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From Binding Proteins to Hormone Receptors?

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Abstract. Phytohormones exert in responsive plant cells specific biochemical and physiological effects. It is a widely held view that phytohormones are first recognized by specific receptors which initiate the transduction of the hormonal signal. While hormone receptors are well studied in many eukaryotes ranging from yeast to man, we are lacking a detailed understanding of phytohormone receptors. Phytohormone binding proteins have been suspected to provide candidates for such receptors. In this review recent progress towards molecular analysis of such proteins and their genes will be summarized.

Plant growth regulators like auxins and cytokinins have been known for quite some time, and numerous physiological responses to each group of growth regulators have been described. Despite their fundamental influence on almost all aspects of plant development little is known of their molecular mode of action (Palme et al. 1991; Klee and Estelle 1991; Jones and Prasad 1992). Physiological effects elicited by auxins include the enhancement of plant growth by cell elongation, the induction of vascular differentiation and the inhibition of lateral bud development; they activate cell division, alter gene expression and affect tropic responses (for review see Davies 1987). Cytokinins are also known to have profound effects on cellular metabolism. They enhance cell division, retard senescence, and enhance nuclear gene expression (for review see Davies 1987). Yet despite the plethora of responses elicited by these substances, their primary recognition by cellular receptor proteins as well as their subsequent cellular transduction pathways are still poorly understood. Here we will describe recent progress from biochemical studies to identify molecular elements of auxin and cytokinin action.

Phytohormone Binding Studies

Over the past decade we have learned from studies of signal transduction pathways in lower and higher eukaryotes that hormones bind to soluble or membrane-associated receptor proteins. As a result of the interaction of a hormone with its cognate receptor, various biochemical events may be initiated leading to the characteristic physiological responses. By analogy, receptor proteins that bind phytohormones have been central elements in most models aiming to explain phytohormone action (Stoddart and Venis 1980; Libbenga and Mennes 1987). Such "receptor" proteins could not only be sites for primary perception of phytohormones like auxins but also be sites for action of herbicides and other synthetic plant growth regulators. What strategies could be applied to identify phytohormone receptors? Obviously biochemical strategies have been successfully used in the past to purify and characterize hormone receptors from a wide variety of organisms, and these successes were largely coupled to refined biochemical technologies as well as to the functional coupling of these receptors to physiological responses. The difficulties to couple binding of phytohormones to subsequent primary cellular responses may explain why it has been so difficult to identify phytohormone receptor proteins. As apparently no phytohormone receptor mutants have been isolated yet and primary responses to phytohormones are still poorly recognized, indirect biochemical methods are still thought to be of value to identify phytohormone binding proteins as potential candidates for receptor proteins. Classical binding of phytohormones to plant proteins or more refined techniques such as photoaffinity labeling could lead to the identification of proteins that might be expected to vary in their binding affinities to the hormone and thus not only represent potential candidates for receptors but alternatively be in-

volved in hormone metabolism or transport. These functions need not be mutually exclusive, as various hormone binding sites with different physiological functions could exist in a given cell type or tissue. Consequently, studies of phytohormone hormone binding sites could clarify aspects of economic use of hormones that finally might lead to a better understanding of the cellular responses to the hormone.

Cytokinin Binding Proteins

The biochemical search for cytokinin binding proteins was initially based on the binding of radiolabeled cytokinins to soluble extracts from various plants. Protein–ligand complexes were analyzed by either gel filtration or equilibrium binding. K_Ds in the range of 10^{-4} to 10^{-6} M were measured (Mitsui et al. 1993). Cytokinin binding proteins with molecular masses ranging from 4.5 to 180 kDa were found in several plants (Tagekami and Yoshida 1975; Erion and Fox 1981; Fox and Erion 1975; Polya and Davis 1978; Moore 1979; Chen et al. 1980; Hamaguchi et al. 1985; Kulaeva et al. 1990; Romanov et al. 1990; Momotani and Tsuji 1992; Mitsui et al. 1993). Whereas a cytokinin binding protein, 4.5 kDa in size, was found to bind to the 40S ribosomal subunits in vitro, other cytokinin binding proteins were suggested to be involved in sequestering of cytokinins (Brinegar et al. 1985, 1988). Recently a cytokinin binding protein complex was purified approximately 700–fold from *Nicotiana sylvestris* having a molecular mass of 130 kDa (Mitsui and Sugiura 1993). This complex was shown to consist of two subunits with molecular masses of 57 and 36 kDa, respectively, and shown to bind cytokinin with a K_D of approximately 2.1×10^{-5} M (Mitsui and Sugiura 1993). The binding constant does not fit the characteristics of a hormone binding with high affinity to its receptor but rather could be interpreted that the hormone binds with low affinity to an enzyme. Low-affinity cytokinin binding to an enzyme could, for example, modulate its kinetic properties or alternatively interfere with the enzymatic reaction catalyzed by the cytokinin binding protein by competing with a substrate or other binding of putative regulators of its activity.

