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The CLAVATA1 Receptor-like Kinase Requires CLAVATA3 for Its Assembly into a Signaling Complex That Includes KAPP and a Rho-Related Protein

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The *CLAVATA1* (*CLV1*) and *CLAVATA3* (*CLV3*) genes are required to maintain the balance between cell proliferation and organ formation at the Arabidopsis shoot and flower meristems. *CLV1* encodes a receptor-like protein kinase. We have found that CLV1 is present in two protein complexes in vivo. One is ~185 kD, and the other is ~450 kD. In each complex, CLV1 is part of a disulfide-linked multimer of ~185 kD. The 450-kD complex contains the protein phosphatase KAPP, which is a negative regulator of CLV1 signaling, and a Rho GTPase–related protein. In *clv1* and *clv3* mutants, CLV1 is found primarily in the 185-kD complex. We propose that CLV1 is present as an inactive disulfide-linked heterodimer and that CLV3 functions to promote the assembly of the active 450-kD complex, which then relays signal transduction through a Rho GTPase.

INTRODUCTION

Receptor-mediated signal transduction for a diverse array of developmental and other signaling events has been well characterized in animal and yeast systems; however, this process remains a mystery in plants. Despite the large number of potential receptors present in the genomes of Arabidopsis and other plants, only a handful of receptors corresponding to mutants with clear developmental defects have been isolated. The first such putative receptor cloned from Arabidopsis, *ERECTA*, encodes a receptor-like kinase (RLK) with a presumed extracellular domain composed of tandem leucine-rich repeats (LRRs) and a potential intracellular serine/threonine protein kinase domain (Torii et al., 1996). *ERECTA* appears to be required for proper organ elongation. *CRINKLY4*, which was cloned from maize, encodes an RLK that contains a predicted extracellular domain with both novel repeats and a region with sequence similarity to the tumor necrosis factor and a predicted intracellular protein kinase domain (Becraft et al., 1996). *CRINKLY4* is required for normal epidermal differentiation in the maize endosperm and leaf. The *CLAVATA1* (*CLV1*) gene from Arabidopsis is similar to *ERECTA* in structure, containing a predicted extracellular domain composed of 21 tandem LRRs and a predicted intracellular protein kinase domain (Clark et al., 1997). Similarly, the putative brassinosteroid receptor gene *BRI1* has been shown to encode an LRR RLK (Li and Chory, 1997).

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Proteins with similar structures have been shown to be involved in pathogen recognition as well. *XA-21* from rice, which provides resistance to *Xanthomonus oryzae* pv *oryzae* race 6, encodes a protein identical in overall structure to CLV1 (Song et al., 1995). The *Cf* family of genes from tomato provides resistance to various strains of *Cladosporium fulvum*, and the genes are similar to *CLV1* in that they encode proteins with predicted LRR extracellular domains; however, they lack intracellular kinase domains (Jones et al., 1994; Dixon et al., 1996). Another LRR RLK, PRK1 from petunia, may function in gamete development (Mu et al., 1994).

How these RLKs perform signal transduction has yet to be fully determined. Several have been shown to encode functional serine or serine/threonine protein kinases (Horn and Walker, 1994; Mu et al., 1994; Williams et al., 1997; Stone et al., 1998). The best-characterized feature of signaling is an interaction of a protein phosphatase, KAPP, with a subset of LRR RLKs. KAPP was identified as binding in a phosphorylation-dependent manner to the kinase domain of RLK5, which is very similar in sequence to CLV1 but of unknown function (Stone et al., 1994). KAPP was subsequently demonstrated to bind to a subset of the LRR RLKs (Braun et al., 1997) and shown to interact in vitro with CLV1 (Williams et al., 1997; Stone et al., 1998). Furthermore, KAPP coimmunoprecipitates with CLV1 from plant extracts (Stone et al., 1998), and both overexpression (Williams et al., 1997) and underexpression (Stone et al., 1998) of KAPP in transgenic plants indicate that KAPP is a negative regulator of CLV1 signaling.

Beyond the interaction of KAPP with a subset of LRR

RLKs, nothing is known about how these receptors function at the cellular level to perform signal transduction. It is unclear whether they form monomers, homodimers, or heterodimers. It is not known what type of ligand(s) interacts with these RLKs or whether ligand binding activates or inactivates signaling. It also is not known what proteins relay the signal from these receptors to downstream targets.

CLV1 signaling is an excellent system in which to address these questions. The phenotype of *clv1* mutants has been examined in detail, and the role of *CLV1* in regulating the balance between proliferation and differentiation at the shoot meristem is well defined (Leyser and Furner, 1992; Clark et al., 1993; Clark, 1997; Laufs et al., 1998). *clv1* mutants gradually accumulate undifferentiated cells at the shoot and flower meristem as the hypothesized result of the failure of these cells to commit properly to organ formation and eventual differentiation. A large allelic series of weak to strong mutant alleles with lesions in both the extracellular domain and kinase domain is available (Clark et al., 1993; Clark, 1997). There are also several other genes that function in the same developmental pathway as *CLV1*, and these may represent other components of the signaling pathway (Clark, 1997). *CLV3* is especially relevant, because *clv3* mutants exhibit phenotypes identical to those of *clv1* mutants, *clv3* dominantly enhances recessive and weakly semidominant *clv1* alleles, and *clv1 clv3* double mutants are indistinguishable from either single mutant (Clark et al., 1995). This evidence indicates that *CLV3* functions in the same pathway as *CLV1. CLV2* also functions in the same pathway as *CLV1* to regulate meristem development (Kayes and Clark, 1998).

A possible target for CLV1, the *WUSCHEL* (*WUS*) gene product, also has been identified. *wus* mutants have phenotypes essentially the opposite of *clv1*; namely, they fail to maintain undifferentiated cells at the shoot and flower meristems (Laux et al., 1996). *wus* mutants are also epistatic to *clv1* mutants, indicating that CLV1 signaling may inactivate the *WUS* gene product, which appears to be a homeodomain protein (Laux et al., 1996; Mayer et al., 1998).

We have sought to investigate how CLV1 signaling is activated in vivo and which proteins associate with CLV1 in response to activation. In this study, we demonstrate that CLV1 is part of a disulfide-linked multimer. We provide evidence that CLV1 is found in two distinct protein complexes. Assembly of the larger CLV1 protein complex, which includes the protein phosphatase KAPP and a Rho GTPase–related protein, requires functional CLV1 and CLV3 proteins. A model discussing the relative functions of the two complexes is discussed.

RESULTS

CLV1 Is Detected in Two Protein Complexes

We first sought to determine the potential association of CLV1 with other proteins in vivo. We have previously described the generation of CLV1-specific polyclonal antibodies (Stone et al., 1998). Antibody specificity was demonstrated by using as a control extract from the *clv1-6* allele, which contains a frameshift mutation that prevents translation of the epitope used for CLV1 antisera production. We then generated detergent-solublized extracts from the vegetable plant cauliflower. Cauliflower is an excellent source of large quantities of meristem tissue, because the cauliflower head is composed primarily of reiterative meristems. Cauliflower is closely related to Arabidopsis, and we have shown previously that both anti-CLV1 and anti-KAPP antibodies recognize appropriately sized proteins from cauliflower extracts (Stone et al., 1998). Expression studies suggest that CLV1 should be expressed in each of the meristems (see below). When cauliflower extracts were subjected to column chromatography and individual fractions were separated by SDS-PAGE and transferred for protein gel blot analysis, we observed two size populations of CLV1 cross-reacting proteins (Figure 1A). The first complex was ~185 kD, whereas the the second was ~450 kD. These sizes are based on comparisons to size standards (Figure 2).

