Arabidopsis RopGAPs Are a Novel Family of Rho GTPase-Activating Proteins that Require the Cdc42/Rac-Interactive Binding Motif for Rop-Specific GTPase Stimulation[1]

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The plant-specific Rop subfamily of Rho GTPases, most closely related to the mammalian Cdc42 and Rac GTPases, plays an important role in the regulation of calcium-dependent pollen tube growth, H_2O_2-mediated cell death, and many other processes in plants. In a search for Rop interactors using the two-hybrid method, we identified a family of Rho GTPase-activating proteins (GAP) from Arabidopsis, termed RopGAPs. In addition to a GAP catalytic domain, RopGAPs contain a Cdc42/Rac-interactive binding (CRIB) motif known to allow Cdc42/Rac effector proteins to bind activated Cdc42/Rac. This novel combination of a GAP domain with a CRIB motif is widespread in higher plants and is unique to the regulation of the Rop GTPase. A critical role for CRIB in the regulation of in vitro RopGAP activity was demonstrated using point and deletion mutations. Both types of mutants have drastically reduced capacities to stimulate the intrinsic Rop GTPase activity and to bind Rop. Furthermore, RopGAPs preferentially stimulate the GTPase activity of Rop, but not Cdc42 in a CRIB-dependent manner. In vitro binding assays show that the RopGAP CRIB domain interacts with GTP- and GDP-bound forms of Rop, as well as the transitional state of Rop mimicked by aluminum fluoride. The CRIB domain also promotes the association of the GAP domain with the GDP-bound Rop, as does aluminum fluoride. These results reveal a novel CRIB-dependent mechanism for the regulation of the plant-specific family of Rho GAPs. We propose that the CRIB domain facilitates the formation of or enhanced GAP-mediated stabilization of the transitional state of the Rop GTPase.

Existing as cycling GTP-bound "on" and GDP-bound "off" forms, G proteins are pivotal switches in eukaryotic signal transduction. Two major classes of signaling G proteins are known: heterotrimeric G proteins and the Ras superfamily of monomeric small GTPases. Among the five families within the Ras superfamily (RAS, RHO, RAB/YPT, ARF, and RAN), RAS and RHO GTPases are considered bona fide signaling proteins. In animals, trimeric G proteins, RAS, and RHO all play an important role in signaling. For example, mammals possess a large number of trimeric G proteins that are formed from the combinations of 20 α-, five β-, and seven γ-subunits (Sternweis, 1996), and thus more than one-third of mammalian pathways are dependent on trimeric G proteins (Sternweis, 1996). In contrast, only two Gα homologs, one Gβ homolog, and no RAS orthologs have been identified in plants (Ma et al., 1990; Weiss et al., 1994; Lee and Assmann, 1999). Loss-of-function Gα mutants in rice are retarded in stem elongation and have reduced seed sizes, but specific pathways controlled by this G protein are unclear.

Plants, however, possess a large family of RHO-related small GTPases termed Rop (Yang and Watson, 1993; Delmer et al., 1995; Winge et al., 1997; Li et al., 1998; Zheng and Yang, 2000b). Studies using constitutively active and dominant-negative rop mutants suggest a pivotal role for Rop in signaling to many important processes in plants, including tip growth, cell polarity formation, cell morphogenesis, H_2O_2 production and programmed cell death, cell wall synthesis, and probably hormone responses (Kawasaki et al., 1999; Potikha et al., 1999; Li and Yang, 2000; Zheng and Yang, 2000a). The role of Rop in pollen tube growth is best studied. Rop acts as a central switch in the pathway leading to tip growth in pollen tubes (Lin et al., 1996; Lin and Yang, 1997; Li et al., 1998, 1999; Kost et al., 1999; Zheng and Yang, 2000a, 2000b). Evidence suggests that Rop signaling controls the formation of tip-focused intracellular calcium gradient and tip-localized calcium entry in pollen tubes and may also control the organization of the actin cytoskeleton (Lin and Yang, 1997; Kost et al., 1999; Li et al., 1999). The tip-localized calcium signaling and the actin cytoskeleton are crucial for tip growth (Malhó et al., 1995; Yang, 1998; Franklin-Tong, 1999a, 1999b; Gibbon et al., 1999; Li et al., 1999). Phosphoinositol phosphate kinase and phosphoinositol 4,5-bisphosphate likely act downstream of Rop to control

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tip growth (Kost et al., 1999). Nonetheless, little is known about the signals that control Rop-dependent pathways and the mechanisms for Rop regulation and action.

