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Antibodies to a peptide from the maize auxin-binding protein have auxin agonist activity

(auxin receptor/ligand-binding site/membrane potential difference)

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ABSTRACT **The major auxin-binding protein in maize membranes is thought to function as a physiological receptor. From earlier information, including the use of site-directed irreversible inhibitors, several of the amino acids likely to form part of the active auxin-binding site were provisionally assigned. Inspection of the amino acid sequence of the auxin-binding protein showed a short region containing all but one of these amino acids. We find that antisera raised against a synthetic peptide encompassing this region recognize all isoforms of the maize auxin-binding protein together with homologous polypeptides in other species. We further find that the antibodies hyperpolarize protoplast transmembrane potential in an auxin-like manner. We conclude that these antibodies display auxin agonist activity and that we have identified an essential portion of the auxin-binding site.**

Auxin binding to microsomal membranes of maize, first reported by Hertel *et al.* (1), has been extensively studied in several laboratories (for review, see ref. 2). The major auxin-binding protein (ABP) has been solubilized from the membranes, purified, characterized (3–5), cloned, and sequenced (6–8); antibodies to ABP have been produced, both polyclonal (3, 5) and monoclonal (5). Several lines of evidence indicate that ABP may act as a receptor; these include correlations between ABP abundance and auxin responsiveness (2, 9) and demonstration of an auxin-dependent response after reconstitution of partially purified ABP in a lipid bilayer (10). Also, anti-ABP IgG was reported to block auxin-induced growth responses (11), although others have been unable to repeat these findings (ref. 4; M.A.V., unpublished observations).

Far more persuasive evidence of the receptor function of ABP has emerged from electrophysiological studies. To explore the putative receptor role of ABP it is necessary to have a reliable functional assay for auxin activity that permits the use of antibodies and other macromolecular probes. In this context, isolated protoplasts have the attraction of providing direct access to the cell surface, eliminating the cuticular and cell wall barriers of intact or excised tissues. It has been found that auxin induces hyperpolarization of the transmembrane potential difference (E_m) of isolated protoplasts (12–15). This response is mediated by proteins immunologically related to maize ABP and located at the plasma membrane surface (13). Its sensitivity, expressed as the auxin concentration required to induce the hyperpolarization maximum, can be manipulated experimentally through several orders of magnitude, increased by incubation of protoplasts with exogenous maize ABP or reduced after incubation with anti-ABP antibodies (13).

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In exploring the function of different ABP domains, we have used the electrophysiological assay to evaluate the biological activity of antibodies raised against a synthetic polypeptide derived from the ABP sequence. We now report that such antibodies display auxin agonist activity and conclude that the selected polypeptide must form an important part of the ligand-binding site of ABP.

MATERIALS AND METHODS

Anti-Peptide Antibodies. The ABP peptide Arg-Thr-Pro-Ile-His-Arg-His-Ser-Cys-Glu-Glu-Val-Phe-Thr was synthesized by standard solid-phase procedures at the Microchemical Facility of the Institute of Animal Physiology and Genetics Research, Babraham, U.K. This peptide was azo-coupled, either to keyhole limpet hemocyanin or to tuberculin-purified protein derivative (Statens Seruminstitut, Copenhagen), through an added N-terminal tyrosine, as described by Bassiri *et al.* (16) but using *o*-tolidine for benzidine. Antisera to these conjugates were raised in rabbits. Conjugate (1 mg) was injected initially in Freund's complete adjuvant, and three boosts (0.5 mg) were administered s.c. at 4-week intervals. Blood was taken 2 weeks after boosting, and serum from the fourth bleeding was used for these experiments. Antiserum D16 was produced by using the hemocyanin conjugate, but antisera with similar properties were also obtained from the tuberculin derivative.