To determine the specificity of these cross-reacting bands, we subjected pooled fractions from the 450- and 185-kD CLV1 complexes to immunoprecipitation by using both the immune and preimmune sera (Figure 1C). After separation by SDS-PAGE, only the proteins immunoprecipitated using the immune serum cross-reacted with the anti-CLV1 antiserum.

To quantify the relative amounts of CLV1 in each complex and to determine the reproducibility of our observations, we developed an ELISA assay using the anti-CLV1 antisera (see Methods). Fractions representing the 450-kD complex and the 185-kD complex from multiple experiments were pooled separately. Aliquots of each pool were tested at various concentrations for the amount of the CLV1 protein. We observed strong reproducibility, as shown in Figure 3. In cauliflower extracts, 65% of CLV1 was detected in the 450-kD complex and 35% in the 185-kD complex.

To determine whether the same complexes were present in Arabidopsis, we collected extract from wild-type Landsberg *erecta* (L*er*) inflorescence apices. We chose this tissue because previous work has demonstrated that *CLV1* mRNA is expressed in shoot and young flower meristems (Clark et al., 1997), and inflorescence apices are the richest source of these tissues. Again, we detected CLV1 in the two size populations, ~450 and ~185 kD, by using protein gel blot analysis (data not shown). ELISA analysis of pooled fractions revealed an identical distribution of CLV1 between the two protein complexes, as was observed from cauliflower extracts, with 65% in the 450-kD complex and 35% in the 185-kD complex (Figure 3).

We also used a dot blotting technique (see Methods) to detect the presence of CLV1 in fractions. By aliquoting fractions directly onto nitrocellulose, we significantly increased the sensitivity of our detection over SDS-PAGE followed by transfer to nitrocellulose. We detected signal on dot blots in

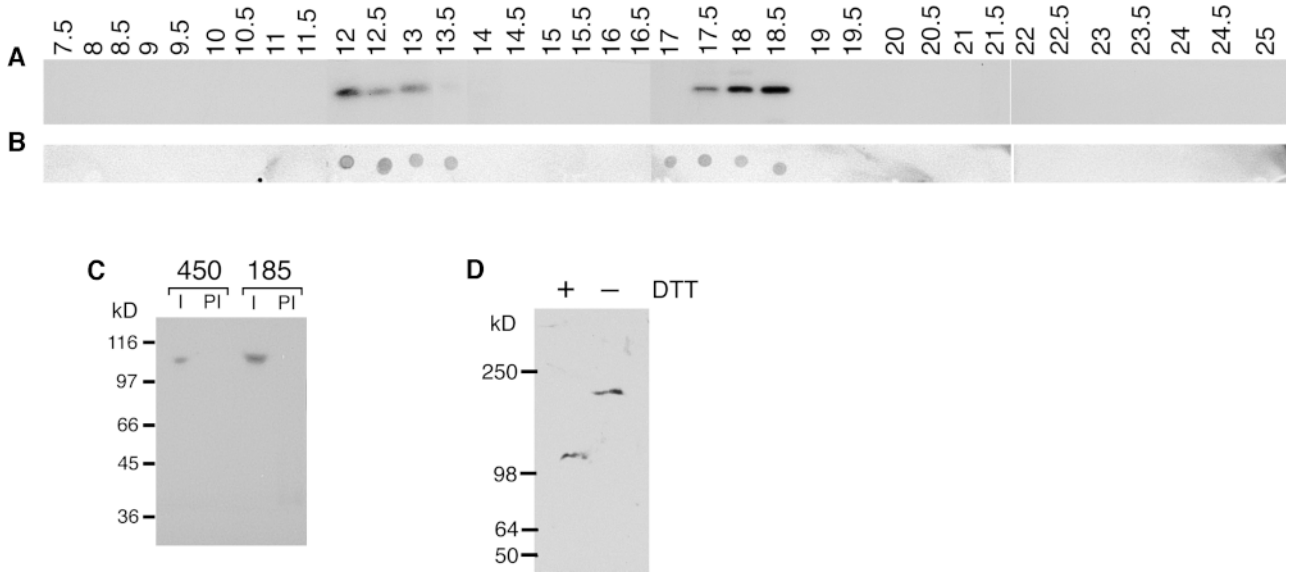

Figure 1. CLV1 Is Present in Two Protein Complexes.

(A) Fractions (0.5 mL) were collected from cauliflower extract separated by column chromatography (see Methods). Each fraction was separated by SDS-PAGE, and the presence of CLV1 was detected by protein gel blot analysis. Numbers refer to the elution volume of the corresponding fraction.

(B) Fractions were collected from wild-type Arabidopsis extracts, as given in **(A)**. The presence of CLV1 was detected by dot blot analysis (see Methods).

(C) Cauliflower fractions containing the 450- and 185-kD CLV1 complexes were pooled and subjected to immunoprecipitation using CLV1 immune (I) and preimmune (PI) sera. The precipitated proteins were separated by SDS-PAGE, and the presence of CLV1 was detected by protein gel blot analysis.

(D) CLV1 is part of a covalently linked multimer. Cauliflower extract was boiled in the presence (+) and absence (–) of the reductant DTT (see Methods). The samples were then separated by SDS-PAGE, and the presence of CLV1 was detected by protein gel blot analysis.

In **(C)** and **(D)**, numbers on the left indicate molecular mass markers in kilodaltons.

the same fractions that gave signal from SDS-PAGE followed by transfer to nitrocellulose (Figure 1B). To determine whether the signal on dot blots was due exclusively to cross-hybridization with the CLV1 protein, we used extract from the *clv1-6* allele for dot blot experiments and detected no signal, indicating that the observed signal from dot blots was due exclusively to CLV1 (data not shown).

CLV1 Is Present as a Disulfide-Linked Multimer

While establishing the conditions for column chromatography of CLV1, we observed that the addition of reducing agents such as DTT to the extraction or column buffer led to the elution of CLV1 exclusively at the monomeric size of ~105 kD. This raised the possibility that the 450- and 185-kD CLV1 complexes depend on covalent disulfide bonds between cysteine residues to retain their assembly. Interestingly, the large majority of plant receptor-like proteins with predicted extracellular LRR domains contain a pair of conservatively spaced cysteine residues both immediately before and after the LRRs (Jones and Jones, 1996).

To test whether the reducing agents were disrupting intramolecular bonds or intermolecular bonds, we boiled cauliflower extract in SDS both in the presence and absence of a reducing agent (DTT). Both samples were then separated by SDS-PAGE and transferred to a nitrocellulose membrane. The anti-CLV1 antisera detected CLV1 at ~105 kD in the sample boiled with DTT but at ~185 kD in the sample boiled without denaturant (Figure 1D). This suggests that CLV1 is linked by intermolecular disulfide bonds to another protein(s), giving a total mass of ~185 kD.

