A Binding Resolution

Prior to the use of Arabidopsis (*Arabidopsis thaliana*) as a model genetic system, there was little progress made in identifying the molecular structures of hormone receptors in plants. Over the past decade with Arabidopsis leading the foray, we now know the structures of the receptors for cytokinins, auxins, gibberellins, and ethylene as well as an ever-increasing litany of receptors for newly discovered hormones and peptides. Despite these great successes, many questions remain, such as whether there are additional receptors for auxin and cytokinin lurking out there as well as the big question of what exactly is (are) the identity of the abscisic acid receptor(s)? This latter question is particularly compelling and is the motivation behind this letter since several high-visibility papers purporting the identity of the abscisic acid receptor have recently appeared and it is unclear which, if any, are bona fide receptors. In particular, this letter will address the question of what should be the bar for sufficient evidence to conclude that a particular protein is, in fact, a plant hormone receptor.

What is a hormone receptor? We provide a set of classic characteristics below. Since plant biology has been dominated in the last 20 years by the application of forward and reverse genetic approaches using Arabidopsis and other plants, let us assume that most ''receptor identification stories'' begin with a mutant phenotype that can be readily explained as an alteration of hormone response, consistent with possible receptor function. While such a result would certainly be a good start, it may also be deceptive. We all know of examples where hormone insensitivity or hypersensitivity is due to changes in hormone metabolism or transport or to changes in a protein that alter the affinity of a separate and distinct receptor that is not itself mutated. Moreover, perturbation of one strand of a signaling network can manifest itself as an alteration in a distant signaling strand, further cause for healthy skepticism. Therefore, an important aspect of all receptor identifications is that the protein should be shown to directly bind the ligand, here the plant hormone, in vitro, in a purified state. Recent advances in heterologous expression of plant proteins offer new avenues of aggressively pursuing this goal, but they also offer dangerous speed bumps that need to be carefully maneuvered, as will be discussed below.

In this letter, we would like to eschew receptor identification via sequence similarity to known receptors in animals since this can be misleading. In fact, all four known plant hormones have receptors that are not at all known in animals, although several are known in microbes (the His kinases for cytokinin and ethylene). On top of being misled by sequence similarities that may be fortuitous or irrelevant to their function, the recent discovery that microbes have actin and other cytoskeletal homologs that have nearly identical three-dimensional structures to the animal proteins (van den Ent et al., 2001), but no sequence similarity at all, highlights the fact that there are so many ways to achieve a particular protein fold. Furthermore, with respect to receptor definition, let us admit that we are using sequence only because it is available and easy, not because it is really what is important.

We propose that a plant protein, whether or not it shares sequence and/or predicted structure similarity to any known class of receptors, must meet strict biochemical criteria for receptor functionality before it is accepted as such. Most of these criteria were established in the 1970s, and they were achievable within the confines of the available technology of the time. We note that these criteria are still standard practice in animal and microbial cell biology and that the plant biology community, authors, readers, and reviewers invite ridicule by not adhering to them, whether through naivety or design. As technology has advanced, the ability to address the receptor problem has become easier, but so does the propensity for false, facile interpretations. We propose that when interrogating a candidate receptor, the following properties should be found before a claim of receptor functionality is made: a ligand binds its cognate receptor reversibly, saturably, selectively, and with a stoichiometry of one or more molecules of ligand per molecule of protein and binding is usually heat sensitive and affected by proteases.

There are a variety of methods to assay binding and programs for analyzing the results; many are commercialized. Equilibrium techniques surpass ''approach to equilibrium'' techniques in reliability but are not always feasible.

(1) Receptors bind ligands with strict structural and steric specificity. Researchers should explore this property with a large set of agonists and antagonists because tests of only a few compounds are not informative. For example, bovine serum albumin binds D-Trp but not L-Trp (Stewart and Doherty, 1973). Another example of an artifact is ^{125}I-glucagon binding to cellulose acetate, which is competed by glucagon and growth hormone but not by six other similar compounds tested (Cuatrecasas, 1975).

(2) Ligand binding to a receptor is saturable, indicating a finite and limiting number of binding sites. Since ligand binding and receptor activation are usually the rate-limiting steps in signal transduction, the number of binding sites should be small. The highly abundant protein ribulose-bisP carboxylase

www.plantphysiol.org/cgi/doi/10.1104/pp.109.136606

binds indole-3-acetic acid saturably (Wardrop and Polya, 1980), but clearly there are no other data, either physiological or genetic, supporting the model that this protein is an auxin receptor.

(3) Receptors have one or more binding sites; therefore, ligands bind receptors with a molar ratio of 1 or greater at equilibrium. Molar ratios substantially below 1 should raise a red flag. Low molar ratios indicate either that the candidate is not a receptor and that the actual binding protein is a minor contaminant or that the proper conditions for native binding or renaturation of the protein were not used.

(4) Ligand binding to its receptor should be reversible and kinetically consistent with reversal of the in planta effect. Ideally, the reversal of a hormone response correlates tightly with the ligand-off rate. A lack of correlation is occasionally insufficient to conclude that a candidate protein is not a receptor because dissociation from the receptor may not be the rate-limiting step. For example, in the case where the natural ligand is a reverse agonist such as ethylene for the ETHYLENE TRIPLE RESPONSE1 family of ethylene receptors, nascent synthesis of new (active) receptors may become the rate-limiting step for the physiological reversal.