IgG fractions were prepared by ammonium sulfate precipitation and anion-exchange chromatography. For affinity purification of D16 IgG, the ABP peptide–tuberculin derivative conjugate was coupled to Reacti Gel 6× (Pierce and Warriner, Chester, U.K.), according to the suppliers' instructions. This matrix was tumbled gently overnight at 4°C with the IgG fraction in phosphate-buffered saline. After centrifugation at 3000 × *g* for 2 min the nonadsorbed fraction was removed, and the gel was washed several times in saline. The affinity-purified IgG was obtained by desorption with 0.5 M acetic acid for 5 min and then immediately neutralized with NH_4OH. Fab fragments were prepared from this fraction and from the total IgG by treatment with pepsin, followed by reduction with cysteine and blocking with iodoacetamide (17). Polyclonal antiserum against purified, native ABP was that described in ref. 5.

ABP Preparations. Microsomal membrane proteins of etiolated 5-day-old maize shoots (*Zea mays*) were precipitated from acetone, solubilized, and fractionated by DEAE-Bio Gel A (Bio-Rad) chromatography (5). The 0.1 M NaCl eluate was loaded onto an affinity column (D. Klämbt and G. Viola, personal communication) consisting of 4-hydroxyphenylacetic acid coupled to epoxy-activated Sepharose 6B (Pharmacia) equilibrated in 10 mM trisodium citrate/5 mM $MgSO_4$,

Abbreviations: ABP, auxin-binding protein; NAA, α-naphthaleneacetic acid; E_m, transmembrane potential difference.

pH 6.0. After incubating by rotation for 15 min, the column was washed with equilibration buffer and then eluted with 50 mM Tris/5 mM $MgSO_4$, pH 9.0. This eluate was loaded onto a Mono Q column (Pharmacia) and eluted with a salt gradient (5).

ABP homologs from etiolated shoots of barnyard grass (*Echinocloa crusgalli*), mung bean (*Vigna radiata*), and pea (*Pisum sativum*) were prepared by ammonium sulfate precipitation (75% saturation) of the soluble proteins that were then dissolved in citrate buffer in preparation for phenylacetic acid affinity chromatography, as described for maize above. The different fractions were precipitated with trichloroacetic acid and subjected to SDS/PAGE and immunoblotting (5) with anti-ABP serum (5) or antipeptide sera.

ABP homologs from tobacco (*Nicotiana tabacum* cv. Xanthi) were prepared from young leaves of 6-week-old plants homogenized in 50 mM Tris/0.1 mM $MgSO_4$/1 mM EDTA/5 mM ascorbate/1 mM dithiothreitol/0.6% poly(vinylpyrolidone), pH 8.0. Membranes were prepared by the method of Shimomura *et al.* (4). After butanol extraction (18) and dialysis against 10 mM Tris/1 mM EDTA, pH 7.0 proteins were equilibrated with 40 ml of QA Trisacryl (Pharmacia) in the same buffer for 4 hr. The resin was then poured into a column, washed in buffer, and eluted with 0.3 M NaCl in the same buffer; the proteins were then concentrated by ultrafiltration (Centriprep 3; Amicon). This concentrate was used directly for SDS/PAGE. Gels were transferred to nitrocellulose membranes and probed with primary antibodies in a hybridization oven at 37°C for 4 hr. Detection was with alkaline phosphatase goat anti-rabbit IgG (Biosys, Compiègne, France) and 5-bromo-4chloro-3-indolyl (BCIP)-NBT reagent (Bio-Rad) in 50 mM Tris acetate/10 mM magnesium acetate, pH 9.5.

