Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3425–3429, April 1995
Plant Biology

Auxins induce clustering of the auxin-binding protein at the surface of maize coleoptile protoplasts

(auxin receptor/plasma membrane/receptor clustering)

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Communicated by Winslow R. Briggs, Carnegie Institution of Washington, Stanford, CA, December 1, 1994

ABSTRACT **The predominant localization of the major auxin-binding protein (ABP1) of maize is within the lumen of the endoplasmic reticulum. Nevertheless, all the electrophysiological evidence supporting a receptor role for ABP1 implies that a functionally important fraction of the protein must reside at the outer face of the plasma membrane. Using methods of protoplast preparation designed to minimize proteolysis, we report the detection of ABP at the surface of maize coleoptile protoplasts by the technique of silver-enhanced immunogold viewed by epipolarization microscopy. We also show that ABP clusters following auxin treatment and that this response is temperature-dependent and auxin-specific.**

The hormone auxin plays a pivotal role in regulating plant growth and development (1). Auxin stimulation implies that the hormone must be recognized (hormone binding) and that its perception must be converted into a physiological response (signal transduction). Many early reports have provided evidence for the binding of auxin to plant membranes, especially the endoplasmic reticulum (ER) (for review, see ref. 2). Subsequent work (reviewed in refs. 1 and 3) has resulted in the isolation and characterization of the major protein (ABP1) responsible for auxin binding in maize (*Zea mays*) coleoptiles. ABP (auxin-binding protein) is a dimeric protein of M_r 44,000 (4–6) which binds either one (4) or two (7) moles of auxin per dimer. Sequencing of cDNA clones for maize ABP (7–10) has indicated a protein of 163 amino acids, 38 of which represent a typical hydrophobic signal peptide at the amino terminus. In addition, ABP has a Lys-Asp-Glu-Leu (KDEL) sequence at its carboxyl terminus and has a single, high-mannose glycan, which is sensitive to endoglycosidase H digestion (5, 11). These are features of proteins that are retained within the lumen of the ER (12) and thus conform with the earlier binding studies on microsomal membranes.

Although the biochemical characteristics of maize ABP are indicative of an ER-resident protein, a number of observations strongly suggest that some of the total cellular ABP is also localized at the cell surface. It has been established both by classical (microelectrode impalement; refs. 13 and 14) and whole-cell patch-clamp (15) electrophysiological methods that auxin causes an increase in H^+ current at the plasma membrane (PM). Since this effect is blocked by antibodies against H^+-ATPase (13) and is further enhanced by the fungal toxin fusicoccin (15), it has been considered that it reflects an activation of the PM-localized H^+-ATPase. Whereas polyclonal antibodies raised against maize ABP (5, 16) prevent these auxin effects (reviewed in ref. 17), antibodies raised against a synthetic peptide corresponding to the putative auxin binding site of ABP induce auxin-like electrophysiological changes at the plasma membrane (15, 18). Two further observations strongly implicate ABP in auxin-related events at the PM: (*i*) the auxin-evoked sensitivity of the hyperpolarization response of tobacco mesophyll protoplasts can be increased when the protoplasts are supplemented with maize ABP (14) and (*ii*) a synthetic peptide corresponding to amino acid residues 151–163 at the carboxyl terminus of maize ABP induces auxin-like changes in K^+-channel currents in the PM of *Vicia faba* guard cells (19).

Crucial to the idea that ABP is indeed functioning as a cell surface receptor for auxin is the actual demonstration of its presence at the PM. Currently there is only one pertinent paper (20) claiming that ABP is transported to the cell surface via the Golgi apparatus. Postembedding immunogold labeling with affinity-purified ABP antibodies depicted ABP at the PM and, in large amounts, in the cell walls of suspension-cultured maize cells. However, the inadequate preservation of ER morphology in this report (20) did not allow a clear allocation of ABP to the ER, which the biochemical data suggest should be the primary intracellular site for ABP (2, 21).