(5) Although RNA can function like a protein and bind some ligands (Breaker, 2008), a very common experiment to determine whether a binding observation is worth pursuing further is to see if the binding factor is heat and protease sensitive. This is not airtight, however, since heat-induced changes in conformation can also lead to gross changes in the physical state of proteins in the mixture and loss of binding might be caused by artifactual changes relevant to a particular method of binding detection (e.g. are heat-induced precipitates still able to be centrifuged or retained using charge-based filter paper binding measurements?). This is still true even when heterologous expression of a protein is used to assay binding, as there is no control supporting the notion that the observed ligand binding is to the recombinantly expressed protein. It is incorrect to use as a control another recombinant protein purified in the same manner as the candidate receptor because there can be different levels of a low-abundance contaminant between purifications.

The Affinities of Agonists Often Correlate with the Half-Maximal Effective Concentration, EC_{50}

The absolute affinities of these ligands should be determined and compared with the potency of the compound in eliciting a physiological or cellular response. A correlation between affinity and potency on its own is insufficient evidence to make claims of receptor functionality. A classic example of an artifact is binding of the plant hormone cytokinin to talcum powder (Sussman and Kende, 1978), which was shown to be saturable, with a binding affinity consistent with the physiological responsiveness of cytokinin. Another example is that opiate drugs bind cerebroside sulfate stereospecifically and with binding affinities that correlate with their biologic potency (Loh et al., 1975). From the opposite side, when a binding affinity is higher than its potency, this finding alone is insufficient evidence to conclude that a candidate protein cannot be a receptor. Deviations from this correlation occur when there are differences in cell uptake and metabolism among compounds in the test set.

Ligands Induce Local or Global Conformational Changes or Recapitulate Ligand Signaling in a Heterologous System

An agonist evokes a conformational change in a receptor that favors its activation state. Advances in technology have made this more easily measured. For example, circular dichroism detects local ligand-induced changes in some protein structures. However, in some cases of ligand-receptor interaction, local structural changes do not occur; rather, ligand binding behaves like chemical glue to cement new protein-protein interactions. This may be viewed as a global change in structure and should be clearly demonstrated for this type of receptor.

Another way to approach this problem is to recapitulate ligand-dependent signaling in a heterologous system. This involves a great deal of serendipity, as the candidate plant hormone receptor must "plug into" the heterologous signaling chain of elements. Ligand binding in this unnatural environment must evoke changes in the receptor that can be rapidly read out (e.g. mitogen-activated protein kinase kinase kinase activation).

Mutations in Putative Hormone Receptors Must Affect Signal Transduction in an Understandable Way

Just as it is important to explore the specificity of ligand binding by measuring affinities for a large set of structurally similar compounds and comparing them with biologic potency, it is critical to explore specificity from the receptor candidate perspective. One goes about this by making mutations that affect hormone binding of a putative hormone receptor. One then must show that changes in the receptor that alter binding consequently alter the cellular or physiological response. In the strictest sense, this is the most onerous of the criteria, as it requires educated guesses about the location of the binding site(s) in order to direct mutations. Computer programs to assess the possible locations are only useful when the three-dimensional structures are known or deduced. In the end, the most persuasive form of evidence is a mutation that alters one amino acid, resulting in loss of binding but no other dramatic changes in protein expression, structure, and stability.

As we began in this letter, loss of function or null alleles of genes encoding the candidate receptor are

easy to come by, and these mutations provide a good start when the phenotype is elimination of some part or all of the known biologic responses of the hormone. However, for the reasons discussed earlier, these genetic observations are only the first act of a long play whose final scene involves biochemical clarity on receptor identification and function.

Concluding Remarks

It is important for reviewers, editors, and readers to adhere to the strictest guidelines for acceptance of reports concerning the attribution of receptor function. In the race to be first, we need to be careful not to sacrifice appropriately robust standards. There are always new discoveries to be made, and since most of science recycles and builds upon previous discoveries, efficient use of our time and energy dictates that the bar for accepting important new discoveries be kept at a level that precludes the need for retractions. At the same time, we do not want the bar so high that we create onerous hurdles in order for a new idea to be put out into the general consciousness. We are not advocating that all of these criteria must be met in a single paper; rather, they must be met simply before the community adopts the functional moniker of receptor for a candidate. Given that there are so many resources available to biologists now to approach problems from several angles (e.g. plant genetics, biochemistry [via heterologous expression], and chemical genomics [i.e. screening for new ligands]), it behooves us to use as many as possible before proclaiming to the rest of the world that our precious plants have told us something new about eukaryotic signaling mechanisms.

Alan M. Jones* and Michael R. Sussman

Departments of Biology and Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599 (A.M.J.); and Department of Biochemistry and University of Wisconsin Biotechnology Center, University of Wisconsin, Madison, Wisconsin 53706 (M.R.S.)

*** Corresponding author; e-mail alan_jones@unc.edu.**

LITERATURE CITED

Breaker RR (2008) Complex riboswitches. Science **319:** 1795–1797

Cuatrecasas P, editor (1975) Hormone Receptors: Their Function in Cell Membranes and Some Problems Related to Methodology, Vol 5. Raven Press, New York

Loh HH, Cho TM, Wu YC, Way EL (1975) Opiate binding to cerebroside sulfate: a model system for opiate-receptor interaction. Life Sci **16:** 1811–1817

Stewart KK, Doherty RF (1973) Resolution of DL-tryptophan by affinity chromatography on bovine-serum albumin-agarose columns. Proc Natl Acad Sci USA **70:** 2850–2852

Sussman MR, Kende H (1978) Cytokinin binding sites in a particulate fraction from tobacco cells. Planta **140:** 251–259

van den Ent F, Amos LA, Lowe J (2001) Prokaryotic origin of the actin cytoskeleton. Nature **413:** 39–44

Wardrop AJ, Polya GM (1980) Co-purification of pea and bean leaf soluble auxin-binding proteins with ribulose-1,5-bisphosphate carboxylase. Plant Physiol **66:** 105–111