Protoplast Isolation and E_m Measurement. Protoplasts were isolated from young leaves of tobacco plants (*N. tabacum* cv. Xanthi, clone XHFD8) and from plants transgenic for the *rolB* gene of *Agrobacterium rhizogenes* (XHFD8-derived clone BBGUS6, C.M., unpublished work), as described (19), except that for BBGUS6 plants the concentration of α-naphthaleneacetic acid (NAA) in the digestion medium was 2 μM instead of 15 μM. The E_m of isolated protoplasts was measured by the microelectrode technique, by using Ag/AgCl/1 M KCl half-cells (see ref. 13 for a detailed description of the procedure). For each experiment, measurements were done on 100-μl samples of the protoplast suspension (5×10^4 protoplasts·ml^{-1}), in the absence of effector (reference condition) or immediately after the addition of different concentrations of NAA or antibody fractions. A mean E_m value and the corresponding SE were calculated for each experimental condition from 15–20 individual measurements. Mean E_m values or effector-induced E_m variations from the reference value (ΔE_m) were then plotted as a function of auxin or antibody concentrations. In each experiment with antibodies, the E_m variation induced by the optimal auxin concentration (3 μM NAA for protoplasts from wild-type plants and 10 pM NAA for protoplasts from *rolB*-transformed plants) was measured (13, 20).

RESULTS

From early studies on membrane protein modification, by site-directed and group-specific reagents and from other approaches, a number of amino acid residues likely to be present at the active site of the receptor were provisionally identified (21, 22). These were as follows: cysteine, histidine (possibly two residues), tyrosine/lysine, aspartate/glutamate, and arginine. Inspection of the deduced amino acid sequence of ABP (6–8) showed a hexapeptide from residues 57–62 containing five of these six residues. This sequence, His-Arg-His-Ser-Cys-Glu, was therefore regarded as a good candidate to form, at least, part of the auxin-binding site of ABP. Accordingly, an oligopeptide of 14 amino acids was synthesized, consisting of this sequence extended in both directions to include a further arginine and a glutamate (see *Materials and Methods* for complete sequence). Antisera raised against this peptide were used to probe immunoblots of maize ABP isoforms (7) and ABP homologs in other species (23). Results with one of these sera (D16), representative of those obtained with other antisera, are shown in Fig. 1*a*. ABP is a glycosylated homodimer with subunits of 22 kDa on SDS/PAGE (5). The major isoform in maize is seen primarily in fractions 9 and 10 in Fig. 1*a*. (The band of slightly lower molecular mass in fractions 8 and 9 is a facile cleavage product of the main isoform; see ref. 5.) A less abundant isoform of similar molecular mass as well as a minor isoform of slightly higher molecular mass can be resolved by high-resolution anion-exchange chromatography (7). Both isoforms appear in fraction 6 in Fig. 1*a*. All these isoforms detected by a polyclonal antiserum produced against native ABP (5) are also recognized by antipeptide serum D16. ABP