Initial attempts to identify and purify this cytokinin binding complex were based on the use of benzyladenine coupled to sepharose. It turned out that hydrophobically bound proteins were very inefficiently eluted from such cytokinin affinity columns. Significant progress was made when conventional chromatographic separations were applied to purify sufficient material of this cytokinin binding protein complex for microsequencing. Amino acid sequence data were obtained after proteolytic cleavage of the 57 kDa subunit. This allowed the construction of sequence-specific oligonucleotides and isolation of corresponding cDNA clones. Most interestingly, clones obtained predict an open reading frame for a polypeptide that shares high homology with a parsley S-adenosyl-L-homocysteine hydrolase (Kawallek et al. 1992; Mitsui et al. 1993). Mammalian S-adenosyl-L-homocysteine hydrolases are major adenosine/cAMP-binding proteins that control the intracellular SAM/SAH ratio and thereby influence the efficiency of biological methylation reactions (Hohman et al. 1985). This suggests an interesting possibility that some aspects of cytokinin action could be mediated through the control of protein or DNA methylation.

Auxin Binding Proteins

The search for auxin binding proteins was similarly based on the analysis of high-specificity binding of auxins to subcellular fractions of plant cells allowing the identification of three different types of membrane-associated auxin binding sites (Hertel et al. 1972; Ray et al. 1977). In maize membranes they were found to be located to the endoplasmic reticulum, the tonoplast, and the plasma membrane (Dohrmann et al. 1978). Indeed, the biological relevance of phytohormone binding has been assessed by several studies showing that specific phytohormone binding to subcellular fractions can be correlated with relevant biological phenomena (Poovaiah 1982; Hicks et al. 1989a).

Early attempts to purify these sites proved to be extremely difficult to biochemically characterize these operationally defined binding proteins. To overcome problems associated with traditional auxin binding studies (e.g., low receptor protein concentration, instability of auxin binding under experimental conditions, denaturation or loss of the auxin binding proteins during purification), auxin specific photoaffinity labeling techniques were found to be useful for identification of auxin binding proteins. Photoaffinity labeling techniques have contributed greatly to identification and structural studies of animal receptor proteins. Photoaffinity ligands covalently label the ligand binding polypeptide and allow receptor molecules to be followed throughout purification under both denaturing and nondenaturing conditions. Although photolabile synthetic cytokinins were prepared as early as 1976 (Theiler et al. 1976; Mornet et al. 1979), they turned out to be of limited use in the identification of cytokinin binding proteins. Initial photoaffinity

labeling experiments using photolabile synthetic auxin analogues were also not successful resulting in signals being overwhelmed by background noise making discernment of radioactive peaks difficult (Jones et al. 1984). More recent improvements of this technique have resulted in a better signal to noise ratio (Hicks et al. 1989a). Special care to ensure the purity of the photoaffinity labeling agent and maximize the specificity of the assay has allowed us to identify and characterize several auxin binding proteins from maize and Arabidopsis membranes (Campos et al. 1991, 1992; Zettl et al. 1991, 1992, 1994; Feldwisch et al. 1992; our unpublished data). Photoaffinity labeling techniques have been applied to identify auxin binding proteins in subcellular fractions obtained from various plants including zucchini, tomato, and maize (Hicks et al. 1989a,b; Jones and Venis 1989; Macdonald et al. 1991; Jacobi et al. 1993; Bilang et al. 1993). Using 5-azido-[7-^{3}H]IAA for photoaffinity labeling we were able to identify three proteins in plasma membranes from maize coleoptiles with molecular masses of 60 kDa (pm60), 58 kDa (pm58), and 23 kDa (pm23). Whereas pm60 and pm58 are not yet characterized, pm23 has been analyzed in detail (Feldwisch et al. 1992). Binding of 5-azido-[7-^{3}H]IAA to pm23 was competed by auxins and functional analogues. A purification scheme allowing purification of pm23 was designed. Homogenous pm23 was obtained from coleoptile extracts after 7000-fold purification. Partial amino acid sequences were obtained for both proteins. The primary amino acid sequence information obtained allowed the synthesis of specific oligonucleotides and subsequent isolation of pm23 specific cDNAs. The proteins predicted from the deduced open reading frames were not found in data bases and represent novel proteins with unknown functions. Several of these auxin binding proteins have meanwhile been characterized in detail and recent progress will be summarized.