In animals and yeast the RHO GTPase switch is controlled by multiple factors (Whitehead et al., 1997). The conversion of the inactive to active form is promoted by guanine nucleotide exchange factors (Whitehead et al., 1997). Following its activation of an effector, the active form returns to the inactive form through an intrinsic GTPase activity, which is stimulated by Rho GTPase-activating proteins (GAPs). Rho GAPs are indispensable for Rho-dependent signal transduction (Ridley et al., 1993; Ridley, 1994), e.g. mutations in the *round* locus encoding a Rac GAP cause cell death in imaginal disc and appendage shortening in *Drosophila* (Agnel et al., 1992). The importance of Rho GAPs in Rho signaling is also reflected by the large number and the structural diversity of Rho GAPs identified from animals and fungi (Lamarche and Hall, 1994). Various Rho GAPs are characterized by the presence of a GAP catalytic domain composed of three conserved subdomains (Lamarche and Hall, 1994; Lancaster et al., 1994). In addition, many Rho GAPs contain multiple signaling domains such as those involved in guanine nucleotide exchange, nucleotide binding, and protein kinase activity (Tan et al., 1993; Lancaster et al., 1994; Homma and Emori, 1995; Lamarche-Vane and Hall, 1998; Tatsis et al., 1998). Roles for these domains in GAPs are not clear.

There are hints that the mechanism for Rop signaling may be distinct from that for yeast and animal RHO GTPases. First, no homologs for conventional Rho guanine nucleotide exchange factors have been identified to date, even though >80% of the Arabidopsis genome has been sequenced. Instead, evidence suggests that Rop directly associates with and may be directly regulated by receptor-like Ser/Thr kinases (Trotochaud et al., 1999). Second, few homologs of RHO effectors found in yeast and animals are known in plants. Third, three lotus Rho GAP-like proteins with a novel structural feature (i.e. the presence of the Rho GTPase-binding Cdc42/Rac-interactive binding [CRIB] domain in their N-terminal region) were recently reported (Borg et al., 1999), and similar sequences are present in various plant expressed sequence tag (EST) and Arabidopsis databases. We have also identified several of these proteins in our yeast two-hybrid screen for Rop-interacting proteins.

In this paper we describe biochemical characterization of one of these Rho GAPs from Arabidopsis designated as RopGAPs. In vitro GAP assays show that RopGAPs specifically stimulate GTP hydrolysis of Rop GTPases, but not Cdc42 GTPases. This Rop-specific GTPase stimulation is dependent on the CRIB domain. Furthermore, the CRIB domain appears to facilitate the formation of or stabilize the transitional state of Rop GTPases. These results provide strong evidence that the signaling by the plant-specific Rop GTPase involves a unique GTPase regulatory mechanism.

RESULTS

Identification of Arabidopsis Genes Encoding Rho GAP-Like Proteins

To identify proteins that interact with the GTP-bound form of Rop, we used a constitutively active rop1At mutant (G15V) as bait in the yeast two-hybrid system (Li et al., 1999). Using this mutant to screen an Arabidopsis seedling library (Kim et al., 1997), we obtained 46 positive clones from approximately 6 million yeast transformants. Eight clones were sequenced and predicted amino acid sequences were used to search the GenBank database. Four of them encode amino acid sequences that exhibit significant similarity to Rho GAPs. These clones fall into three distinct genes designated as *RopGAP1*, *RopGAP2*, and *RopGAP3*.