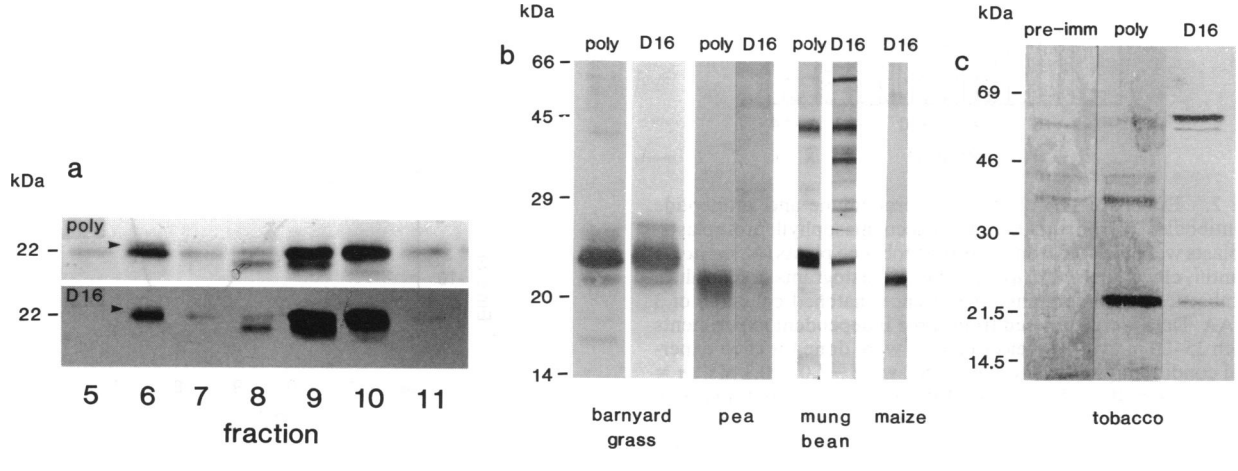

FIG. 1. Antipeptide serum D16 recognizes ABP isoforms and homologs on blots. (*a*) Isoforms of maize ABP separated by Mono Q chromatography and SDS/PAGE are identified by sera raised in rabbits against purified ABP (poly) and against the ABP peptide–keyhole limpet hemocyanin conjugate (D16). The major isoform in maize is collected primarily in fractions 9 and 10 (eluted at 0.17 and 0.185 M NaCl, respectively); less abundant isoforms appear in fraction 6 (0.125 M NaCl), arrowheads. (*b* and *c*) Blots probed with D16 serum or anti-ABP (poly), showing ABP homologs in barnyard grass, pea, mung bean (*b*), and tobacco (*c*). For comparison, crude maize microsomes probed with D16 serum are shown in *b*. Preimmune (pre-imm) sera did not recognize any band in the ABP area (22–24 kDa) in any species.

homologs of 22–24 kDa have been detected in several other species—barnyard grass, mung bean, pea (ref. 23; Fig. 1*b*), and tobacco (Fig. 1*c*) with antiserum against native maize ABP. The presence of these homologs is also shown by antiserum D16 (Fig. 1 *b* and *c*). Antiserum D16 also recognizes a band at ≈60 kDa, but information on this protein is lacking. A band at 40 kDa is recognized by both sera in some species, such as mung bean, where sequence homologies with the 22-kDa ABP have been shown by epitope mapping (24).

The ability of serum D16 to recognize ABP isoforms and homologs indicates that the peptide against which it was raised is highly conserved. If the peptide does, in fact, form part of the ligand-binding site of an auxin receptor, antibodies in the antipeptide serum might recognize the binding domain sufficiently accurately to generate a hormone-like response (25). To examine this hypothesis we used the auxin-dependent membrane-hyperpolarization response of tobacco mesophyll protoplasts. Fig. 2*a* illustrates that 3 μM NAA shifted the distribution of protoplast E_m toward more negative values. The antipeptide D16 antibody applied to the same protoplast suspension at 10 nM IgG exhibited a similar hyperpolarizing effect, whereas preimmune IgG had no significant effect on E_m (Fig. 2*b*).

To test further the hypothesis that the hyperpolarization induced by the antipeptide antibodies could correspond to an auxin agonist activity, two approaches were used: (*i*) The dose–response curve of E_m to the antibodies (Fig. 3*b*) was compared with that induced by NAA (Fig. 3*a*). Fig. 3*b* shows that protoplasts exposed to D16 IgG fraction responded as if to auxin with a typical bell-shaped dose–response curve and a maximal hyperpolarization of −5 mV. Preimmune IgG applied in the same concentration range did not modify E_m (Fig. 3*b*). (*ii*) We compared the reactions to the antibodies of protoplasts prepared from untransformed plants and from plants transgenic for the *rolB* gene from *A. rhizogenes*. The rationale of this comparison is that the dose–response curve for E_m is shifted to lower auxin concentrations when protoplasts from plants transformed with *A. rhizogenes* are used; this shift reflects an increased sensitivity of the electrical response to auxin (13, 20). Within the Ri transferred DNA (T-DNA), the single *rolB* gene increases auxin sensitivity up to 10^5-fold (20). Fig. 4*a* illustrates that a maximal hyperpolarization of protoplasts from *rolB*-transformed plants was obtained with 10 pM NAA, whereas the optimal NAA concentration was 3 μM for protoplasts from untransformed plants. Protoplasts from *rolB*-containing plants responded to D16 IgG similarly to those from untransformed plants (Fig. 4*b*) but with a sensitivity increase of five orders of magnitude. This sensitivity shift is almost precisely that seen in response to auxin (Fig. 4*a*). Neither wild-type nor transformed protoplasts show any response to IgGs from preimmune D16 (Fig. 4*c*). Fab fragments of D16 are approximately twice as active as the IgG fraction from which they were derived (Fig. 4*d*). In all cases the amplitudes of the responses to IgG and Fab fractions are closely comparable with the response to an optimal auxin concentration tested in the same experiment (see legend to Fig. 4). We further find that auxin-like activity is confined to the D16 IgG fraction affinity purified on an ABP–peptide–agarose matrix (12.5% of total IgG), the remaining bulk IgG fraction being completely inactive (Fig. 4*e*). Essentially similar results were obtained with the antipeptide serum D51 raised against the ABP peptide–tuberculin protein conjugate (data not shown).

