes 5 and 6) or ABP1 purified from maize (Fig. 1A, lanes 7 and 8). The antibodies did not recognize ovalbumin, further demonstrating that high mannose sugar-type epitopes were not recognized (data not shown). Note that when partially purified ABP1 was probed with the affinity-purified antibodies, two bands were observed (Fig. 1A, lanes 7 and 8): one at 22 kD and a minor one at 42 kD. Presumably, the 42-kD band is an aggregate formed during purification of ABP1. As shown in Figure 1C, higher resolution gels reveal that the protein in BMS extracts recognized by anti-ABP1 antibodies is slightly larger than the ABP1 purified from maize shoots. The difference in mobility was not due to differences in the samples (ionic, protein concentration, etc.) because doublet bands were observed when ABP1 and a BMS extract were run together (Fig. 1C). The size of the BMS ABP1-related protein is approximately 24 kD, and as yet we do not know whether the difference in mobility is due to modification (e.g. glycan differences) or whether the 24-kD protein in BMS cells is an isoform of ABP1.

Jones and Venis (1989) and Hesse et al. (1989) demonstrated the presence in maize of a 24-kD protein similar to ABP1. Jones and Venis showed that this 24-kD protein binds auxin, and Hesse et al. showed that a 24-kD protein shares amino-terminal sequence identity with ABP1. Furthermore, Napier and Venis (1992) showed that antibodies directed against ABP1 from maize recognize a 24-kD protein as the major antigen in barnyard grass and mung bean.

In addition to the indirect evidence mentioned above, there are four observations supporting the conclusion that the 24-kD protein in BMS cells is the homolog of ABP1 found in maize shoots. (a) Affinity-purified monospecific antibodies using maize ABP1 as the ligand recognize *only* the 24-kD protein in BMS cell crude extracts (Fig. 1). (b) Affinity-purified monospecific antibodies using a fusion protein of LacZ-ABP1 as the ligand (Jones et al., 1991) recognize *only* the 24-kD protein in BMS cell crude extracts (e.g. see Fig. 15). (c) Antibodies directed against a fusion protein made from the *mal* E gene fused to the ABP1 cDNA expressed in *Escherichia coli* recognize *only* the 24-kD protein in BMS cell crude extracts (S. Orsulic and A.M. Jones, unpublished data). (d) Photoaffinity labeling of crude BMS cell extracts using 7-[^{3}H]5-azidoindole-3-acetic acid reveals a labeled 24-kD protein and *not* a 22-kD protein as in maize shoots (D. Klambt and A.M. Jones, unpublished data). We will refer to this 24-kD protein in BMS cells simply as ABP, not ABP1.

ABP1 Is Localized in the Cell Wall and at the Plasma Membrane

Maize Coleoptile Cells

Ultrathin L.R. White sections of embedded coleoptile tissue were probed with monospecific anti-ABP1 antibodies and indirectly labeled with anti-rabbit IgG coupled to colloidal gold. Colloidal gold particles uniformly labeled the cell wall (Figs. 2–4), indicating that ABP1 apparently exists within the cell wall. Label was also seen associated with the secretory system (Figs. 2–4 and 6). Immunological control reactions using preimmune IgG resulted in a complete absence of colloidal gold labeling of the cell wall (Fig. 5). Because ABP1 is a high-mannose glycoprotein (Hesse et al., 1989) that could elicit antiglycan antibodies that would be isolated in the monospecific anti-ABP1 antibody preparations, we tested for glycan reactivity on the grids in the same manner as we did for the immunoblots (cf. Fig. 1). Ultrathin sections of coleoptile tissue mounted on grids were treated with sodium periodate and borohydride to oxidize surface glycans, thus disrupting the exposed glycan epitopes. Immunocytochemical analysis of periodate-treated coleoptile epidermal cells resulted in disperse colloidal gold labeling of the cell wall (Fig. 6), indicating that ABP1 localization within the cell wall is not the consequence of glycan cross-reactivity. ABP1 is localized at the plasma membrane of coleoptile epidermal cells, which is best visualized in slightly plasmolyzed cells (Fig. 2, arrows) where the cell membrane can be distinguished from the adjoining wall. In such cells the gold particles appeared to be distributed along the cell membrane. Gold label located immediately below the cell surface was consistently observed. Label associated with the plasma membrane indicates that ABP1 is localized to the plasma membrane or is part of an endomembrane system that is closely appressed to the plasma membrane. In either case, this localization is consistent with its postulated site of action (Barbier-Brygoo et al., 1991).

The Golgi apparatus was consistently seen to contain gold label, but the mild fixation required to retain immunoreactivity precluded visualization of specific Golgi compartments (Fig. 3). At this resolution it always appeared that gold labeling was uniform throughout the Golgi. Conventional electron microscopic analysis of osmium tetroxide-fixed coleoptile cells revealed good preservation of structures at high resolution (Fig. 7). Label was rarely observed in the vacuole or nucleus.