Recently we have used silver-enhanced immunogold viewed by epipolarization microscopy (SEIG-EPOM) to visualize elicitor binding at the surface of protoplasts prepared from suspension-cultured cells (22). This technique has been particularly successful in the detection of cell surface antigens in leukocytes (23) but has, in part due to inadequate protection of the PM during protoplast preparation, not previously been used by plant cell biologists. With this method we now demonstrate the presence of ABP at the surface of the PM of maize coleoptile protoplasts. Further, we show that ABP clusters in response to auxin treatment. This effect is not evoked by inactive auxin analogs and appears to be restricted to ABP.

MATERIALS AND METHODS

Plant Tissue and Preparation of Protoplasts. Apical 1.0-cm segments were excised from the shoots of 6-day-old dark-grown *Zea mays* L. (cv. Mutin; KWS Saatzucht, Einbeck, Germany) seedlings and gently abraded with diatomaceous earth to remove the cuticle. After decapitation, the coleoptiles were separated from the primary leaves and briefly washed in distilled water. Coleoptile tissue was transferred to 100-ml Erlenmeyer flasks, covered with 20 ml of protoplasting medium, and vacuum infiltrated for 10 min. The protoplasting medium consisted of 1.5% cellulase (Yakult Honsha, Tokyo), 0.5% macerozyme R-10 (Yakult Honsha), 0.1% pectolyase Y-23 (Seishin, Tokyo), 0.1% kanamycin sulfate, 2% bovine serum albumin (BSA) (fraction V; Biomol, Hamburg, Germany), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM sodium ascorbate, and 0.35 M mannitol and was heat-pretreated to inactivate proteases (22). Tissue was incubated in this medium at 26°C in

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Abbreviations: ABP, auxin-binding protein; ER, endoplasmic reticulum; PM, plasma membrane; SEIG-EPOM, silver-enhanced immunogold viewed by epipolarization microscopy; IAA, 3-indole acetic acid; BSA, bovine serum albumin.

a reciprocally shaken water bath. After 3 hr of incubation, coleoptile protoplasts were harvested by centrifugation at 80 × *g*, for 2 min and washed by suspension and centrifugation at 100 × *g*, for 5 min in 0.5 M mannitol/1 mM $CaCl_2$.

Isolation of PM and Western Blotting. Maize coleoptile segments (35 g, fresh weight) were homogenized at 4°C in a medium containing 250 mM sorbitol, 3 mM EDTA, 1 mM dithiothreitol, aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), and 0.7 μM pepstatin in 25 mM Hepes (pH 7.8) with 25 mM bistrispropane. After filtration through Miracloth and centrifugation at 8000 × *g* for 20 min, the homogenate was centrifuged at 100,000 × *g* for 60 min to obtain a total membrane pellet. PM (200 μg) was isolated from this fraction by two-phase partitioning (24). Threefold purified PM was subjected to SDS/12% PAGE and then to Western blotting according to standard procedures. Bound antibodies were visualized with an ECL kit (Amersham).

SEIG-EPOM Procedure. Visualization of cell surface antigens was done essentially as described (22), except that because of the size and starch content of the maize protoplasts, it was found necessary to stabilize them by an initial mild prefixation before exposure to the antisera. This was done in two 1-hr stages at 20°C. The protoplasts were first exposed to 0.1% (vol/vol) glutaraldehyde/2% (wt/vol) paraformaldehyde/1 mM $CaCl_2$/0.4 M mannitol/25 mM potassium phosphate buffer, pH 7.0, and then, without washing, to 0.01% (wt/vol) OsO_4/50 mM potassium phosphate buffer, pH 7.0. In control experiments this prefixation protocol was shown to have no significant effect on the subsequent visualization of cell surface antigens. After two 10-min washes in Tris-buffered saline (TBS: 50 mM Tris/0.9% NaCl, pH 7.5) the protoplasts were suspended for 30 min at 20°C in blocking solution [3% BSA plus 0.2% acetylated BSA (BSA-C; Biotrend, Cologne, Germany) in TBS] before incubation for 60 min at 20°C in primary antibody solution. Unbound antibodies were removed by four 10-min washes in TBS containing 1% BSA. Antibody-decorated protoplasts were then incubated for 1 hr at 20°C in a solution of gold-coupled secondary antibody solution and then washed with 1% BSA in TBS. Subsequently the protoplasts were fixed for 12 hr at 4°C in aqueous 1% glutaraldehyde, washed four times for 10 min in double-distilled water, and finally suspended (in the dark) for 15 min at 25°C in silver-enhancing solution, made up exactly according to the maker's instructions (Biogenzia Lemania, Bochum, Germany). After four 10-min washes in double-distilled water, the protoplasts were investigated by reflection (epi)polarization microscopy with an Axiovert 35 microscope (Zeiss) equipped with a ×63/1.25 Plan-Neofluar Ph3 Antiflex objective.