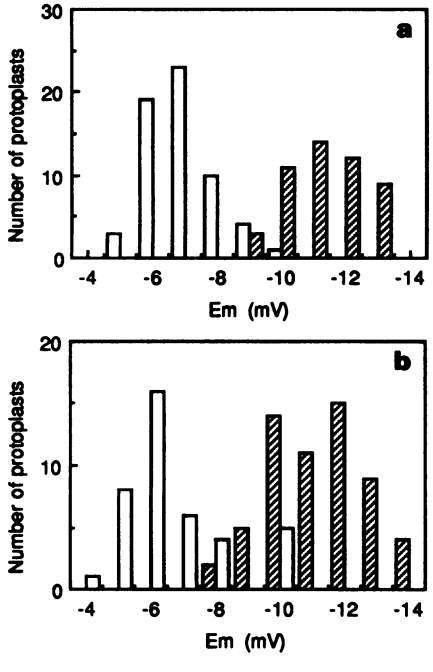

FIG. 2. Effects of NAA (*a*) and of preimmune and antipeptide D16 antibodies (*b*) on the E_m of tobacco mesophyll protoplasts. Protoplasts were isolated from wild-type tobacco plants (*N. tabacum* cv. Xanthi, clone XHFD8). (*a*) Distribution histograms of E_m values in the absence (open columns) or presence (hatched columns) of 3 μM NAA. Data were gathered from three independent experiments in which 15–20 individual measurements were done for each experimental condition. Mean E_m values ± SE were −7.0 ± 0.1 mV (n = 60) and −11.4 ± 0.1 mV (n = 49) for the series without and with NAA, respectively. The NAA-induced variation was −4.4 mV. (*b*) Distribution histograms of E_m values with 10 nM preimmune IgG (open columns) or 10 nM D16 IgG (hatched columns). Data were gathered from two (preimmune IgG) or three (D16 IgG) independent experiments, in which 20 individual measurements were done for each experimental condition. Mean E_m values ± SE were −6.7 ± 0.2 mV (n = 40) and −11.3 ± 0.2 mV (n = 60) for the series with preimmune and D16 IgG, respectively. The D16-induced variation was −4.6 mV.