Formation of the 450-kD Complex Requires Functional CLV1 and CLV3

To determine whether mutations in CLV1 affected the ability of the protein to assemble into either of the complexes, we analyzed CLV1 size fractionation from extracts of *clv1-1*,

clv1-4, *clv1-8*, and *clv1-10* plants. The *clv1-4* and *clv1-8* alleles contain missense mutations in the LRR domain, and it had been speculated that these alterations disrupt ligand binding (Clark et al., 1997). Dot blot analysis indicated that the clv1-4 and clv1-8 proteins are found in three size populations: the 450- and 185-kD complexes and an ~105-kD complex, which is the predicted size of a CLV1 monomer (Figures 2 and 4B). ELISA analysis of *clv1-4* and *clv1-8* extracts revealed nearly equal amounts of protein in each complex (Figure 3), indicating an ~50% reduction in the amount of 450-kD complex compared with the wild type.

clv1-1 and *clv1-10* both contain missense mutations in the kinase domain. *clv1-1* exhibits an intermediate clv1 phenotype (Clark et al., 1993), whereas *clv1-10* exhibits a strong phenotype and is, in fact, an intragenic enhancer of *clv1-1* (Pogany et al., 1998). The biochemical activity of these mutants reflects their phenotype: the clv1-1 protein retains reduced kinase activity when expressed in *Escherichia coli*, whereas the clv1-10 protein exhibits no kinase activity (A.E. Trotochaud and S.E. Clark, unpublished data). When we examined the size fractionation of the mutant proteins, we observed a similar correlation between the mutant phenotype and an alteration in complex formation. *clv1-1* extracts exhibited a significant increase in the amount of protein observed in the 185-kD complex (78%) and a corresponding reduction in the amount of the 450-kD complex, whereas the clv1-10 protein was found exclusively in the 185-kD complex (Figures 2 to 4A).

We next sought to determine the role of CLV3 in the assembly of the CLV1 complexes. *CLV3*, as mentioned above, displays identical mutant phenotypes and functions in the same pathway as does *CLV1* (Clark et al., 1995). We have speculated that *CLV3* encodes the ligand for CLV1. We sought to determine whether CLV3 functions upstream or

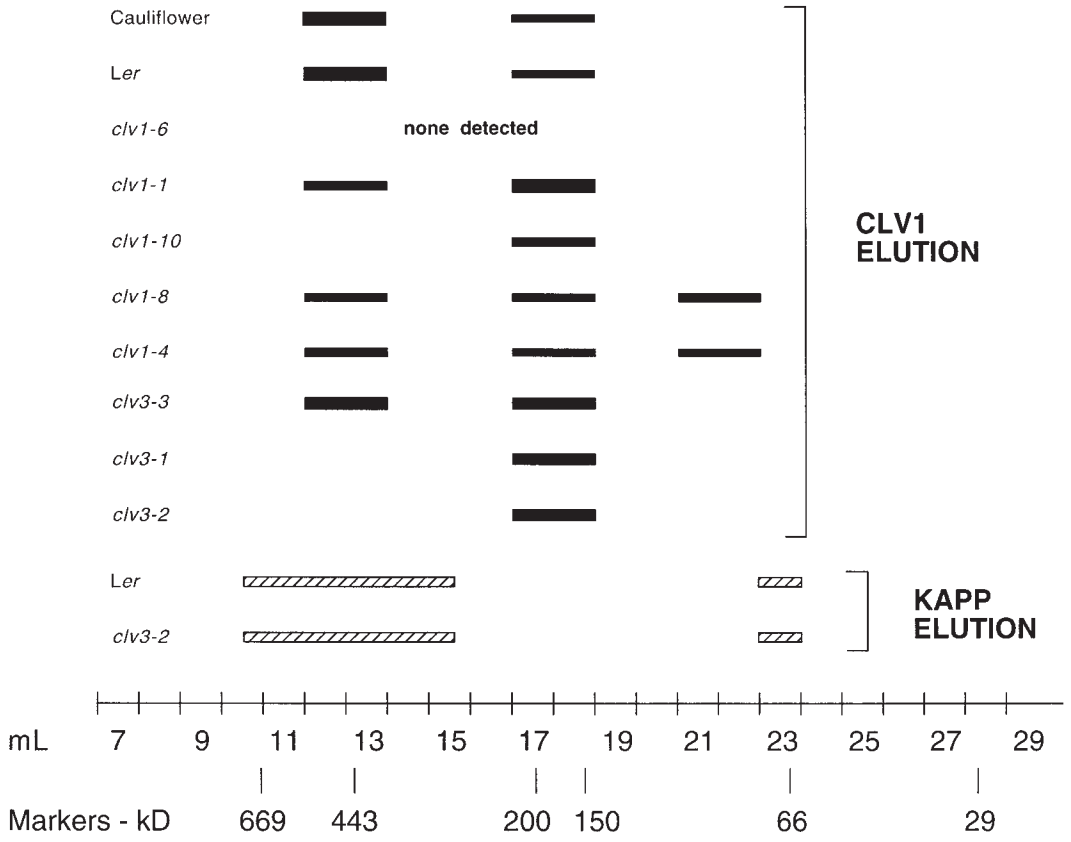

Figure 2. Elution of CLV1 and KAPP in Wild-Type and Various Mutant Backgrounds.

Extracts from cauliflower plants and wild-type (L*er*), *clv1-6*, *clv1-1*, *clv1-10*, *clv1-8*, *clv1-4*, *clv3-1*, *clv3-2*, and *clv3-3* Arabidopsis plants were subjected to column chromatography. Fraction numbers and the elution position of molecular mass markers are indicated at bottom in kilodaltons. In each 0.5-mL fraction, the presence of CLV1 was detected by using protein gel blot and/or dot blot analysis. For each genotype, the fractions in which CLV1 protein was detected is indicated by bars. The height of the bars indicates the relative amount of CLV1 protein present in that size population based on ELISA analysis (see Figure 3 and Methods). Fractions from wild-type and *clv3-2* extracts were also analyzed for the elution of KAPP protein (hatched bars).

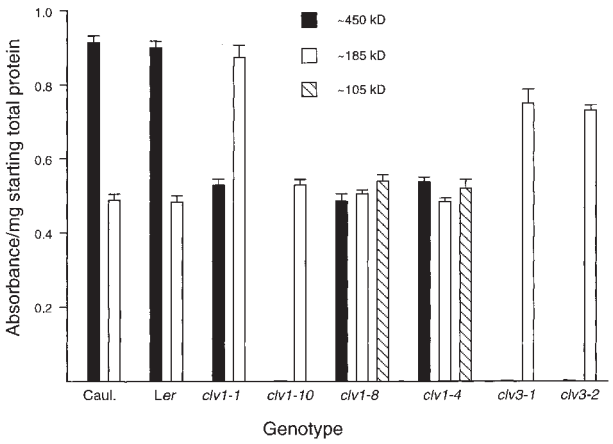

Figure 3. Relative Amounts of CLV1 in Various Complexes.

Extract was isolated twice each from wild-type (L*er*) and mutant Arabidopsis and cauliflower (Caul.) plants. A portion of each extract was separated twice by column chromatography. For each chromatographic separation, fractions corresponding to the 450- and 185-kD CLV1 complexes were pooled. For *clv1-4* and *clv1-8*, fractions corresponding to the predicted CLV1 monomer were pooled as well. Each pool was measured using an ELISA assay (see Methods) at three different concentrations, and the background was subtracted. Bars represent the absorbance averaged for the 12 readings (two extracts for two separations for three readings), with the standard error indicated. For *clv1-4*, only three fractionations were performed.

downstream of CLV1 by examining the effects of *clv3* mutations on CLV1 complex formation. If CLV3 functions upstream of CLV1 or at the same point, then it should alter the activation of CLV1, which could be observed as a change in the assembly of CLV1 into an active protein complex. If, on the other hand, CLV3 functions downstream of CLV1, then the biochemical activity of CLV1 should be unaffected. To test these hypotheses, we analyzed the size fractionation of CLV1 in *clv3-1* and *clv3-2* plants by using both dot blots and ELISA assays. For both alleles, CLV1 is found exclusively in the 185-kD complex (Figures 2 to 4C). We also examined CLV1 complex formation in the *clv3-3* mutant, which has a very weak phenotype and is likely a partial loss-of-function allele (data not shown). Based on ELISA assays of fractionated *clv3-3* extracts, CLV1 is equally distributed between the 450- and 185-kD complexes (Figure 2).