BMS Cells

Because we used maize BMS cells as a model system to study secretion of ABP, we thought it necessary to establish the location of ABP in these cells as well and to determine whether the distribution is significantly different from that observed in coleoptile epidermal cells. An immunocytochemical assay of BMS cells showed the localization of ABP clearly at the plasma membrane (Figs. 8 and 9, arrows) and within the cell wall as shown for coleoptile epidermal cells. Label was observed in the Golgi apparatus (Fig. 10) and in plasmodesmata (Figs. 11 and 12). Immunological control assays using preimmune IgG were devoid of gold particle labeling at the cell surface and over the wall (Fig. 13). The immunocytochemical observations confirmed that in BMS cells, as in coleoptiles, ABP is secreted into the cell walls. Specific labeling of the nuclear envelope was observed in only a few cells (data not shown). The labeling of the Golgi apparatus of BMS cells (Fig. 10) is consistent with the apparent secretion of ABP described in detail below.

Specific Secretion of ABP

The labeling of the Golgi apparatus by affinity-purified ABP1 antibodies suggested to us that movement of ABP to the plasma membrane and cell wall space may occur through the normal default secretory path, and it seemed possible that ABP could be detected in the medium of BMS cultures. To test this, culture medium was collected at different times after transferring BMS cells to fresh medium, and the proteins in the media were precipitated and subjected to SDS-PAGE and immunoblot analysis using anti-ABP1, anti-BiP, anti-PDI, and anti-KDEL peptide antibodies. As shown in Figure 14, A and B, essentially a single band with the same molecular mass as ABP was observed, and the abundance of this band detected in the media increased with time. In contrast, anti-BiP antibodies did not detect any polypeptide in the medium with mobility corresponding to that of BiP, thus demonstrating that BiP, another ER protein that contains an ER retention signal, is not secreted from the cells. Also, PDI, an ER protein containing a KDEL carboxy terminus, is shown not to be secreted (Fig. 14C). In these analyses, we base our conclusions on the relative amount of ABP, BiP, and PDI in the medium versus, respectively, ABP, BiP, and PDI in the cell. Note that the amount of ABP in the medium compared to the amount in the cell is much greater than the same comparison using BiP or PDI. The use of separate antibodies precludes the comparison of the amount of ABP , BiP, and PDI to each other. The amount of ABP and BiP in the cell represented by the 48-h sample (Fig. 14, A and B) did not change during the 72-h time course.

As shown in Figure 14E, serum directed against a KDEL-containing peptide was used to probe proteins in the medium that have a KDEL carboxy terminus. Anti-KDEL antibodies recognized purified ABP1 as expected and several proteins in BMS cell extracts. In particular, bands having molecular masses similar to those of ABP and PDI are noted by the lower and upper arrows, respectively. However, essentially a single protein having the molecular mass of ABP was recognized in the medium, suggesting that this secreted protein contains the KDEL epitope.

Brefeldin A is a fungal toxin that inhibits vesicular transport from the ER to the Golgi (Pelham, 1991). Brefeldin A prevents non-clathrin-coated vesicle assembly in the ER and Golgi complex, resulting in disruption of anterograde movement (ER → Golgi) and redistribution of the Golgi into the ER (Lippincott-Schwartz et al., 1989). Brefeldin A effects have recently been localized to the trans-Golgi network as well (Lippincott-Schwartz et al., 1991; Wood et al., 1991), and a model describing the mode of action via cytosolic G-proteins and coatomers (e.g. ADP ribosylation factors) binding to membrane sites has been proposed (Klausner et al., 1992). We used brefeldin A to study the pathway of the secreted ABP in BMS cultures. Cells were washed in fresh medium,