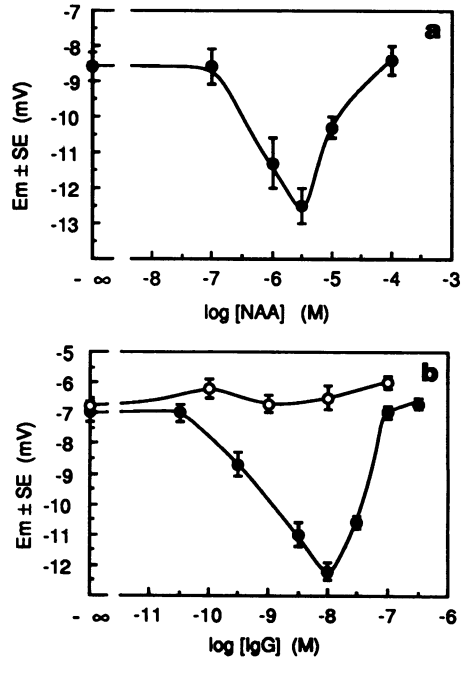

FIG. 3. Dose–response curves of the E_m of tobacco mesophyll protoplasts to NAA (*a*) and to preimmune (○) and antipeptide D16 (●) antibodies (*b*). Protoplasts were isolated from wild-type tobacco plants (*N. tabacum* cv. Xanthi, clone XHFD8). Mean E_m values were obtained for each experimental condition from 15 (*a*) or 20 (*b*) individual measurements, and the corresponding SE values were calculated.