Because none of the cDNA clones are full-length sequences we searched the Arabidopsis database for predicted complete coding sequences. *RopGAP1* is identical to the EST clone 142H15T7 and the hypothetical gene MWD9.12 encoding a predicted polypeptide of 466 amino acid residues. The *RopGAP1* clone obtained from the two-hybrid screen lacks the N-terminal 89 residues. The full-length sequence for the *RopGAP2* gene encoding a predicted polypeptide of 424 amino acid residues is found in the bacteria artificial chromosome (BAC) clone T4I9.2. *RopGAP2* from two-hybrid screen lacks the N-terminal 115 amino acids. Two overlapping cDNA clones obtained from the two-hybrid screen encode RopGAP3. The predicted RopGAP3 polypeptide shown in Figure 1A is derived from these two cDNA clones and the BAC clone T4A3.7. The BLAST search also identified two additional RopGAPs that we designate RopGAP4 (BAC clone F24K9.16) and RopGAP5 (BAC clone T27G7.4).

The alignment of the predicted amino acid sequences for the five RopGAPs shows several conserved structural domains (Fig. 1). The central region contains a GAP-like domain (residues P179–E341 in RopGAP1) that shares 70% identity among different RopGAPs and about 27% identity with various Rho GAP domains from animals and yeast (Fig. 1B). It contains the typical three subdomains in all Rho GAPs and it has the invariant Arg (residue 202 for RopGAP1) required for GAP catalytic activity (Rittinger et al., 1997; Leonard et al., 1998; Scheffzek et al., 1998). The GAP-like domain is most similar to the p50 rhoGAP, which preferentially activates Cdc42 GTPase (Lancaster et al., 1994).

It is interesting that all RopGAPs contain a CRIB motif found in several Cdc42/Rac effector proteins