The ERabp Family

A binding site (site I) from maize initially thought to be membrane associated has been most thoroughly studied. To purify this auxin binding protein, protocols were developed by several groups allowing purification to homogeneity of a major 22 kDa protein as well as several isoforms (Venis 1977, 1987; Shimomura et al. 1987; Napier and Venis 1990; Hesse et al. 1989; Palme et al. 1990). The major ER-located auxin binding protein from maize coleoptiles (Zm-ERabp1, for *Zea mays* ER-located auxin binding protein) has an apparent molecular weight of 22 kDa. Equilibrium auxin binding assays as well as auxin-specific photoaffinity labeling techniques demonstrated that this protein binds various auxins including 1-naphthylacetic acid (1-NAA) and indole-3-acetic acid (IAA) (Palme et al. 1990). The protein that was isolated by chromatography on 1-naphthylacetic acid or phenylacetic acid bound to aminohexyl-Sepharose has an apparent molecular mass of 20 kDa. Equilibrium dialysis showed that the protein binds 1-NAA with a K_D of 2.4×10^{-7} M. Scatchard analysis revealed one auxin binding site per protein for this phytohormone (Shimomura et al. 1986; Palme et al. 1990). Its primary structure was independently deduced from different cDNAs isolated from maize (Hesse et al. 1989; Inohara et al. 1989; Tillmann et al. 1989). Meanwhile, additional members of this family were isolated from maize as well as from *A. thaliana* (Hesse et al. 1989; Yu and Lazarus 1991; Palme et al. 1992; Schwob et al. 1993; Shimomura et al. 1993). All these proteins contain an N-terminal hydrophobic signal sequence that appears to be responsible for the uptake of these proteins into microsomes. This was confirmed by in vitro studies which demonstrated that both the maize as well as the Arabidopsis ERabp's are translocated into ER-derived microsomes and cotranslationally glycosylated (Palme et al. 1992; Campos et al. 1994). Biochemical experiments reported by Hesse et al. (1989) and Palme et al. (1990) clearly established that this auxin binding protein is a luminal component of the ER. This observation is consistent with the absence of hydrophobic sequences that could be responsible for membrane insertion and by the presence of a C-terminal tetrapeptide sequence, -Lys-Asp-Glu-Leu (-KDEL), previously reported for other proteins to be a retention signal for the endosplasmic reticulum (Pelham 1990). The signal K/HDEL is recognized as being responsible for retrieval of proteins from a post-ER salvage compartment in several eukaryotic organisms and has apparently a similar function in plants.

Although the primary sequences of the proteins encoded by this gene family do not fit the structural requirements of "animal" receptor proteins, Zm-ERabp1 is considered to be an "auxin receptor." The basis for this belief originally was a correlation of auxin binding profiles with the cellular pattern of auxin-stimulated elongation growth. In addition, light-induced changes in cell elongation were related to a modulation of the number of this binding site (Walton and Ray 1981). Recent experiments using antibodies raised against Zm-ERabp1 demonstrated a specific inhibition of electrical responses at the plasma membrane of tobacco protoplasts (Barbier-Brygoo et al. 1989, 1991). This was further confirmed by analyzing the electrical response to

maize protoplasts using the patch clamp technique (Rück et al. 1993). It was found that after a short lag phase, indole-3-acetic acid induced an outwardly directed current that was dependent on the concentration of the auxin applied to the protoplasts. This current was inhibited by the application of antibodies directed against the Zm-ERabp1 protein. Furthermore, Venis et al. (1992) demonstrated that antibodies to a peptide from Zm-ERabp1 that probably harbor the auxin binding site, have auxin agonist activity and, most interestingly, these antibodies were able to induce the outward current in the absence of auxin (Rück et al. 1993). The question, however, arises as to how a putative receptor located in the lumen of the endoplasmic reticulum could be involved in the perception of exogenous auxins and affect phenomena such as cell expansion and membrane hyperpolarization. A number of observations indeed imply that ERabp plays a role in these phenomena. These data indicate that the Zm-ERabp1 protein may be a site for auxin perception through which the activity of the plasma membrane H^+ATPase is activated and modulated. The answer may well be that although the ERabp-encoded proteins can be found to be associated with the ER, they can apparently, under circumstances that still await elucidation, go through the secretory pathway and appear on the plasma membrane. That such a remarkable movement of a luminal ER auxin binding protein to the cell surface might indeed occur and have functional significance, was first suggested by experiments (Barbier-Brygoo et al. 1991) showing that ERabp was involved in the perception of the auxin signal at the plasma membrane. Indeed, incubation of tobacco mesophyll protoplasts with exogenous Zm-ERabp1 increased their sensitivity to hyperpolarization by external auxins through several orders of magnitude, whereas incubation of the protoplasts with antibodies directed against Zm-ERabp1 decreased their sensitivity. This interpretation was recently supported by Jones and Herman (1993) studying the subcellular distribution and secretion of an ABP in maize coleoptiles and suspension culture cells by microscopic immunochemistry and immunochemical assays.