CLV1 mRNA Is Expressed in Mutant Apices

To determine whether the reduction of CLV1 protein levels observed in *clv3* mutants was the result of a reduction in *CLV1* mRNA expression, we examined *CLV1* transcript accumulation in *clv3* plants. In wild-type plants, *CLV1* mRNA is expressed in a broad group of cells in the center of the shoot meristem (Clark et al., 1997; Figure 5A). *CLV1* expression is restricted to the subepidermal layers of the meristem, primarily the L3 layer. In the flower meristem, *CLV1* expression is first detected at stage 2 and also in the central subepidermal cells. *CLV1* continues to be expressed until the

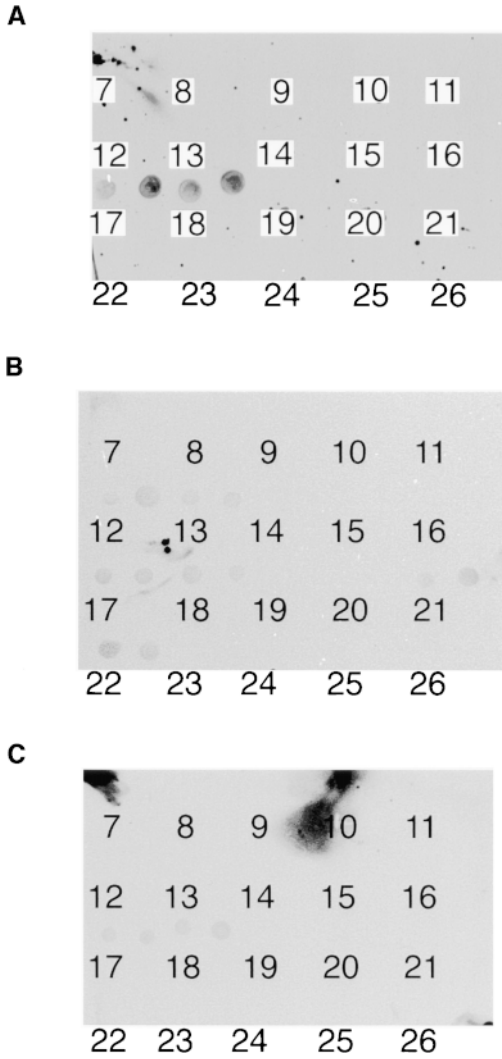

Figure 4. Alterations in CLV1 Complex Assembly in Mutant Backgrounds.

Extracts from plants were separated by gel chromatography. The presence of CLV1 in 0.5-mL fractions was determined by dot blot analysis. Note the absence of CLV1 in the 450-kD complex (fractions 12 to 13.5) in *clv1-10* and *clv3-2* plants, whereas a portion of the clv1-8 protein is found at monomeric size (fractions 21 to 22.5). Numbers on the blots represent fraction numbers (cf. with Figures 1 and 2).
(A) *clv1-10.*
(B) *clv1-8.*
(C) *clv3-2.*

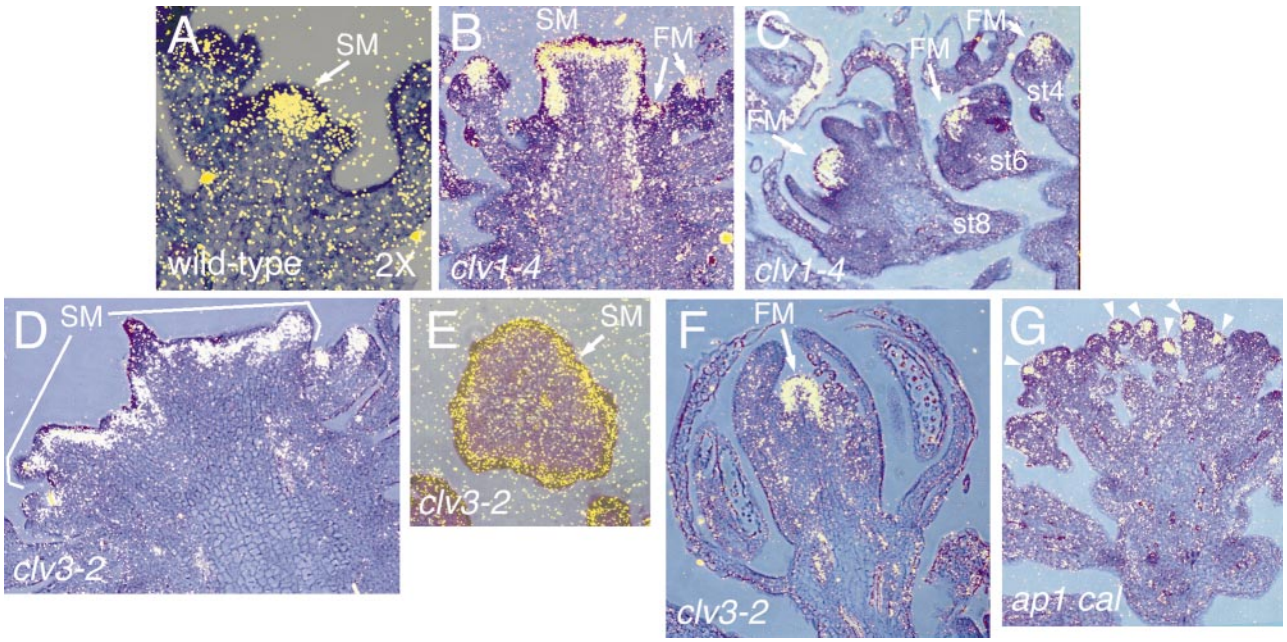

Figure 5. *CLV1* Is Expressed in Ectopic Meristems and throughout Enlarged Meristems.

RNA in situ hybridization using an antisense *CLV1* probe was performed as described by Clark et al. (1997) with wild-type and various mutant inflorescence tissue.

(A) Longitudinal section through a wild-type inflorescence shoot meristem.

(B) Longitudinal section through a *clv1-4* inflorescence shoot meristem.

(C) Section through the center of a stage 4 (st4), stage 6 (st6), and stage 8 (st8) *clv1-4* flower meristem (all stages according to Smyth et al., 1990).

(D) Longitudinal section through a *clv3-2* inflorescence shoot meristem. Note the gap in *CLV1* expression at the very center of the meristem.

(E) Transverse section through a *clv3-2* shoot meristem.

(F) Section through the center of a stage 12 *clv3-2* flower meristem.

(G) Section through an *ap1 cal* inflorescence. Arrowheads indicate meristems expressing *CLV1*; the other *ap1 cal* meristems exhibit *CLV1* expression in other section.

All images are combined bright-field and dark-field exposures, where the yellow grains indicate the signal from hybridization of the antisense *CLV1* probe. All images are shown at the same magnification, except in **(A)**, which is at twice (2×) the magnification relative to the other images. FM, flower meristem; SM, shoot meristem.

formation of carpel primordia in the center of the flower meristem at stage 6. *clv1* and *clv3* mutants form enlarged shoot meristems (Clark et al., 1993, 1995). When *CLV1* RNA in situ hybridizations were performed with enlarged *clv3* apices, we observed *CLV1* expression throughout the shoot meristem (Figures 5D and 5E). In irregularly shaped *clv3* meristems, we occasionally observed a gap in *CLV1* expression (Figure 5D). *clv3* plants develop flowers that retain a meristematic region throughout flower development, and *CLV1* expression was retained in these mutant flower meristems throughout their development (Figure 5F). A similar pattern of expression was observed in *clv1* plants (Figures 5B and 5C). *apetala1 cauliflower* (*ap1 cal*) double mutants form reiterative meristems in the place of flowers very similar to the vegetable cauliflower (Bowman et al., 1993). We detected *CLV1* expression in each of the *ap1 cal* meristems (Figure 5G). Thus, *CLV1* mRNA was expressed at levels equal to or greater than that of wild-type plants in the *clv1*, *clv3*, and *ap1 cal* mutants.