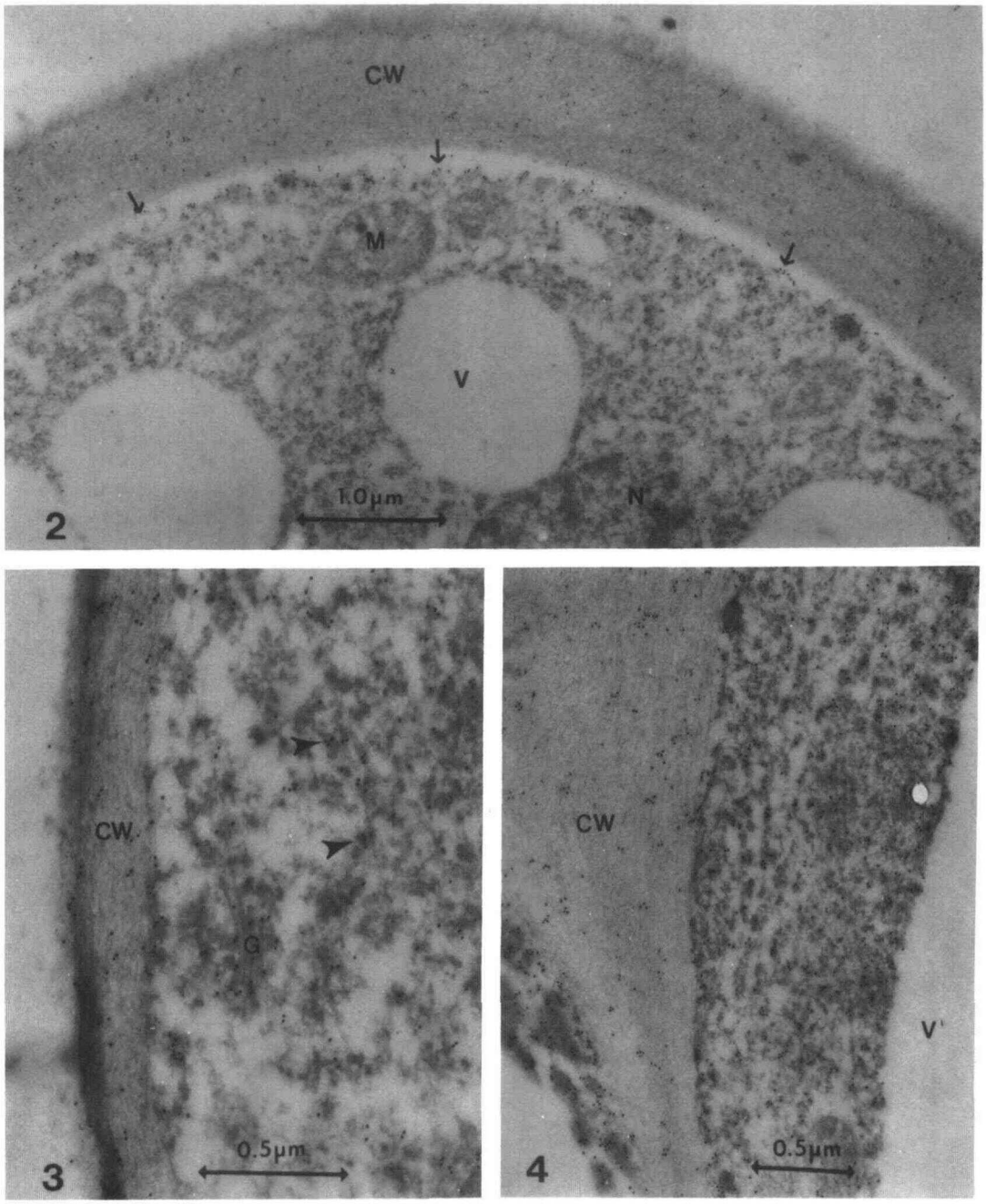
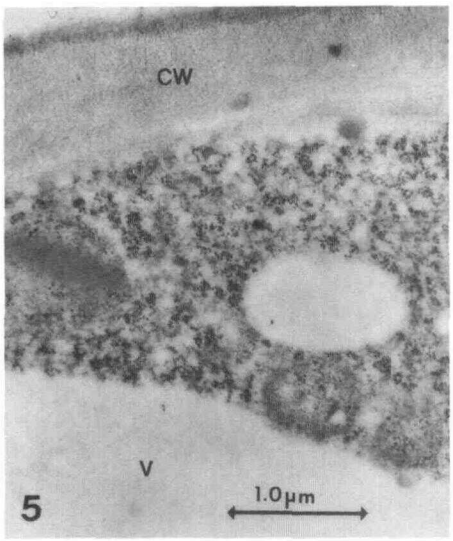
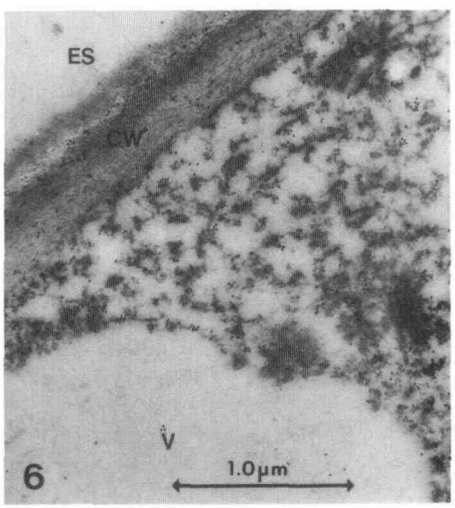


Figure 2. Immunocytochemical localization of ABP1 in a coleoptile epidermal cell. Gold particles are distributed throughout the cell wall (CW) facing the exterior space (ES). The cell shown in this micrograph is slightly plasmolyzed as a consequence of the preparative procedure to expose the cell surface. The surface of the plasma membrane is labeled with gold particles (arrows). The segments of ER are labeled with a few gold particles. The mitochondria (M), vacuole (V), and nucleus (N) are either devoid of gold particles or have a few scattered particles within the normal background of the assay method. ×21,600.

Figure 3. The localization of ABP1 in the Golgi apparatus (G) of a coleoptile epidermal cell is shown. Additional gold particle labeling is localized on the rough membranes distributed in the cytoplasm (arrowheads). The cell wall (CW) is labeled with gold particles dispersed throughout the wall. ×40,500.

Figure 4. Another coleoptile cell is shown that exhibits dense gold particle labeling of the entire endomembrane system. In contrast, the adjacent vacuole (V) is devoid of gold particles. Although the endomembrane system of this cell is more densely labeled at this plane of section than the adjacent epidermal cells, the gold particle density on the cell wall (CW) is very similar to cells exhibiting lower density of endomembrane label. ×27,000.