Antibodies. Three types of primary antibodies were employed for the SEIG-EPOM procedure, each diluted 1:250 in wash solution (1% BSA in TBS): IgG fractions of polyclonal antibodies raised against maize ABP1 (5) or against a synthetic peptide corresponding to the auxin-binding site of ABP (D16; ref. 18) or monoclonal antibodies recognizing epitopes at the carboxyl terminus (MAC 256) or close to the amino terminus (MAC 257) of maize ABP1 (5, 21). Two types of 1-nm-gold-conjugated secondary antibodies (Biocell Laboratories) were used: goat anti-rabbit IgG for the polyclonal antibodies and goat anti-rat IgG for the monoclonals. These antibodies were presented at a dilution of 1:500 in wash solution containing additionally 0.1% BSA-C. ABP1 was prepared from maize shoots by ion-exchange and affinity chromatography (18). For Western blotting primary antibodies were presented at a dilution of 1:1000 (polyclonals) or 1:10 (monoclonal hybridoma supernatants).

RESULTS

Visualization of ABP at the PM of Maize Coleoptile Protoplasts. Maize coleoptile protoplasts decorated with ABP antibodies and then processed by the SEIG-EPOM method revealed a dense labeling at the outer surface of the PM (Fig. 1 *a* and *b*). Counting the number of point light sources in cap (pole) views and extrapolating to the total surface of the protoplast according to the formula previously derived for this purpose (22) led to a total number of around 1200 ABP-binding loci per cell (Table 1). Of these, around 400 represented nonspecific binding of IgGs as judged by control incubations with preimmune IgG (Fig. 1*c*; Table 1). Other control incubations confirmed the validity of these observations. Thus, when the ABP polyclonal antibodies were presented in the presence of a molar excess of free ABP, the number of punctate light sources was reduced to a similar extent (Fig. 1*d*; Table 1). When the protoplasts were exposed to the secondary antibody solution alone, very few punctate light sources were visible at the surface of the PM (Fig. 1*e*). Protoplasts treated with carboxypeptidase A prior to incubation with the ABP antibodies showed a reduction in the number of punctate light sources to around the level seen with preimmune IgG (Fig. 1*g*; Table 1). Protoplasts which were challenged with neither primary nor secondary antibody solutions but were otherwise processed identically for SEIG-EPOM, including the silver enhancement step, were almost without any light reflections (Fig. 1*f*). However, undecorated protoplasts from maize coleoptiles, in contrast to other protoplasts (22), did show a diffuse background reflectance. This resulted from silver reduction caused by residual amounts of the fixatives used for stabilizing the protoplasts. Despite extensive experimentation (varying aldehyde and OsO_4 concentrations; subsequent aldehyde reduction with borohydride; microwave fixation) we have been unable to eliminate this technical deficiency. Unstabilized maize coleoptile protoplasts