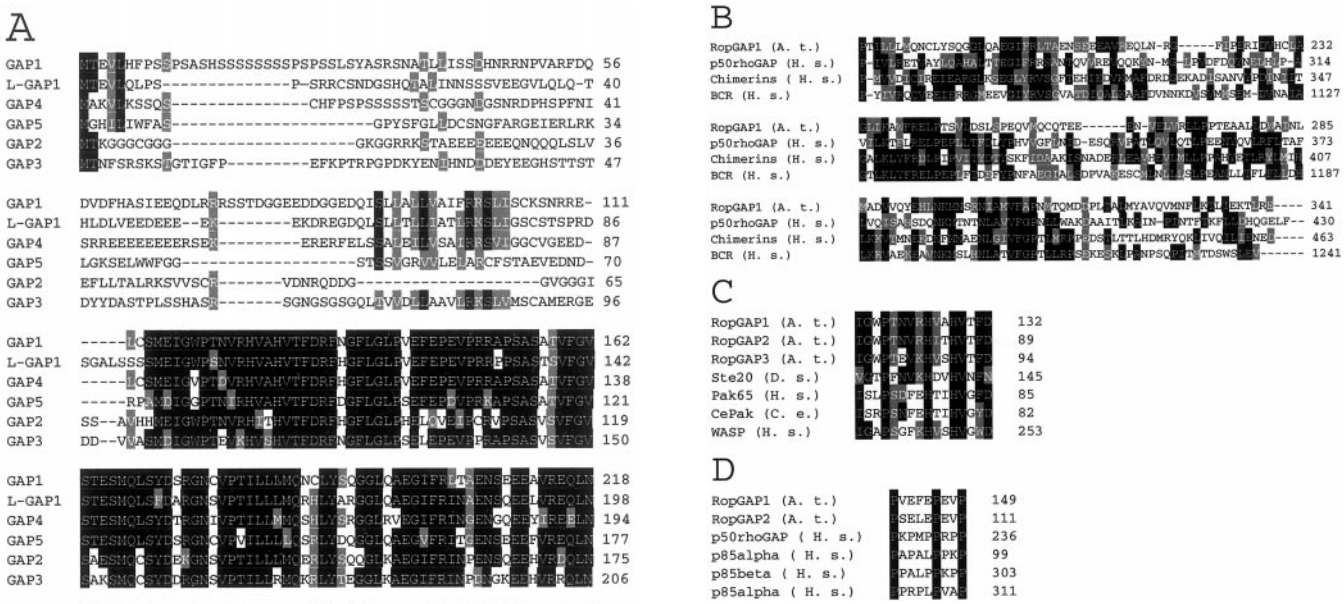

Figure 1. Comparison of predicted amino acid sequences between five RopGAPs and their conserved domains and motifs. Amino acid sequences for RopGAP1, RopGAP2, and RopGAP3 are predicted from a combination of two-hybrid clones, EST clones, and genomic sequences from the Arabidopsis database as described in text; RopGAP4 and RopGAP5 are annotated by the Arabidopsis Genome Sequencing Project. The lotus RopGAP is as described previously (Borg et al., 1999). Sequence alignment was performed by using the Clustal W program. The conserved motifs or domains shown were obtained from the GenBank database by using the BLAST search. A.t., Arabidopsis; D.d., *Dictyostelium discoideum*; S.c. *Saccharomyces cerevisiae*; H.s., human; C.e., *Caenorhabditis elegans*. A, Alignment of predicted amino acid sequences of RopGAPs. B, Alignment of the GAP-like domain from RopGAP1 with various Rho GAPs. C, Alignment of the CRIB-like motif with known CRIB motifs from Cdc42/Rac effector proteins. D, Alignment of src homology domain 3-binding motifs from RopGAPs and other signaling proteins.

(Burbelo et al., 1995; Fig. 1C). None of the known animal and fungal GAPs contains this motif. The CRIB motif and the GAP domain are joined by another conserved region having a consensus sequence for src homology domain 3-binding motifs (Pxxxx-PxxP or PxxPR; Ren et al., 1993; Fig. 1D). However, C- and N-terminal regions of RopGAPs are quite divergent among different RopGAPs. Three homologs of RopGAPs from lotus have been recently reported (Borg et al., 1999), and sequences related to RopGAPs are also present in rice and maize EST databases. RopGAP1 and lotus Rac GAP1 share the first and the last five amino acid residues in the N termini and C termini, respectively, suggesting that they might be orthologs.

CRIB Motif Enhances RopGAP-Mediated Stimulation of Rop GTPase Activity

We were interested in the potential function of the CRIB-like domain in RopGAPs because CRIB motifs are found in Cdc42/Rac effector proteins in yeast and animals (Burbelo et al., 1995). Mammalian CRIB motifs are known to inhibit Rho GAP activity by competing with GAPs by binding to Rho GTPases (Zhang et al., 1997). Thus we sought to determine whether the CRIB-like domain in RopGAPs has a similar role in inhibiting GAP activity. We examined the effects of CRIB domain deletion on the GTPase-stimulating activity of RopGAPs (Fig. 2A). The activation of Rop GTPase activity by RopGAP was determined by mea-