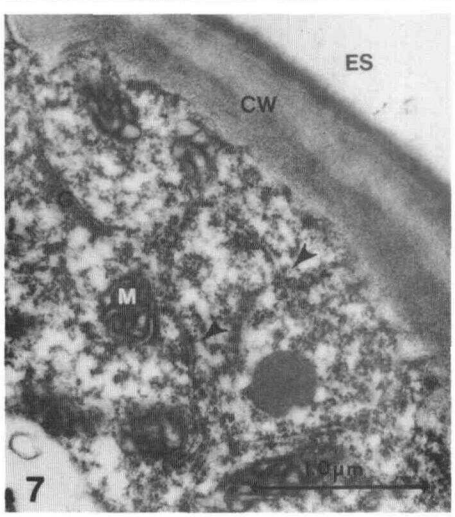


Figure 5. A portion of a coleoptile epidermal cell labeled from an immunological control reaction is shown. Portions of the cell including the cell wall (CW) and endomembrane components are devoid of gold particles in the control assays using preimmune IgG. ES, Exterior space; V, vacuole. ×21,600.

then transferred to a medium containing 200 nM brefeldin A and cultured for 24 h. Cells grown under these conditions appeared normal by light microscopy. Media were sampled and subjected to SDS-PAGE and immunoblot analysis with anti-ABP1 antibodies, and it was found that brefeldin A-treated cells secreted dramatically less ABP than the control cells (Fig. 14D).

In contrast to the effects of brefeldin A on secretion of ABP, monensin greatly increased the secretion of ABP in a concentration-dependent fashion, as shown in Figure 15. Monensin has variable effects on plant cells, causing an increase in protein secretion by some cells, whereas in others, secretion is blocked (Craig and Goodchild, 1984; Bowles et al., 1986; Melroy and Jones, 1986).

The effect of auxin on the cellular versus extracellular distribution of ABP was investigated using BMS cells and is shown in Figure 16. These cells were cultured in the presence or absence of 2,4-D for 72 h, and the amounts of ABP in the medium and washed cells were quantitated by immunoblot analysis. Cells from which the 2,4-D was withdrawn for 72 h, despite having increased their fresh weight per mL of culture as much or slightly more than control cells, had about 50% less ABP in the cell extracts and about 200% more ABP extracellularly than cells cultured continually in the presence of 2,4-D. This difference cannot be attributed to a nonspecific loss of ABP due to a leaky membrane because 2,4-D-starved cells accumulated auxin and benzoic acid as well or slightly better than control cells (data not shown). Because auxin and benzoic acid accumulation is a process requiring not only an intact membrane but also a cellular metabolism capable of hyperpolarizing the membrane through a pH differential, we conclude that the 72-h exposure to hormone-free medium had no deleterious effect on membrane integrity.

DISCUSSION

In this paper we show, using affinity-purified antibodies against ABP1, that an ER-localized ABP can also be found, in part, at the plasma membrane and in the cell wall. Secretion into the cell wall is probably via the secretory system. This observation is important because the cell wall is a known site of action for auxin-induced growth, and it raises the possibility that ABP1 has a direct role in the cell wall.

There are indirect data showing that ABP1 may be involved in mediating auxin-induced growth, probably in concert with

Figure 6. This micrograph shows the immunocytochemical assay of a coleoptile epidermal cell that was periodate treated. Although the glycan epitopes were destroyed, the cell wall (CW), Golgi apparatus (G), and endomembranes are labeled with gold particles. This control assay demonstrates that the immunocytochemical localization of ABP1 is not the consequence of glycan immunoreactivity. ES, Exterior space. ×27,000.

Figure 7. A conventional electron micrograph of an osmium-post-fixed coleoptile epidermal cell is shown. Osmium postfixation results in superior preservation of membranous components including the ER (arrowheads), shown as short segments and vesicles, and the Golgi apparatus (G). Other organelles and structures shown include mitochondria (M), vacuole (V), and the cell wall (CW) facing exterior space (ES). ×27,000.