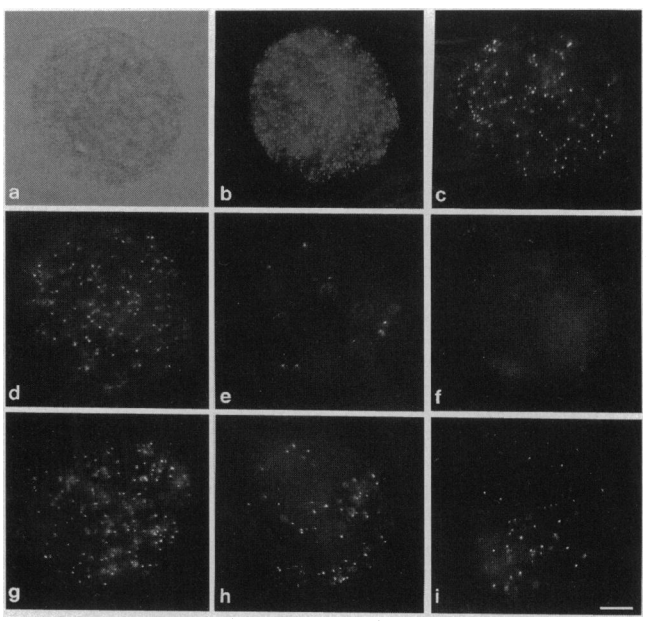

FIG. 1. ABP-binding loci at the surface of maize coleoptile protoplasts as visualized by SEIG-EPOM (pole-cap views are depicted). (*a* and *b*) Epidermal protoplast incubated first with maize ABP1 polyclonal antibodies (1 hr at 4°C) and then with 1-nm-gold-conjugated secondary antibodies. After glutaraldehyde fixation the decorated protoplasts were silver-enhanced and viewed with normal light (*a*) and reflection polarized light optics (*b*). (*c–f*) Control incubations of protoplasts with preimmune serum (*c*), ABP antibodies plus 100 nM exogenous ABP (*d*), secondary antibody without primary antibody (*e*), or neither primary nor secondary antibodies (*f*). (*g*) Protoplast prepared as in *a–f* but incubated for 1 hr at 4°C with 0.1% carboxypeptidase A before exposure to ABP antibodies and subsequent SEIG-EPOM. (*h* and *i*) Protoplasts incubated with the ABP monoclonal antibodies MAC 256 (*h*) and MAC 257 (*i*). (Bar = 20 μm; ×215.)

Table 1. Quantitation of SEIG-EPOM-visualized ABP-binding loci at the surface of maize coleoptile protoplasts

Treatment	Protoplast diameter, μm	Total no. of binding loci	Density of binding loci, μm^{-2}	P (n)*
D16 antibodies	38 ± 8	816 ± 404	0.18 ± 0.12	
ABP antibodies	39 ± 10	1182 ± 589	0.25 ± 0.17	
ABP antibodies + 100 nM exogenous ABP	37 ± 8	478 ± 263	0.11 ± 0.07	<0.001 (2–3)
Preimmune IgG	35 ± 8	364 ± 224	0.09 ± 0.07	<0.001 (2–4)
Proteolysis,† then ABP antibodies	39 ± 7	245 ± 132	0.05 ± 0.03	<0.001 (2–5)

At least 50 epidermal protoplasts (recognized by the presence of small amounts of anthocyanins in their vacuoles) were counted per treatment. All incubations with antibodies were 1 hr at 4°C.
*Statistical analysis according to Mann and Whitney (25).
†Protoplasts were suspended in 0.5 M mannitol/1 mM $CaCl_2$/0.1% carboxypeptidase A and incubated for 1 hr at 4°C before washing and exposure to the primary antibody solution.

did not survive the SEIG-EPOM procedure, bursting at the latest during the silver enhancement step.

We also exposed maize coleoptile protoplasts to the ABP monoclonal antibodies MAC 256 and 257. In neither case were >200 punctate light sources visible at the surface of the PM (Fig. 1 *h* and *i*). Since this reflects a labeling density somewhat less than the preimmune control for the ABP polyclonal antibodies, we also consider this as representing nonspecific antibody binding. We therefore infer that the two epitopes against which the monoclonal antibodies are directed (carboxyl terminal and near the amino terminus, respectively; ref. 21) are occluded when ABP is attached to the PM. This suggestion is reinforced by the failure of a polyclonal antiserum raised against a carboxyl terminal peptide of ABP1 to give more than a background signal (data not shown). It is also supported by the observation that in Western blots of denatured maize coleoptile PM proteins separated by SDS/PAGE, a single polypeptide (22 kDa) is recognized by both the monoclonal antibodies and the ABP polyclonal antibodies (Fig. 2).