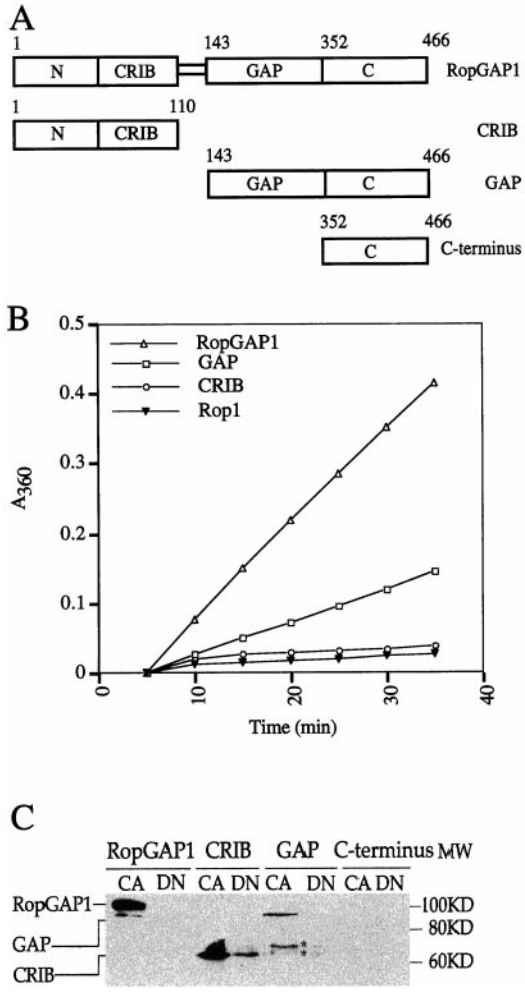

Figure 2. Effects of removing the CRIB domain from RopGAP1 on its GTPase stimulation and interaction with Rop1At. A, RopGAP1 and deletion mutants used in GAP assays. All deletion constructs except for CRIB are derived from RopGAP1. We used a CRIB domain from RopGAP3 because the corresponding region from RopGAP1 was unstable when expressed as a fusion protein with MBP in *Escherichia coli*. However, we have shown that the CRIB domain from either RopGAP has very similar properties of interaction with Rop1At using yeast two-hybrid interaction assays (data not shown). Numbers above each construct indicate amino acid residues as shown in Figure 1A. B, The activation of Rop GTPase by RopGAP1 and deletion mutants. The full-length RopGAP1 and deletion mutants shown in A were fused to the C terminus of MBP, and the fusion proteins were expressed in *E. coli*, purified through maltose-conjugated agarose, and used for GAP activity assays as described in text. GST-Rop1At fusion was used as a GTPase substrate for RopGAPs. Each reaction contains 500 nM MBP fusion proteins and 1 μM GST fusion proteins. The release of phosphate from the GST fusion proteins was monitored by spectroscopy every minute after the single turnover reaction was initiated. C, Analyses of RopGAP-Rop1At interactions by in vitro binding assays. The MBP fusion protein containing RopGAP1 and deletion mutants shown in A were used for in vitro binding assays to test their interactions with GST-Rop1At fusion proteins as described in text. The MBP fusion proteins were "pulled down" with GST-Rop1At fusion proteins that were bound to glutathione-conjugated beads, used for western blotting, and detected with anti-MBP antibodies. CA, Constitutively active rop1At mutant; DN, dominant negative rop1At mutant.

suring the rate of phosphate release from the GTP-bound glutathione *S*-transferase (GST)-Rop1At fusion protein in the presence of RopGAP fused with maltose-binding protein (MBPs). All of our GAP assays including those for various RopGAP1 mutants described below were repeated two to three times using the same preparation of *E. coli*-expressed and affinity-purified proteins. Consistent results were obtained in each repeat.

As shown in Figure 2B, GST-Rop1At fusion proteins have very weak intrinsic GTPase activity. However, 500 nM of the full-length RopGAP1-MBP fusion protein stimulates Rop1At GTPase activity by at least 70-fold within 30 min. Equal amounts of *E. coli*-expressed MBP protein by itself have no effects on Rop1At GTPase activity (data not shown). These results clearly demonstrate that RopGAP1 function as an active GAP for Rop GTPase, as expected from the presence of the Rho GAP-like domain. Similar results have also been shown for a lotus RopGAP (Borg et al., 1999).

It is surprising that a truncated RopGAP1 mutant lacking the CRIB domain had greatly reduced GAP activity on Rop1At compared with the full-length RopGAP1. This suggests that the N-terminal region containing the CRIB motif does not inhibit RopGAP activity, rather it is required for the full GAP activity of RopGAP1 on Rop1At. Furthermore, the CRIB-containing domain by itself had no GAP activity on Rop1At (Fig. 2B). Similar deletion mutations for RopGAP3 produced the same effects on GAP activity (data not shown). Because the N-terminal region outside of the CRIB motif has limited sequence similarity between RopGAP1 and RopGAP3, these results suggest that the CRIB motif in the N-terminal region is likely required for the full GAP activity in RopGAPs.