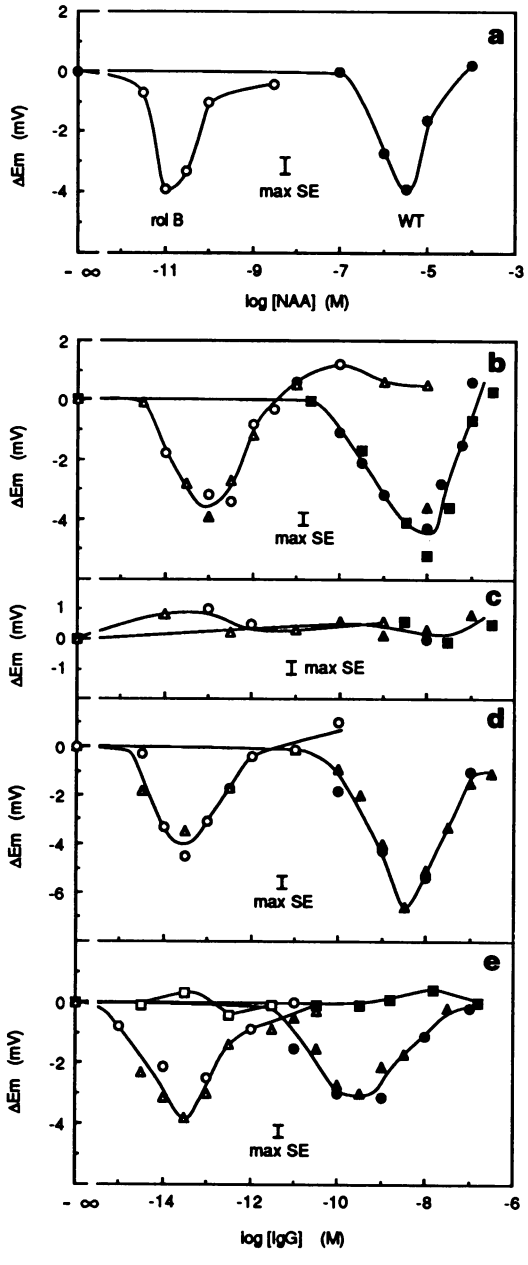

FIG. 4. Auxin agonist activity of antipeptide antibody D16. Protoplasts were isolated from wild-type tobacco plants (*N. tabacum* cv. Xanthi, clone XHFD8, closed symbols) and from plants transgenic for *rolB* of *A. rhizogenes* (XHFD8-derived clone BBGUS6, open symbols). (*a*) Dose–response curves of the E_m of tobacco mesophyll protoplasts to NAA. A mean E_m value was obtained for each experimental condition from 15 individual measurements, and NAA-induced E_m variations (ΔE_m) from the reference value were plotted as a function of NAA concentration; maximal SEs (max SE) are indicated. Data are given from one representative experiment among 16 independent experiments. (*b–e*) Dose–response curves of E_m to antipeptide D16 IgG (*b*), preimmune IgG (*c*), Fab fragments from antipeptide D16 IgG (*d*), and Fab fragments from antipeptide D16 IgG (*e*) affinity-purified on a peptide–agarose matrix (○, ●, △, ▲) and nonadsorbed remaining bulk D16 IgG (□, ■). Twenty individual measurements were done in each experimental condition, and antibody-induced E_m variations (ΔE_m) from the reference value were plotted as a function of antibody concentration. Maximal SEs (max SE) are indicated. Different symbols represent independent experiments. For each experiment, the mean E_m value in the absence of effector (reference value, E_{m0}) and the E_m variation (ΔE_m NAA) induced by the optimal NAA concentration—i.e., 3 μM and 10 pM for XHFD8 and BBGUS6 protoplasts, respectively—were as follows: (*b* and *c*) ●, E_{m0} = −7.1 mV and ΔE_m NAA = −4.0 mV; ▲, E_{m0} = −6.8 mV and ΔE_m NAA = −3.9 mV; ■, E_{m0} = −7.0 mV and ΔE_m NAA = −5.2 mV; ○, E_{m0} = −8.3 mV and ΔE_m NAA = −4.4 mV; △, E_{m0} = −8.1 mV and ΔE_m NAA = −4.5 mV. (*d*) ●, E_{m0} = −7.3 mV and ΔE_m NAA = −5.3 mV; ▲, E_{m0} = −7.2 mV and ΔE_m NAA = −5.9 mV; ○, E_{m0} = −7.8 mV and ΔE_m NAA = −4.8 mV; △, E_{m0} = −5.3 mV and ΔE_m NAA = −3.6 mV. (*e*) ●, E_{m0} = −4.5 mV and ΔE_m NAA = −3.8 mV; ▲ and ■, E_{m0} = −5.2 mV and ΔE_m NAA = −3.6 mV; ○, E_{m0} = −5.8 mV and ΔE_m NAA = −3.1 mV; △ and □, E_{m0} = −5.0 mV and ΔE_m NAA = −4.3 mV.

DISCUSSION

Several antisera from different laboratories, raised against whole maize ABP, have recently been evaluated with an epitope-mapping kit, and all antisera were found to recognize at least two of three major epitopes clustered around the glycosylation site (24). Two of these epitopes are also conserved in barnyard grass and mung bean, accounting for the cross-species recognition by ABP antisera (23). All three epitopes are well separated from the polypeptide region synthesized for production of D16 serum and related antisera.

This region was identified on the basis of deductions from early experiments on auxin binding to microsomal membranes, designed to address the likely environment of the ligand-binding site. We would expect the ligand-binding site of a hormone receptor to be highly conserved. Recognition by the antipeptide sera of all known maize ABP isoforms as well as homologous polypeptides in other species (Fig. 1) is, therefore, one essential property of antibodies that are directed against the auxin-binding domain.

The physiological activity of the antipeptide antibodies demonstrated here by hyperpolarization of tobacco mesophyll protoplasts is similar, in many respects, to the characteristic hyperpolarization induced by auxins (13). Compared with the auxin-induced electrical responses described for cells or organs (27, 28), the reaction of tobacco protoplasts is characterized by their low basal polarization (see legends of Figs. 2–4). This depolarized state could result from electrical leakage at the insertion point of the microelectrode and/or electrolyte leakage from the electrode tip and consequent rise in anionic conductance at the plasma membrane (15, 20), as well as from voltage-insensitive, but La^{3+}-sensitive, Ca^{2+} conductance (29). Despite this low basal potential, the absolute amplitude of the auxin-induced hyperpolarization of protoplasts is comparable with that reported for oat coleoptiles in equivalent experimental conditions (−6 mV hyperpolarization induced by 10 μM indol-3-yl acetic acid in 10 mM external K^+, ref. 27). Furthermore, the relative amplitude of this hyperpolarization, ≈70% of basal potential, together with low variability, leads to an easy distinction between control and treated protoplasts, as shown by the histograms of Fig. 1*a* (see also ref. 13) and makes the protoplast response to auxin highly significant. The finding that auxin rapidly modulates anion channels of *Vicia faba* guard cell protoplasts (30) through a direct interaction of the hormone with the external face of the plasma membrane is in agreement with our previous experiments indicating that auxin-responsive proteins are located at this face (13, 26, 31). Auxin also induces a slightly delayed increase in pump current through the H^+-ATPase (30), confirming involvement of the proton pump in the reaction of plasma membranes to auxin already shown in tobacco (31, 32). Although we do not know the relationship between the protoplast electrical response and longer-term auxin effects on growth, comparison between several auxins and physiologically inactive analogues (12, 13) shows that the hyperpolarization response does represent an auxin-specific phenomenon and can, therefore, be used as a functional assay for auxin activity.