KAPP Is Associated with the 450-kD CLV1 Complex

The protein phosphatase KAPP is a likely candidate for a component of one or both of the CLV1 complexes. We and others have previously shown that KAPP and the CLV1 kinase domain interact in vitro and that this interaction requires an active CLV1 kinase domain, suggesting that KAPP specifically binds the phosphorylated form of CLV1 (Williams et al., 1997; Stone et al., 1998). We also have shown that KAPP and CLV1 coimmunoprecipitate from cauliflower extracts (Stone et al., 1998). We first generated polyclonal anti-

bodies to the kinase interaction domain of the KAPP protein. The resulting antisera recognized a single ~65-kD protein from Arabidopsis extracts, which is the predicted size of KAPP (Figure 6A). This cross-reactivity is lost when extracts from transgenic plants in which KAPP has been cosuppressed are used, indicating that the antibody is specific for KAPP (data not shown).

Using the anti-KAPP antisera, we examined the size fractionation of KAPP by using the same protocol used for size fractionation of CLV1. We observed that the majority of KAPP was found in a large size range from ~300 to ~670 kD, which includes but is much broader than the CLV1 450-kD complex (Figures 2 and 6C). A smaller portion of KAPP was found at the monomer size of KAPP, which is ~65 kD. These data suggest that the coimmunoprecipitation we previously observed was between KAPP and CLV1 in the 450-kD complex. To confirm that KAPP was specifically associated with the 450-kD CLV1 complex, we subjected the cauliflower extract to column chromatography, and fractions containing each of the CLV1 complexes were pooled separately. From each pool, CLV1 was immunoprecipitated using the anti-CLV1 antisera, and the immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and tested for the presence of KAPP protein by using the anti-KAPP antisera. As shown in Figure 6B, only the immunoprecipitation of the 450-kD complex produced cross-reactivity to the anti-KAPP antisera, indicating that KAPP is associated only with the 450-kD CLV1 complex.

The broad range of KAPP elution from 300 to 670 kD is consistent with the hypothesis that KAPP associates with a number of different RLKs. Work with KAPP by Walker and colleagues has demonstrated that KAPP will bind in vitro to a subset of LRR kinases (Braun et al., 1997). To test whether KAPP elution in the 300- to 670-kD range was due to association with RLKs other than CLV1, we examined the size fractionation of KAPP from extracts of *clv3-2* plants. KAPP was still found in the 300- to 670-kD size range, despite the fact that these plants lack the 450-kD CLV1 complex (Figures 2 and 6D). This suggests that the majority of KAPP association in the 325- to 600-kD range is independent of CLV1.

A Rho GTPase–Related Protein Is Associated with the 450-kD CLV1 Complex

Because we and others have demonstrated that KAPP is a negative regulator of CLV1 signaling (Williams et al., 1997; Stone et al., 1998), it was still unclear how CLV1 signal is relayed to downstream targets. Many animal receptor kinases associate with a small GTPase of the Ras superfamily to relay the signal to downstream targets (often a mitogen-activated protein kinase [MAPK] pathway). Although there is no evidence for a Ras ortholog in plants, they do have a family of small GTPases that fall into the Rho subfamily and that have been termed Rop (Winge et al., 1997; Li et al., 1998). Several Rops are expressed specifically in pollen tubes and have been shown to be required for pollen tube growth (Lin et al., 1996; Lin and Yang, 1997). An initial clue to a potential role for Rops in RLK-dependent signaling came from two-hybrid screens designed to identify Rop-interacting partners. Using Arabidopsis Rop1At as bait, we isolated two Arabidopsis genes encoding polypeptides with extensive similarity to serine/threonine protein kinases (data not shown). Thus, we sought to determine whether a Rop is possibly associated with CLV1. To do so, we used an affinity-purified polyclonal antibody generated against the Rop1Ps protein from pea (Lin et al., 1996). This antibody cross-reacts with at least five Arabidopsis Rop isoforms closely related to Rop1Ps and is likely to react with all Arabidopsis Rop isoforms (H. Li and Z. Yang, unpublished data). Cauliflower extract was immunoprecipitated with the anti-CLV1 antisera. The precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Using the anti-Rop antisera, we detected a strongly cross-reacting band at ~25 kD, the predicted size of Rop, indicating the presence of a Rop-like polypeptide in the precipitated proteins (Figure 7A).

To determine with which CLV1 complex the Rho-related protein was associated, we fractionated cauliflower extract by column chromatography, and the 450- and 185-kD complexes were isolated separately. Each complex was subjected to immunoprecipitation with the anti-CLV1 antisera and separated by SDS-PAGE for protein gel blot analysis. The anti-Rop antisera cross-reacted only with proteins precipitated from the 450-kD complex, suggesting that Rop is exclusively associated with the 450-kD complex (Figure 7B). This was confirmed by assessing the ability of CLV1 and Rop to coimmunoprecipitate from wild-type, *clv1-10*, *clv3-2*, and *clv1-4* extracts. We precipitated total extracts from each genotype with anti-CLV1 antisera and tested the precipitate for the presence of a Rho-related protein by protein gel blotting (Figure 7C). We did not detect CLV1/Rop coimmunoprecipitation in extracts from *clv3-2* and *clv1-10.* These mutants lack the 450-kD complex.

DISCUSSION

CLV1 Is Present in Two Distinct Complexes

We have observed that CLV1 is present in plant extracts in two protein complexes, one of 450 kD and one of 185 kD. A critical question is whether these correspond to bona fide complexes within the plant. Perhaps CLV1 is normally found only in the 450-kD complex, and the 185-kD complex is a breakdown product due to our purification scheme.

Several lines of evidence suggest that the latter possibility is not the case. (1) We repeatedly observed the same proportion of CLV1 in the 450- and 185-kD complexes (note the small error bars in Figure 3). This is true regardless of whether we used the extract fresh or subjected it to freezing

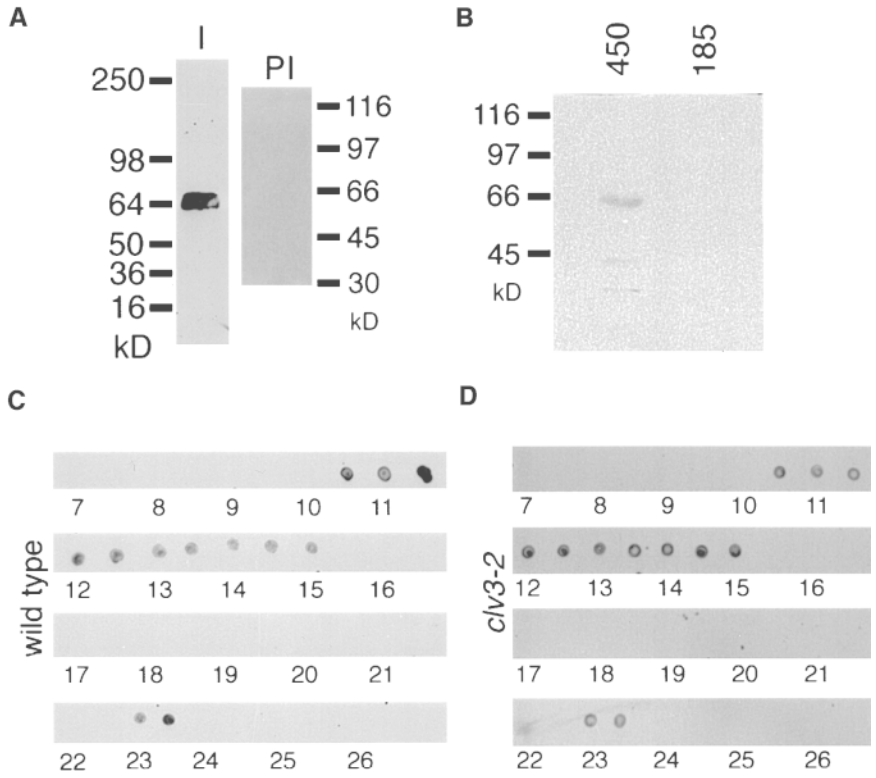

Figure 6. The 450-kD CLV1 Complex Includes KAPP.