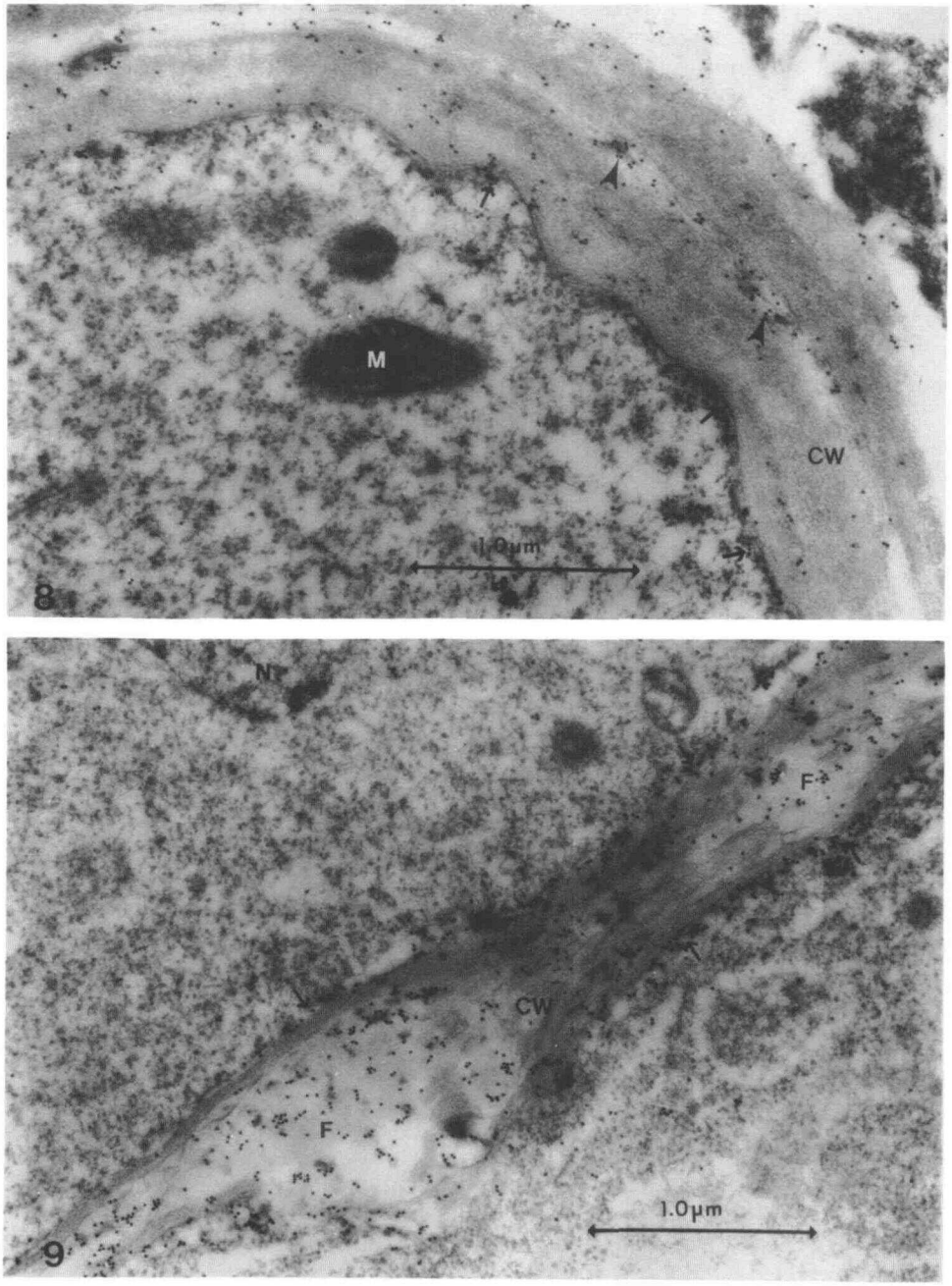


Figure 8. The immunocytochemical localization of ABP in a BMS cell is shown. The gold particle distribution in the cell wall (CW) facing exterior space is nonuniform and appears to be most concentrated where there are cracks in the CW (arrowheads). Gold particles also are concentrated along plasma membrane (arrows). Note that there are few gold particles localized on the intracellular membranes. M, Mitochondria. ×33,750.

Figure 9. The localization of ABP in the cell wall (CW) between two adjacent BMS cells is shown. Gold particles densely label the fractured region of the wall (F), whereas the intact wall region of the wall exhibits little label. Gold particles are also concentrated along the plasma membrane (arrows). The nucleus (N), mitochondria, and cytoplasmic membranes exhibit few if any gold particles. ×37,750.