Auxin Effects on PM as Demonstrated by SEIG-EPOM. Maize coleoptile protoplasts, when challenged at 4°C with auxin agonist D16 antibodies (15, 18), instead of the ABP antibodies also showed a significant punctate labeling of the PM after SEIG-EPOM (Fig. 3*a*). Labeling density was statistically somewhat less than with the ABP antibodies (Table 1) but could be effectively blocked by the inclusion of 10 μM IAA during incubation with D16 (compare Fig. 3*a* with Fig. 3*b*).

Presenting ABP antibodies to maize coleoptile protoplasts in the presence of 10 μM IAA at 4°C for 1 hr gave rise to a labeling density for ABP similar to that seen when auxin was absent (compare Fig. 3*c* with Fig. 1*b*). The same result was obtained when protoplasts were incubated for 2 hr at 4°C in the presence of 10 μM IAA before application of ABP antibodies and subsequent processing for SEIG-EPOM (data not shown).

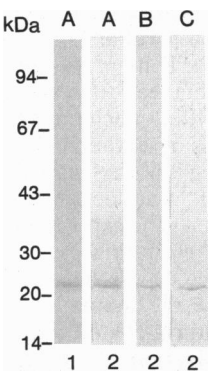

FIG. 2. Western blots of phase-partitioning-purified PM (lanes 2; 20 μg of protein) from maize coleoptiles probed with ABP1 polyclonal antibodies (lanes A), ABP monoclonal antibody MAC 256 (lane B), or ABP monoclonal antibody MAC 257. Lane 1 (0.2 μg of protein) has authentic maize ABP1 as a reference.

However, when protoplasts were incubated at 25°C in the presence of 10 μM IAA, a dramatic change in the distribution of cell surface ABP labeling was observed (compare Fig. 3*c* with Fig. 3*e*). The punctate light sources were clustered (we estimate around 120 ± 60 clusters per cell) and the numbers of nonclustered point light sources decreased to levels equivalent to nonspecific antibody labeling (286 ± 142). Each cluster had a diameter of ≈4 μm, but, since a ×100 Plan-Neofluor Antiflex objective is not produced by Zeiss, we were unable to resolve the fine structure of the clusters. Clustering was first observed after 30 min of IAA treatment at 25°C but reached completion after a further 30–40 min. Protoplasts incubated in 10 μM IAA for 4 hr at 25°C closely resembled those treated for only 1 hr.

Protoplasts incubated at 25°C in the absence of IAA did not show clustering of ABP (Fig. 3*d*). Since clustering of ABP was evoked by the synthetic auxin analog 1-naphthaleneacetic acid (Fig. 3*f*), but not by the potent antiauxin 2-naphthaleneacetic acid (Fig. 3*g*) or benzoic acid (Fig. 3*h*), clustering appears to be auxin specific. Moreover, it is a phenomenon which is also restricted to ABP, since an IAA-induced clustering of other surface antigens was not observed in identical experiments performed with elicitors on soybean and parsley protoplasts (22) and with a putative fusicoccin receptor antiserum (26) on maize coleoptile protoplasts (data not shown). Clustering of ABP, as visualized with ABP polyclonal antibodies, could also not be observed when protoplasts were treated with D16 antibodies (IgG or Fab fragments) at 25°C.