(A) Immune antisera (I), but not preimmune sera (PI), which was produced to the kinase interaction domain of KAPP (see Methods), detected a single ~65-kD protein in extracts from cauliflower plants.
(B) Immunoprecipitation of the 450-kD complex, but not the 185-kD complex, using the anti-CLV1 antibody coprecipitated KAPP, as detected by protein gel blot analysis. The lower molecular mass bands may represent proteolytic degradation products of the full-length KAPP protein.
(C) and **(D)** Extracts from wild-type and *clv3-2* Arabidopsis plants, respectively, were separated by gel chromatography. The KAPP elution profile was identical in each genotype, as detected by dot blot analysis.
In **(A)** and **(B)**, numbers on the left and right indicate molecular mass markers in kilodaltons. In **(C)** and **(D)**, numbers represent fraction numbers (cf. with Figures 1 and 2).

and thawing (data not shown) or whether we isolated the extract from cauliflower or Arabidopsis. Each extraction requires a somewhat different purification protocol. (2) The nature of the lesions in various *clv1* mutant alleles corresponded well with their effects on the CLV1 complexes. *clv1-4* and *clv1-8*, both of which contain mutations in the LRR region, led to the accumulation of the monomeric clv1 protein. The effects of *clv1-1* and *clv1-10* mutations on the protein complexes correlated well with both their phenotypes and biochemical defects. *clv1-1*, which displays an intermediate phenotype and has reduced kinase activity, had a reduction in the proportion of the 450-kD complex, whereas the *clv1-10* mutant, which displays a strong phenotype and has no detectable kinase activity, exhibited a complete absence of the 450-kD complex. (3) The *clv3-1* and *clv3-2* mutants, which have wild-type CLV1 protein present, only had the 185-kD complex. In *clv3-1*, *clv3-2*, and *clv1-10* plants, there was no 450-kD complex to break down into a 185-kD complex. (4) There is tremendous precedence from animal systems of receptors associating with proteins only in response to a signal. Thus, if CLV1 acts as a receptor, then one would expect that some of the CLV1 protein would have been activated, whereas other CLV1 protein would have remained inactive. Taken together, these points provide a strong but indirect argument for the in vivo significance of the two complexes.

Complex Components

The 185-kD complex is entirely, or almost entirely, composed of CLV1 and another protein(s) linked together by disulfide bonds. We propose that the conserved cysteine pairs that flank the LRRs are involved in these intermolecular bonds. Given the conserved nature of the cysteine residues,

this would imply that a common feature of LRR RLK function in plants is disulfide dimerization or multimerization. The ethylene receptor ETR1, which shares similarity with the bacterial two-component sensors, is also a disulfide-linked dimer when expressed in yeast (Schaller et al., 1995). The S-linked glycoprotein, which is a secreted protein involved in pollen recognition in Brassica stigmas, also is found as a disulfide-linked dimer (Doughty et al., 1998). In animals, the type II transforming growth factor β receptor (also a receptor serine/threonine protein kinase) can form homodimers in the absence of ligands (Chen and Derynck, 1994; Henis et al., 1994), whereas the insulin growth factor receptor is found in a disulfide-linked tetramer (Cheatham and Kahn, 1995). One possibility is that the 185-kD complex represents a CLV1 homodimer or a heterodimer with a different receptor (Figure 8). A heterodimer may be more likely, because the size of the complex (~185 kD) is significantly smaller than the predicted size of a CLV1 homodimer (~210 kD). The 185-kD complex does not require CLV3 or a functional CLV1 kinase domain for assembly. However, alterations in the LRR region may reduce dimerization efficiency, as evidenced by the accumulation of clv1-4 and clv1-8 monomers.

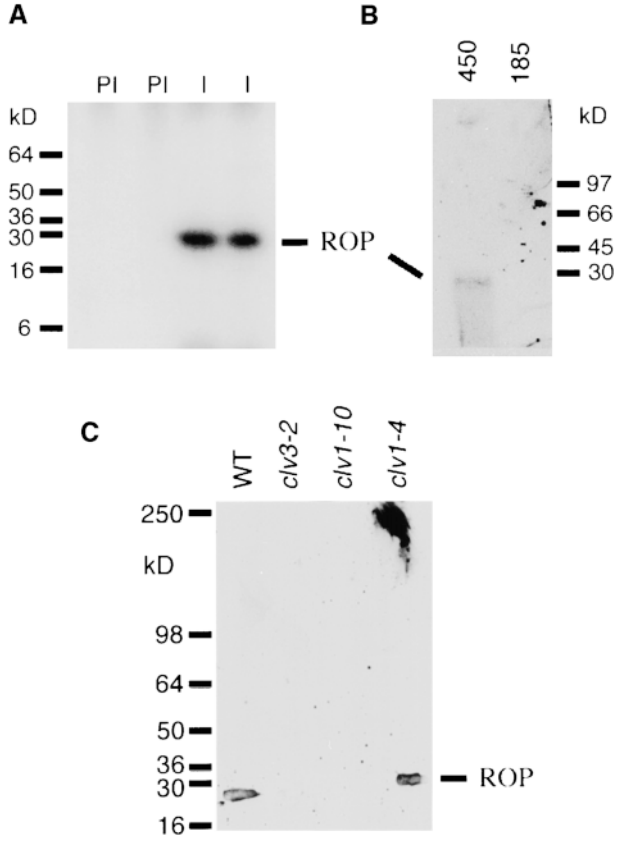

Figure 7. The 450-kD Complex Includes a Rho-Related Protein.

(A) Cauliflower extract was immunoprecipitated using immune (I) and preimmune (PI) anti-CLV1 sera. The precipitated proteins were subjected to protein gel blot analysis using affinity-purified anti-Rop1Ps antisera, which detected an ~25-kD protein (**[A]** and **[B]**) in immune reactions only. Each reaction was performed in duplicate.

(B) The 450- and 185-kD CLV1 complexes from cauliflower extract were separated by column chromatography before immunoprecipitation with anti-CLV1 antisera. Protein gel blot analysis of the precipitated proteins using the anti-Rop1Ps antisera reveals a cross-reacting protein only in the 450-kD complex.

(C) Extracts from wild-type, *clv3-2*, *clv1-10*, and *clv1-4* plants were immunoprecipitated using the anti-CLV1 antisera, and the precipitated proteins were subjected to protein gel blot analysis using the anti-Rop1Ps antisera.

Numbers to the left and right of blots indicate molecular mass markers in kilodaltons.