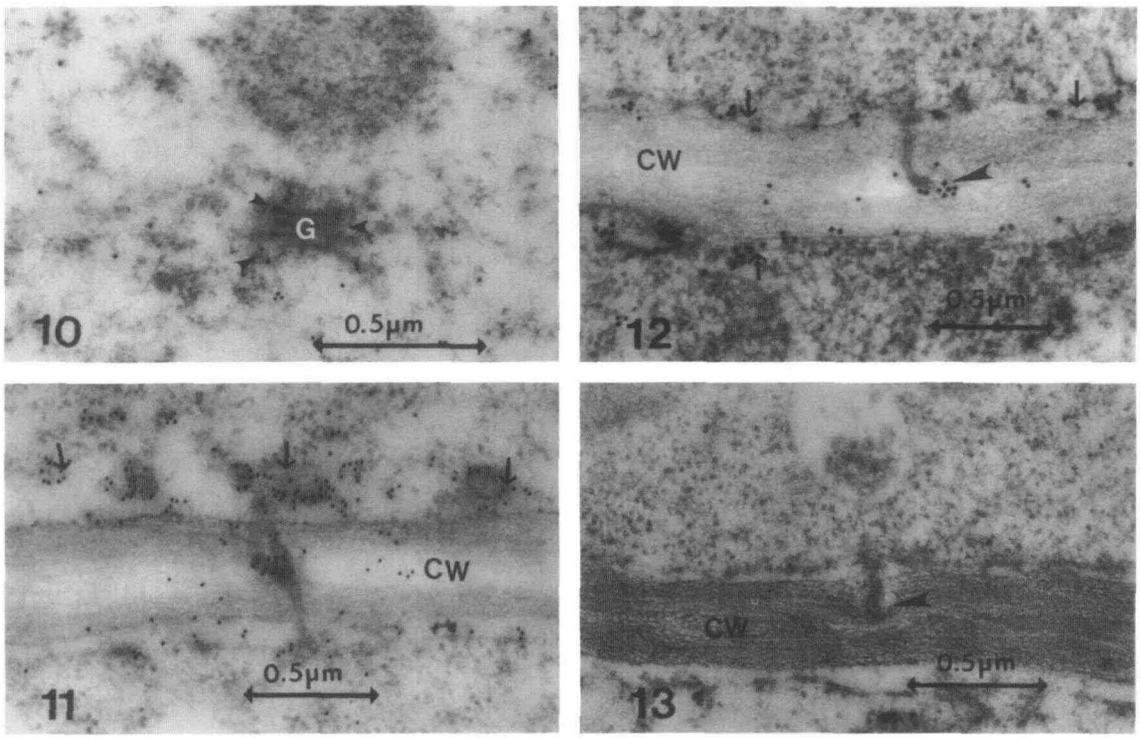


Figure 10. The localization of ABP in the Golgi apparatus (G) of a BMS cell is shown. Gold particles are localized across the entire cisternal stack of the Golgi (arrowheads). ×40,500.

Figure 11. Localization of ABP in the BMS cell wall is shown. Gold particles are concentrated in the central region of the plasmodesmata, indicating ABP accumulation. ABP is also distributed along the plasma membrane (arrowheads) of both adjacent BMS cells. CW, Cell wall. ×33,750.

Figure 12. Localization of ABP in BMS cell walls (CW) is shown. For reference, the arrowhead indicates a plasmodesmata. Additional ABP is localized along the plasma membrane of both adjacent cells (arrows). ×33,750.

Figure 13. An immunological control assay of a BMS cell is shown. Note that the plasmodesmata (arrowhead) and plasma membrane are completely devoid of gold particles. CW, Cell wall. ×33,750.

other auxin receptors (discussed by Jones and Prasad, 1992). For example, the distribution of ABP1 along the length of the maize mesocotyl correlates with auxin-inducible growth (Jones et al., 1989). Also, the auxin-binding specificity profile for ABP1, studied in crude extracts and designated site I auxin binding, roughly parallels the specificity of growth activity determined for a large set of compounds (Ray, 1977). Finally, antibodies against ABP1 are able to block auxin-induced hyperpolarization measured by microelectrodes inserted into evacuolated tobacco mesophyll protoplasts (Barbier-Brygoo et al., 1989). One of the puzzling aspects of the latter finding has been how antibodies to an ER protein are able to block the auxin response if the antibody recognition occurs at the plasma membrane of the protoplasts. To be able to conclude that ABP1 is the receptor mediating the hyperpolarization response, Barbier-Brygoo and coworkers had to postulate that the tobacco ABP1 homolog was present and acting at the plasma membrane. Our data now provide direct evidence that at least some of the total pool of ABP1 in maize and an immunochemically related protein in BMS cells is indeed located at the plasma membrane and within the cell wall. Moreover, ABP, which retains its KDEL carboxy terminus, can be readily detected in the culture medium of BMS cells.

The etiolated cereal shoot has been a useful model system to study the physiology and biochemistry of auxin-induced growth, and for this reason we have included the maize coleoptile in our study. However, the coleoptile is not amenable to a study of protein secretion, so we turned to a cereal cell culture for secretion experiments. To extend the coleoptile results to cultured cells, we first showed the same immunolocalization of ABP in BMS cells as for ABP1 in the coleoptile epidermal cells. Our secretion data obtained with BMS cells indicate that ABP secretion is sensitive to brefeldin A. Among proteins with carboxy-terminal KDEL, only ABP was secreted by the maize cells. We conclude that the presence of ABP in the media of BMS cultures is not due to cell death and breakage or some unique property of cultured cells and that cells of organized tissues of the cereal shoot also secrete ABP.

Five lines of evidence support the idea of specific secretion of ABP in BMS cells: (a) ABP is localized to the Golgi apparatus and at the plasma membrane and cell wall. (b) Relative to BiP and PDI, the ratio of ABP in the medium versus the cell is much greater. (c) Brefeldin A blocks the accumulation of ABP in the medium. (d) Anti-KDEL peptide antibodies recognize a single protein secreted into the medium corresponding to ABP and no others, such as BiP and PDI, two other ER proteins. (e) Auxin can affect the relative