NPA-Binding Proteins

It is generally assumed that polar auxin transport by carrier proteins plays an important role in controlling auxin concentrations in various tissues. Little is known about the molecular elements required for auxin transport through the various tissues of a plant (for review see Rubery 1990). Binding and inhibitor studies, using active auxins or auxin transport inhibitors, as well as electrophysiological evidence, point to the presence of auxin uptake and efflux carriers localized on the plasma membrane of responsive plant cells (Felle et al. 1991). Binding studies have indicated that in addition to unspecific uptake of undissociated IAA across the membrane, an H^+/IAA^--influx-carrier must be involved in IAA uptake. In addition, evidence for an auxin efflux carrier was provided (Rubery 1990). This efflux carrier was found to be inhibited by phytotropins such as 1-N-naphthylphtalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA). NPA, for example, inhibits polar auxin transport, affects root growth and abolishes gravitropic responses. Such responses to NPA in vivo correlate with an in vitro inhibition of auxin efflux from PM vesicles isolated from zucchini or maize resulting in a net accumulation of IAA.

NPA binding studies using high-specific binding of [^{3}H]NPA to maize microsomes or plasma membranes led to the identification of NPA binding sites, which were operationally called *NPA receptors*. It has been proposed that NPA binding proteins could be components of an auxin efflux carrier or, alternatively, regulatory elements of the auxin efflux carrier. Although NPA binding activities were detected in membrane vesicles from various plants by binding of radioactively labeled NPA, such assays were not sensitive enough to allow isolation and molecular characterization of NPA binding proteins. We, therefore, aimed to develop a photoaffinity probe for auxin carrier proteins and synthesized a tritiated and light-sensitive NPA-analogue, 5′-azido-[3,6-$^3H_2$]NPA ([$^3H_2$]N_3NPA) (Zettl et al. 1991, 1992). After incubation of plasma membrane vesicles with [$^3H_2$]N_3NPA and exposure to UV light, we observed specific photoaffinity labeling of a protein with an apparent molecular mass of 23 kDa. Pretreatment of the plasma membrane vesicles with indole-3-acetic acid or with the auxin transport inhibitor 2,3,5-triiodobenzoic acid strongly reduced specific labeling of this protein. This 23 kDa protein was also labeled by the addition of 5-azido-[7-^{3}H]IAA to plasma membranes prior to exposure to UV light. The 23 kDa protein was solubilized from plasma membranes using 1% Triton X-100. In addition, we have found a protein of related molecular mass in soybean root nodule symbiosome membranes by photoaffinity labeling with both 5-azido-[7-^{3}H]IAA and [$^3H_2$]N_3NPA (Jacobi et al. 1993). Soil bacteria such as *Rhizobium* or *Bradyrhizobium* can induce root nodules on host plants resulting in fixation of atmospheric nitrogen into organic nitrogen. Although most of the factors responsible for nodule development are not yet known, changes in phytohormone ratios are

thought to play an important role in this process. In particular it has been shown that phytotropins like NPA or TIBA can induce the formation of root nodules and, moreover, such nodules contain higher amounts of IAA. Our finding that tritiated indole-3-acetic acid bound reversibly with a K_D of 1×10^{-6} M to nodule – specific symbiosome membranes suggested the presence of specific auxin binding proteins in these membranes. The only protein found after photoaffinity labeling was a 23 kDa protein, and labeling was specifically completed for not only by unlabeled IAA and 1-NAA, but also by NPA, quercetin, and rutin, known inhibitors of auxin transport. Similar to the maize 23 kDa protein, the symbiosome-specific 23 kDa protein was solubilized by Triton X-100 and Triton X-114 suggesting that this protein could be a component of the auxin transport system.