The 450-kD CLV1 complex appears to contain the same disulfide-linked CLV1 multimer that comprises the 185-kD complex. This conclusion is based on the observation that boiling total protein extract in the absence of reducing agent gives rise to a single ~185-kD covalently linked complex, suggesting that this disulfide-linked multimer is found in both complexes. The 450-kD complex also contains the protein phosphatase KAPP. Because KAPP binds specifically to the phosphorylated forms of various kinase domains, CLV1 in the 450-kD complex is likely phosphorylated and is likely not phosphorylated at the KAPP binding site in the 185-kD complex. Because KAPP is a negative regulator of CLV1 signaling (Williams et al., 1997; Stone et al., 1998) and has been shown to be capable of dephosphorylating CLV1 in vitro (Williams et al., 1997), a plausible scenario is that KAPP binds to CLV1 when phosphorylated (activated?) and functions to attenuate CLV1 signaling.

Another component of the 450-kD complex is a Rho GTPase–related protein, the plant subfamily of which has been termed Rop. Direct observation that this class of small GTPases is involved in receptor-mediated signal transduction in plants has not been reported previously, and neither has there been any direct evidence for the association of a Rho with an RLK in any system. In animals, the binding of Ras to RLKs is mediated by linker proteins (Simon et al., 1993). Although we show that a Rop-related protein is associated with the CLV1 complex, it remains to be determined whether a similar linker protein is involved. In fact, the 450-kD complex very likely contains additional proteins. If we use the sizes of the various known components and assume a constant stoichiometry, then the combined predicted size of the CLV1 disulfide-linked multimer (185 kD), KAPP (65 kD), and Rop (25 kD) is ~175 kD short of the 450-kD mass of the larger CLV1 complex. One possibility is that the ligand for the extracellular domain and linker protein(s) between CLV1 and Rop comprise a portion of the mass that is unaccounted for. Another possible component could be the *CLV2* gene product, because *CLV2* functions in the same pathway as *CLV1* (Kayes and Clark, 1998). There may even be additional proteins associated with either complex in vivo

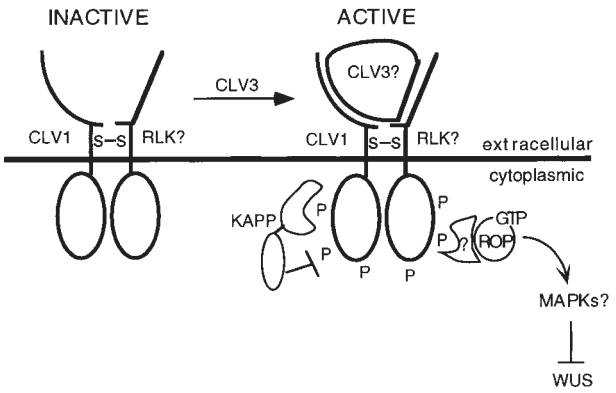

Figure 8. Model for the Composition and Function of the CLV1 Protein Complexes.

We propose that the 185-kD complex contains CLV1 disulfide linked (S-S) with another RLK in a heterodimer and that this complex is inactive. The model predicts that upon binding CLV3, the complex becomes active, which leads to phosphorylation of the kinase domains as well as association with downstream factors, including KAPP and Rop. The activation of Rop activates a signal cascade, perhaps via a MAPK pathway, to inactivate the WUS protein. Question marks indicate areas of uncertainty, as explained in the text.

that are not bound tightly enough to allow for stable association during our purification scheme.

Distinguishing the Active and Inactive Forms of CLV1

If CLV1 functions as a receptor that relays positional information, then one would expect that a portion of cells would be in a position to activate CLV1, whereas other cells would be in a position in which CLV1 was not activated. Thus, one would expect a portion of CLV1 protein to be in an active state and a portion to be inactive, corresponding to the different cellular populations, respectively. We propose that the 185-kD CLV1 complex represents the inactive form of CLV1, whereas the 450-kD complex represents the active form of CLV1 (Figure 8). Several features of the complexes are consistent with this hypothesis. (1) The 450-kD complex is significantly reduced or completely absent from plants carrying loss-of-function *clv1* alleles. (2) The 450-kD complex likely contains the phosphorylated form of CLV1 (see above), and kinase activity is required for CLV1 activity. (3) The putative downstream signaling factor Rop is associated only with the 450-kD complex. (4) Mutations in *CLV3*, which functions in the same pathway as does *CLV1*, lead to the reduction or complete absence of the 450-kD complex correlated with the severity of their phenotype.

If CLV1 functions on the flanks of the meristem, as we have inferred from our analysis of *clv1* mutants, then the cells on the flanks of the meristem should contain the active form of CLV1 (i.e., CLV1 should be in the 450-kD complex), whereas cells in the center of the meristem should contain the inactive form of CLV1. If this is the case, then some of the differences in the relative amounts of 450- and 185-kD complexes between the wild type and those mutants with both complexes (*clv1-1*, *clv1-4*, *clv1-8*, and *clv3-3*) may be the result of changes in the relative amounts of active and inactive cell types.

The Role of CLV3

In genetic terms, *CLV3* is required for *CLV1* function. Thus, we have speculated previously that *CLV3* could code for a component of the CLV1 signaling pathway. The evidence presented here suggests that CLV3 indeed functions in the CLV1 signaling pathway and that it functions either upstream or at the same point in the signaling pathway. This still leaves open several possible roles for CLV3. An obvious possibility is that *CLV3* encodes the ligand for CLV1 and that the binding of CLV3 to the 185-kD CLV1 complex leads to CLV1 activation, phosphorylation, and association with other signaling factors. A related hypothesis is that CLV3, although not the ligand, is required for the production or release of the ligand. Another possibility is that CLV3 is part of the 185-kD complex and is required for CLV1 function or for ligand binding. If this is so, then CLV3 would either have to be covalently linked to CLV1 or be very small, because the denaturation of the 185-kD complex does not significantly change its size. Antibodies raised to CLV3 could distinguish between these possibilities. These hypotheses would predict, respectively, that CLV3 is associated with the 450-kD complex only, is never associated with CLV1, or is associated with both the 185- and 450-kD complexes. These experiments await the identification of the *CLV3* gene.

Downstream Targets

Although we have no direct evidence for the identity of targets for CLV1 signal transduction, there are possible candidates based on precedents in other systems. Our observation that a Rop isoform may interact with the active form of CLV1 indicates that CLV1 may relay signal transduction through this small GTPase. In many cases, members of the Ras superfamily relay signals through a MAPK pathway (Lim et al., 1996). Plants contain a family of genes for each of the members of the MAPK pathway (Hirt, 1997) and even a Raf-like homolog that is involved in ethylene signaling (Kieber et al., 1993). Thus, the Rop GTPase could associate with the CLV1 kinase domain when CLV1 is activated via phosphorylation. An activated Rop could in turn activate a MAPK pathway. Demonstrating this mechanism for signaling will require identifying both the specific Rop isoform with which CLV1 interacts and potential MAPK component isoforms that are present within meristem tissue.

A Paradigm for LRR RLK Signaling in Plants

It is critical to establish an understanding of receptor signaling in plants to decipher the roles that the large family of RLK-encoding genes play in development, defense responses, and other processes. Given the conservation of many of the structural features of LRR receptor-like proteins involved in development and defense response, as well as those of unknown function, it is a strong possibility that these proteins share many common features of signal transduction. Thus, our observations that CLV1 forms an inactive disulfide-linked multimeric complex, which upon activation associates with KAPP and a Rho-related protein, may describe fundamental properties of LRR RLK signaling in angiosperms. A potential in vivo association of KAPP with RLKs other than CLV1, as suggested by both previous in vitro experiments (Braun et al., 1997) and our in vivo evidence (Figure 6), is consistent with this notion. A detailed analysis of a second RLK may do much to test this idea.