DISCUSSION

Maize ABP1 has three immunodominant domains lying between residues 85 and 100, and all polyclonal antisera raised so far against whole ABP recognize at least two of them (27). The ABP antibodies used in this investigation recognize all three. Although only a single polypeptide with an apparent molecular mass typical of ABP1 is seen in Western blots of purified PM, non-ABP IgGs in the antibody population may contribute to nonspecific binding at the surface of maize coleoptile protoplasts. However, as judged by SEIG-EPOM the degree of nonspecific binding is considerably lower than that obtained with the ABP antibodies. Based on control experiments, including competition with exogenous ABP, we regard the punctate light sources made visible at the surface of anti-ABP-decorated protoplasts to be an authentic representation of ABP located at the surface of the PM. Assuming 50 pmol of total ABP per gram of coleoptile tissue (2), uniformly distributed (3) among cells averaging 20 μm × 20 μm × 50 μm, then even if PM ABP is only 1% of the total, we can expect ≈6000 ABP molecules per cell surface. For reasons previously given (22) we cannot be certain that an individual point light source corresponds to a single ABP monomer/dimer, but we note that the number of binding loci made visible by the SEIG-EPOM method (>1000 per cell) is within this estimate of PM ABP.

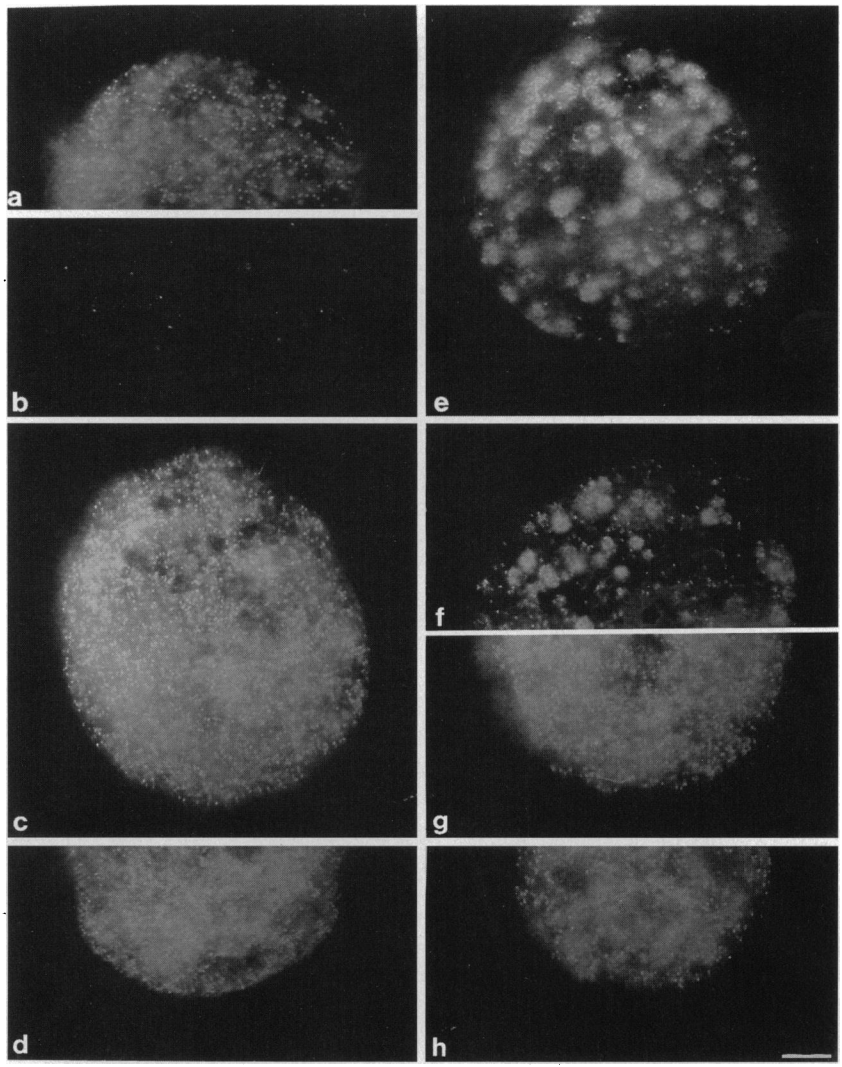

FIG. 3. Auxin effects on ABP distribution at the surface of maize coleoptile protoplasts. (*a*) Protoplast incubated with auxin agonist D16 antibodies (1 hr at 4°C). (*b*) As for *a*, but with 10 μM 1-napththaleneacetic acid in addition to D16. (*c*) Epidermal protoplast treated with 10 μM 3-indoleacetic acid (IAA) (1 hr at 4°C) before incubation with ABP antibodies. (*d*) Protoplast incubated at 25°C for 1 hr in the absence of IAA. (*e*) As for *d*, but in the presence of 10 μM IAA. (*f*) As for *e*, but with 10 μM 1-naphthaleneacetic acid instead of IAA. (*g*) As for *e*, but with 10 μM 2-naphthaleneacetic acid. (*h*) As for *e*, but with 10 μM benzoic acid. (Bar = 20 μm; ×350.)

Although not giving any indication as to how the ER retention mechanism for ABP is overcome, the results presented here provide strong evidence for the presence of ABP at the surface of the PM in maize coleoptile protoplasts. In addition, our results clearly show that the distribution of ABP changes in response to auxin.

Although current opinion (28) generally recognizes that ABP is a true auxin receptor, it is also thought that, in order for the hormone stimulus to be transduced, a PM-localized ABP "docking protein" may exist (29, 30), since ABP contains no obvious transmembrane domain. However, since ABP can be recognized at the surface of the PM in the absence of IAA, ABP and its docking protein must be in continuous association with one another. Signal transduction might then occur via an auxin-induced conformational change involving the ABP/docking protein interface. A short, KDEL-containing carboxyl-terminal peptide of ABP