Antibodies raised against whole maize ABP preparations do not themselves affect the membrane potential of tobacco protoplasts but inhibit auxin-induced hyperpolarization (13,

31). In contrast to this antagonist activity, D16 IgG behaves as an auxin agonist closely reproducing the biological activity of NAA in the protoplast assay (Figs. 2–4). In particular, not only does the antibody elicit a similar bell-shaped curve with amplitude comparable to that seen with NAA, but the dramatic increase in sensitivity shown by protoplasts from plants transgenic for *rolB* is almost identical for both NAA-induced and D16 IgG-induced responses. D16 IgG was raised against a restricted polypeptide but is, nevertheless, polyclonal. Such heterogeneous, bivalent antibodies could activate cellular responses by inducing receptor microclustering (33), without necessarily recognizing the hormone-binding site. However, the activity of the monovalent Fab fragments of D16 IgG (Fig. 4*d*) allows us to rule out such "non-specific" activation.

In a few experiments D16 Fab preparations significantly promoted growth of abraded pea sections (data not shown), but we were unable to obtain consistent growth stimulation. Possibly, the extended β-sheet structure of antibody Fab fragments may not be conducive to ready penetration of cell wall pores (34), or the fragments may not be able to bind effectively to ABP with the wall present.

Unlike anti-ABP serum (35), D16 serum does not immunoprecipitate native ABP, nor does it inhibit auxin binding to solubilized ABP (M.A.V., unpublished data). While at first sight this may appear paradoxical, it seems that the conformation of ABP under these *in vitro* conditions is such that the epitopes recognized by anti-ABP sera (24) are exposed on the protein surface, whereas the auxin-binding region is buried. This explanation is consistent with observations that unlike native ABP, denatured ABP can be immunoprecipitated by D16 serum and adsorbed by D16–agarose (M.A.V., unpublished work). On the other hand, in the *in vivo* (protoplast) situation the auxin-binding site is clearly accessible to D16 IgG. This interpretation agrees with earlier evidence that *in vivo* and *in vitro* conformations of ABP are not identical (36).

The potent auxin agonist activity of our antipeptide sera in the protoplast assay is most readily accounted for by assuming that the peptide embraces a substantial or critical portion of the auxin-binding domain of the receptor and that binding of agonist antibodies to this domain elicits the identical activation to that induced by auxin binding. We cannot exclude the possibility that agonist activity arises from a conformational change evoked by antibody binding to an allosteric or other site distinct from the auxin-binding site. However, the rationale of ABP polypeptide selection as well as the fidelity with which antipeptide antibodies reproduce the electrophysiological activity of auxins on protoplasts (Figs. 2–4), makes the simpler explanation—namely, that the antibodies are directed at the ligand-binding site—more probable. The likely importance of this region is reinforced by recent information showing that the sequence is largely conserved in a different maize ABP cDNA (37), in *Arabidopsis* (38), and in strawberry (C. M. Lazarus, personal communication), whereas the maize epitopes recognized by ABP antisera are less fully conserved. The significance and characteristics of this region can now be explored more completely by site-directed mutagenesis. Furthermore, our results reinforce evidence that auxin receptors are accessible at the exterior face of the plasma membrane (13, 26) and provide a basis for the use of antipeptide antibodies as impermeant molecules exhibiting auxin activity.

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