METHODS

Protein Extraction

Cauliflower (*Brassica oleracea*) meristem tissue (50 g) was ground in a prechilled blender (Waring) with 100 mL of extraction buffer (50 mM Hepes, pH 7.4, 10 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg mL^{-1} aprotinin, 10 μg mL^{-1} chymostatin, and 1 μg mL^{-1} leupeptin). The ground extract was filtered through one layer of Miracloth (Calbiochem, La Jolla, CA). The extract was then centrifuged (3000*g* for 10 min at 4°C) repeatedly until all flocculate was removed from the supernatant, which was then stored at −20°C. Before use, the extract was centrifuged at 100,000*g* for 1 hr at 4°C, and the supernatant was then filtered through a 0.2-μm filter.

Extract from plants (*Arabidopsis thaliana*) was generated in a similar fashion, with the following changes. Inflorescence apices from ~250 plants were placed in 15 mL of extraction buffer and disrupted in a homogenizer (Polytron; Brinkman Instruments, Westbury, NY) three times for 10 sec each at the level 4 setting.

Antisera Generation

Anti-CLV1 antisera were generated as described previously (Stone et al., 1998). For anti-KAPP antisera, an 861-bp fragment was amplified from a KAPP cDNA by using the KAPP Ab N1 (5′-CCCGGAAAGCCATGGCGATATTTTT-3′) and KAPP Ab C1 (5′-AATTCTAGATCATTTGATCTGAGGCCACACCAA-3′) primers. The resulting fragment was cloned into the pMalK vector (New England Biolabs, Beverly, MA) and expressed in *Escherichia coli.* The expressed protein was purified using amylose resin (New England Biolabs) and separated by SDS-PAGE. The excised protein bands were used for antisera production in rabbits (Cocalico Biologicals, Reamstown, PA).

The production and affinity purification of anti-Rop antisera were previously described (Lin et al., 1996).

Gel Filtration

Gel filtration was performed using a fast protein liquid chromatography system (Pharmacia) in HR 10⁄30 columns (Pharmacia) containing Superose 6 resin (Pharmacia) in 50 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, and 10% glycerol. Fractions were collected every 0.5 mL after the exclusion volume. All gel filtration was performed at room temperature.

Protein Gel Blot Analysis

Protein samples separated by SDS-PAGE were transferred to a nitrocellulose filter with a Bio-Rad semidry transfer cell for 20 min at 12 V. Nitrocellulose filters were blocked overnight in 2.5 g of dry milk per 100 mL of TBST (100 mM Tris, pH 7.54, 0.9% NaCl, and 0.5% Tween-20) and washed three times in TBST. Filters were incubated with 1:1000 dilution of KAPP UM206 antisera, CLV1 UM174 antisera, or anti-Rop antisera for 2 hr. Each filter was washed three times in TBST and incubated for 1 hr with a 1:30,000 dilution of BioRad goat anti–rabbit IgG horseradish peroxidase conjugate. Each filter was again washed three times in TBST and then incubated for 1 min in 2 mL of oxidizing reagent plus 2 mL of luminol reagent from a Super-Signal chemiluminescent kit (Pierce, Rockford, IL). Filters were then exposed to film for 1 to 2 min.

We also used a dot blot procedure in which we assayed for the presence of CLAVATA1 (CLV1) or KAPP by spotting 1 μL of each fraction onto nitrocellulose. The nitrocellulose was allowed to dry for 20 min. CLV1 and KAPP detection on the dot blot filters was performed in the same manner as for protein gel blots.

Disulfide Bond Detection

The gel filtration fractions containing the 450- and 185-kD complexes were pooled, and two 50-μL aliquots were removed. Fifty microliters of 2 × sample buffer (20% glycerol, 4% SDS, 30 mM Tris, pH 6.8, and 1% bromophenol blue) was added to both samples, and 1 mM DTT was added to only one sample. The aliquots were boiled for 5 min and separated on a 4 to 15% Tris-HCl polyacrylamide Ready Gel (Bio-Rad). The protein was transferred to nitrocellulose and used for protein gel blotting with the anti-CLV1 antibody.

ELISA Assay

The gel filtration fractions containing the 450- and 185-kD complexes were pooled separately and used as the antigen in an ELISA assay. Ninety-six-well ELISA plates (Corning; Corning, NY) were coated with 40, 50, 60, and 70 μL of antigen and incubated overnight at room temperature. After each step, the plates were rinsed three times with deionized water. Each well was filled with blocking buffer (0.017 M $Na_2B_4O_7{\cdot}10H_20$, 0.12 M NaCl, 0.05% Tween 20, 1 mM EDTA, 0.25% BSA, and 0.05% NaN_3) and incubated for 30 min at room temperature. The anti-CLV1 antibody was diluted to 50 ng/mL with blocking buffer, and each well was filled with 50 μL of the solution and incubated for 2 hr at room temperature. Protein A–alkaline phosphatase conjugate was diluted to 250 ng/mL in blocking buffer, and 50 μL was added to each well. The plates were incubated for 2 hr at room temperature. Seventy-five microliters of NPP substrate

solution (3 mM *p*-nitrophenyl phosphate, 0.05 M Na_2CO_3, and 0.05 mM $MgCl_2$) was added to each well and incubated for 4 hr at room temperature. The NPP hydrolysis was measured on a microtiter plate reader at 405 nm.

ELISA analysis of *clv3-3* extracts, along with a wild-type control, were performed as above with the following differences. After incubation with the anti-CLV1 antibody, goat anti–rabbit IgG horseradish peroxidase conjugate (Bio-Rad) was diluted 1:3000 in blocking buffer, and 50 μL was added to each well. The plates were incubated for 2 hr at room temperature. Equal volumes of TMB solution and peroxide solution from the ImmunoPure TMB substrate kit (Pierce) were combined according to the manufacturer's instructions, and 100 μL of the mixture was added to each well. One hundred microliters of 2 M H_2SO_4 was added to stop the reaction. The absorbance was read at 450 nm.

Immunoprecipitations

One hundred and eighty-five microliters each of CLV1 UM174 antiserum and preimmune serum were dialyzed to 0.1 M $NaHCO_3$/0.5 M NaCl in 10,000 molecular weight dialysis membrane. $A_{280\ nm}$ absorption was measured to determine protein concentration. Dialyzed serum was cross-linked to CnBr-activated Sepharose 4 Fast Flow beads (Pharmacia), according to the manufacturer's recommendations. Cross-linked beads were stored in TSA solution (0.01 M Tris, pH 8.0 [at 4°C], 0.14 M NaCl, and 0.025% NaN_3) at 4°C.

Fifty milliliters of cauliflower extract was incubated with ~5 mL of swelled (1 g dry weight) preimmune coupled beads for 2 hr at 4°C. The reaction was centrifuged at 1000*g* for 1 min at 4°C. Half of the supernatant was then incubated with immune beads for 2 hr at 4°C and the other half with fresh preimmune beads for 2 hr at 4°C. Both reactions were centrifuged at 1000*g* for 1 min, and the supernatant was removed. Beads were washed one time each in wash buffer (0.01 M Tris, pH 8.0, 0.14 M NaCl, 0.025% NaN_3, 0.5% Triton X-100, and 0.5% SDS) and Tris–Triton X-100–NaCl buffer (50 mM Tris, 0.1% Triton X-100, and 0.5 M NaCl), pH 8.0 and pH 9.0. The beads were eluted twice with 5 mL of triethanolamine solution (50 mM triethanolamine, pH ~11.5, 0.1% Triton X-100, and 0.15 M NaCl) into tubes containing 0.2 volumes of 1 M Tris, pH 6.7. Elutions were concentrated to ~100 μL in Centricon 3000 columns (Amicon, Beverly, MA), and 30 μL each of preimmune and immune elutions were separated by SDS-PAGE for protein gel blot analysis.

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