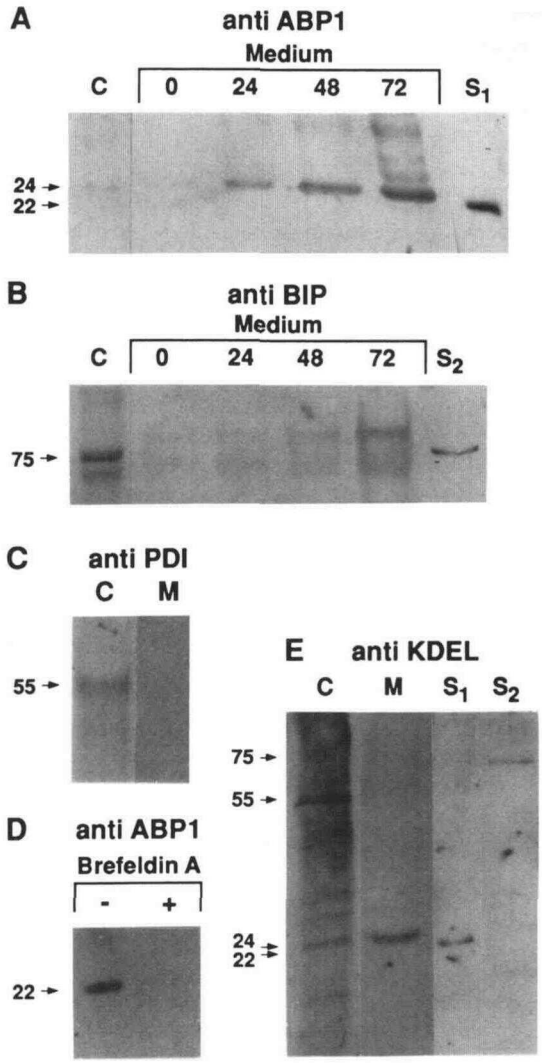


Figure 14. Presence of ABP in the culture medium of BMS cells is shown. Samples of BMS cells or media were collected and subjected to SDS-PAGE (14%) and immunoblot analysis using different antisera. A and B, Cells (C) were harvested 48 h after transfer and media sampled at the indicated times; immunoblots were made with antisera against ABP1 (A) or maize BiP (B). Protein in the media was precipitated with TCA and acetone before SDS-PAGE as described in the "Materials and Methods." C, PDI in BMS cell crude extract (48 h, C) and medium (M) was compared by immunoblot analysis using anti-PDI. D, Washed BMS cells were cultured in the presence (+) or absence (−) of 0.2 μM brefeldin A. Media were collected 72 h after the cells were transferred to media with or without brefeldin A and subjected to SDS-PAGE and immunoblot analysis using anti-ABP1 antiserum. E, Cells (C) and medium (M) collected 48 h after transfer were subjected to SDS-PAGE and immunoblot analysis using antiserum directed against a KDEL peptide. The upper and lower arrows indicate the positions of PDI and ABP, respectively. S_1 and S_2 in all panels refer to ABP1 and BiP standards, respectively. In all panels, molecular mass in kD is indicated.

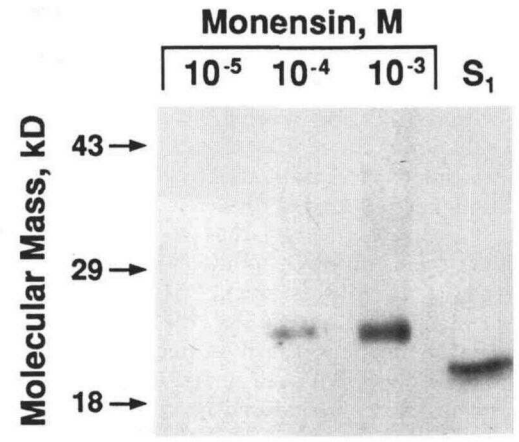


Figure 15. The effect of monensin on secretion of ABP to the medium of BMS cell cultures is shown. Cells were washed as described in "Materials and Methods" and transferred to fresh medium containing the indicated concentration of monensin. Proteins in the media were concentrated by TCA and acetone precipitation. Each lane contains the equivalent of 1 mL of medium. Samples were subjected to SDS-PAGE (14%) and immunoblot analysis using antiserum against ABP1 that had been antigen purified using a fusion protein made from a *lacZ* and *ABP1* gene fusion expressed in *E. coli* as described by Jones et al. (1991). S_1 is the purified maize ABP1 standard.

distribution of the steady-state levels of ABP between the cell and medium. Furthermore, detection of ABP in the medium indicates that the ABP is free to diffuse away from the plasma membrane and cell wall space where it can be observed.

Secretion of ER proteins can occur in some cells when cellular calcium levels have been perturbed (Booth and Koch, 1989), but where this phenomenon has been observed, all

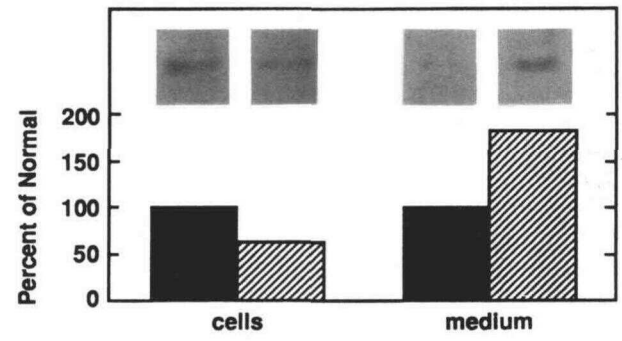


Figure 16. The effect of auxin on the secretion of ABP in BMS cell cultures is shown. Cells were washed in the appropriate media as described in "Materials and Methods" and then cultured for 72 h in medium containing (solid bars) or lacking (shaded bars) 10 μM 2,4-D. Cells and media were sampled and subjected to immunoblot analysis. The amount of ABP in cells or medium was quantitated by scanning blots and integrating the volume of the bands. The average is shown in the histogram with a cut out of a typical band from the immunoblot above the bars. Variance, noted as the sum of the SE of repeated scans of several lanes of the two treatments, never exceeded 10%.