Identification of a β-Glucosidase

As plants lack the structures analogous to the specific endocrine organs of animals for spatially localized hormone production, it is reasonable to expect different principles to apply to the organization of the production, movement, and sequestration of phytohormones. Recent insights into such processes demonstrate the involvement of various glycosidases that can metabolize phytohormones (for review see Spena et al. 1993; Palme and Schell 1993). Glycosidases probably alter the cellular concentrations of active forms of phytohormones and thus are likely to play an important role in the control of plant development. That release of active hormones from intracellular pools of inactive conjugates may be an important mechanism underlying hormone action in plants is further supported by our observation that a gene coding for a glucosidase capable of hydrolyzing phytohormone conjugates was recently isolated (Brzobohaty et al. 1993). Protein extracts from maize coleoptiles grown in the dark were photoaffinity labeled with 5-azido-[7-^{3}H]IAA and a 60-kDa protein, termed *p60,* was thus identified (Campos et al. 1992). This protein was initially detected in the postribosomal supernatant, indicating that it might be present in the cytosol of intact cells. p60 was also detected in protein extracts prepared after solubilization of microsomal fractions. In both cases, labeling of p60 was strong and no other protein present in the extract was labeled. To demonstrate the specificity of photoaffinity labeling of p60, competition studies were performed using various unlabeled auxin analogues. Physiologically active natural and synthetic auxins significantly reduced the incorporation of 5-azido-[7-^{3}H]IAA into p60, whereas compounds specific for either the indole ring such as L-tryptophan, or the aromatic ring system, or radical scavengers such as *p*-amino-benzoic acid, did not complete the labeling of p60.

p60 was purified from maize coleoptile microsomes and microsequencing studies were performed after proteolytic digestion. The primary amino acid sequence of p60 already revealed similarities to β-glucosidases. Extensive amino acid sequence analysis of this protein allowed the construction of several synthetic oligodeoxynucleotide probes which were used to isolate a cDNA clone coding for a protein related to p60. The cDNA, named Zm-p60.1, corresponded to a mRNA a single open reading frame. When the Zm-p60.1 primary sequence was compared with other amino acid sequences available in protein data bases, similarities were observed to other β-glucosidases from archaebacteria, eubacteria, and eukaryotes. As indicated by the amino acid alignments Zm-p60.1 is most closely related to the β-glucosidase family, but also partly to the phospho-β-glucosidases from *Escherichia coli* and phospho-β-galactosidases from lactic bacteria. This was confirmed by the analysis of the substrate specificity of this enzyme showing a very distinct pattern of substrate specificity. In particular, it was interesting to find that Zm-p60.1 specifically cleaves biologically inactive hormone conjugates (i.e., cytokinin-O- and -N3-glucoside) releasing active cytokinins (Brzobohaty et al. 1993).

The fact that Zm-p60.1 releases active cytokinins from inactive conjugates suggests that this protein could play a pivotal role in the germination process by controlling the release of free cytokinin. To test whether Zm-p60.1 expression was able to influence plant growth, tobacco protoplasts were transiently transformed with Zm-p60.1. These protoplasts acquired the ability to use exogenous cytokinin glucosides to initiate division. Further immunocytochemical analysis of maize seedling roots localized Zm-p60.1 to meristematic cells suggesting that Zm-p60.1 is a glucosidase capable of supplying the developing embryo with biologically active cytokinins (Brzobohaty et al. 1993).

One of the most attractive features of a model explaining plant growth control could be based on the action of β-glucosidases. Particularly attractive is the simplicity by which developmental adaption to environmental cues could be provided. Auxin and cytokinin conjugates have been found to be broadly distributed in plants. The activity of phytohormone-specific β-glucosidases could be easily regulated by environmental as well as endogenous factors. Thus, phytohormone-specific β-glucodosidases might provide a link between environmental

stimuli and the activation of phytohormones in precise locations of the plant. Although these ideas are far from being proven, they open a promising area of research in plant development. We hope that future investigations will contribute to define the importance of phytohormone-specific β-glucosidases in the control of developmental processes in plants.

Perspective

For a long time the "site I" specific auxin binding site was the only one amenable to biochemical and physiological analysis. Now several additional phytohormone binding proteins have been isolated and can be used for molecular and electrophysiological studies. We expect that analysis of the genes coding for these proteins will contribute to disclose some of the aspects of phytohormone perception.

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