has been shown to evoke an auxin-like response (19), suggesting that this region of the ABP molecule may interact with the docking protein or other signal transduction elements and hence be unavailable for antibody interaction. This would account for the failure of the monoclonal antibody MAC 256 or the polyclonal antibodies against a carboxyl-terminal peptide to recognize PM-bound ABP. We presume that the amino terminus, which contains the epitope for MAC 257, is also conformationally unavailable to the antibody. On the other hand, the auxin-binding domain of ABP (recognized by D16) appears to be exposed, consistent with the earlier electrophysiological evidence (15, 18).

Although the auxin agonist antiserum D16 can hyperpolarize protoplasts in an auxin-like manner (18), it failed to induce ABP clustering. This observation is not necessarily in contradiction to the auxin specificity of the clustering phenomenon. It is perfectly conceivable that, for steric reasons, ABP cannot cluster when tagged with a much larger molecule such as an antibody. However, this observation also leads to the conclusion that ABP clustering is not essential for signal transduction leading to inereased H^+ translocation (15, 18). Other auxin-induced effects—e.g., on gene expression—could nevertheless be dependent on ABP clustering.

In animal cells many receptor–ligand interactions at the PM have as a consequence the internalization of the receptor–ligand complex in clathrin-coated vesicles, followed by the dissociation and degradation of the ligand and subsequent recycling of the receptor back to the PM (31). Is it therefore possible that ABP-clustering might be a prelude to receptor-mediated endocytosis? For the moment we can only speculate on this, but we draw attention to the facts that ligand binding often induces the clustering of cell surface receptors in animal

cells (e.g., refs. 32 and 33), that this occurs more slowly than many hormone-induced biochemical events (34), and that such clustering has also been recorded both by electron microscopy (35) and by light microscopy with fluorescently labeled conjugates (34). In addition, the clustering we observe is temperature-dependent (Fig. 2 *a* and *d*), as are receptor clustering in animal cells (34) and receptor-mediated endocytosis in plants (36). Although the ABP clusters (≈4 μm; Fig. 2 *d* and *e*) appear far larger than the diameter of coated pits (≈100 nm), they are similar in size to clusters of animal hormone receptors visualized by methods of comparable resolution (34).

This work was supported by the Deutsche Forschungsgemeinschaft (Göttingen) and by the Agricultural and Food Research Council and the Biotech program of the European Economic Communities (East Malling). We thank Dr. Richard Napier (East Malling) for the monoclonal antibodies and for useful discussions throughout this work, Claudia Terschüren (Göttingen) for protoplast preparations, and Heike Freundt for helping with the preparation of the manuscript.

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