reticuloplasmins including BiP and PDI were secreted. This suggested that calcium disrupted the retention system in a general manner or somehow allowed the loss of the KDEL retention sequence. In the case of rat exocrine pancreatic cells, PDI secretion occurred even though the KDEL carboxy terminus was intact (Yoshimori et al., 1990). Because other reticuloplasmins were found to be secreted by the pancreatic cells, it is not clear whether these cells were defective in their ER retention system. However, for the BMS cells used in our study, specificity for ABP is evident; therefore, we are left with the possibility that secretion of ABP1 and its presence at the plasma membrane is part of its mode of action.

Napier and Venis (1990) used a monoclonal antibody to ABP1 to map a hormone-induced conformational change (Shimomura et al., 1986) possibly within 10 residues of the carboxy terminus. Binding of their antibody MAC256 to ABP1 in a sandwich ELISA was sensitive to several auxins and structurally similar nonauxins, qualitatively parallel to the activities in growth assays. For example, a concentration of 100 μM napthalene-1-acetic acid inhibited 50% of MAC256 binding, whereas the less active auxin napthalene-2-acetic acid inhibited binding by 15%. Napier and Venis concluded that the epitope for MAC256 was located at the carboxy terminus, which includes KDEL, because MAC256 recognized a slightly smaller protein (21 kD), presumably a breakdown product, which contained the mature amino-terminal ABP1 sequence (22 kD) determined by sequence analysis.

Their observation using ABP1 in solution supports the hypothesis that auxin binding may be involved in regulating the retention mechanism. But there is also reason to speculate that auxin could modulate putative ABP uptake into cells. Cross (1991) proposed that ABP1 cycles in and out of cells and that this movement could be a part of ABP action in regulating growth. Recently, Letourneur and Klausner (1992) identified a di-leucine motif (roughly DKQTLL) that is involved in the endocytic movement of the CD3-γ subunit of the T-cell antigen receptor, a membrane protein containing an ER retention sequence. ABP1 has a sequence (G^{108}KGTLL) similar to the di-leucine motif. This putative endocytosis signal in ABP1 is found in a conserved region of the polypeptide and is adjacent to residues that Venis et al. (1992) proposed to constitute the auxin-binding pocket. This raises the possibility that auxin binding could alter recognition of the di-leucine motif and, therefore, modulate movement of ABP into the cell after it is has been secreted. This is consistent with our observation that cells cultured in the absence of auxin have a higher level of ABP in the medium. However, based on our current understanding of the mechanism of signaling via this di-leucine motif, the presence of a di-leucine motif on ABP1 is inconsistent with the idea that presentation of the endocytosis signal is on the cytoplasmic side of a transmembrane protein because ABP is not a transmembrane protein and would be on the extracytoplasmic side of the plasma membrane after secretion.

There are several known examples of ligand-regulated redistribution of receptors. For example, the glucocorticoid receptor translocates from the cytosol to the nucleus upon binding of glucocorticoid (Picard and Yamamoto, 1987), insulin receptor endocytosis is insulin mediated (Marshall, 1988), and the retinol-binding protein requires retinol for assembly in the ER and for secretion (Ronne et al., 1983). In each of these cases, the mechanism of ligand-induced redistribution is understood at the molecular level. In contrast, the mechanism by which auxin alters the ratio of cellular versus extracellular ABP is not understood but is likely to be different from known mechanisms because ABP contains a carboxy-terminal KDEL.

The secretion of ABP is unique because it possesses a carboxy-terminal KDEL retention sequence that has been shown to be necessary and sufficient for retention in the ER lumen of animal cells (for review see Pelham, 1989). The secretion of proteins from plant cells is widely assumed to be a default process, occurring because of the absence of specific targeting information (for review see Chrispeels, 1991). With the exception of ABP, all known plant proteins containing a KDEL or HDEL carboxy terminus are not secreted (two thiol proteases, Akasofu et al., 1989; Tanaka et al., 1991; BiP and PDI, this work). This also holds true for recombinant phytohemaglutinin, phosphophinotricin acetyl transferase, and vicilin, where a KDEL or HDEL carboxy terminus was added (Herman et al., 1990; Denecke et al., 1992; Wandelt et al., 1992). The results presented in this paper suggest that the carboxy-terminal KDEL is retained on the secreted ABP; therefore, the secretion of ABP probably does not require the posttranslational removal of the KDEL carboxy terminus. This raises the question of whether in plant cells the KDEL signal is sufficient for ER retention and whether retention of special ER proteins, such as ABP1, is conditional.

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