To confirm that the CRIB motif is critical for the regulation of GAP activity we created point mutations on the highly conserved His-125 residue within the CRIB motif of RopGAP1 (Fig. 3A). The corresponding residue has been shown to be critical for the interaction of Cdc42/Rac effectors with GTPases (Abdul-Manan et al., 1999; Mott et al., 1999). As shown in Figure 3B, a single His mutation (H125Y) caused dramatic reduction of GAP activity on Rop1 GTPase. A double mutation and several triple mutations within the CRIB motif produced a similar effect as the single mutation (data not shown). The extent of GAP activity reduction caused by the point mutations is less than that caused by the CRIB deletion mutants described above. This may be due to some protein degradation that occurred for the deletion mutant (see Fig. 2C described below) or an additional requirement of sequences outside of the CRIB motif in the N-terminal region for the full GAP activity. Nonetheless, these results clearly establish a critical role for the CRIB motif in the positive regulation of RopGAP activity.

We next asked whether the CRIB-dependent mechanism for GAP activity regulation is specific for the Rop subgroup of Rho GTPases or is general for different Rho GTPases. We investigated the ability of RopGAP1 to stimulate Cdc42 GTPase, because the GAP domain is most similar to p50 rhoGAP, which preferentially activates Cdc42 GTPase. As shown in Figure 4, the full-length RopGAP1 fusion protein had a weak GTPase-stimulating activity on a human Cdc42. MBP alone does not alter Cdc42 GTPase activity (data not shown). The removal of the CRIB-containing domain from RopGAP1 enhanced RopGAP1-mediated Cdc42 GTPase activation by at least 2-fold, whereas the deletion RopGAP1 mutant lacking the CRIB domain had the identical GAP activity on Cdc42 and Rop1At. Furthermore, the GAP domain, when mixed with an equal molar ratio of the CRIB domain, had identical GAP activity on Cdc42 as the full-length RopGAP1, indicating that the Arabidopsis CRIB motif competes with the GAP domain for binding to Cdc42, as the mammalian CRIB motif does (Zhang et al., 1998). Therefore, these results indicate that the CRIB domain provides a mechanism that underlies the specific stimulation of Rop GTPase activity by RopGAPs.

The CRIB Domain Interacts with GTP- and GDP-Bound Rop and Enhances Binding of RopGAPs to Rop

To gain insights into the mechanism by which the CRIB motif regulates RopGAP activity we investigated the role of the CRIB motif in the modulation of RopGAP-Rop interaction. We examined the interaction of Rop with the full-length RopGAP1 and different deletion mutants fused with MBP (see Fig. 2A). The MBP fusion proteins were "pulled down" with constitutively active or dominant-negative rop1At mutants fused with GST and detected with anti-MBP antibodies. Two micrograms of each fusion protein was used in this assay. Western-blot analyses using anti-Rop antibody or anti-MBP antibody confirmed that equal amounts of fusion proteins were used in each assay (data not shown).

As shown in Figure 2C, the full-length RopGAP1-MBP (approximately 105 kD) and GAP-MBP (approximately 90 kD) interact specifically with constitutively active rop1At, but not dominant-negative rop1At. In a similar manner, most mammalian Rho GAPs specifically interact with the GTP-bound Rho GTPases (Lamarche and Hall, 1994). However, GAP-MBP has a drastically lower capacity to interact with constitutively active Rop1At than the full-length RopGAP1-MBP does. In the GAP-MBP reaction constitutively active rop1At was also associated with two lower M_r proteins, which probably resulted from partial degradation of the GAP-MBP fusion protein. It is surprising that CRIB-MBP interacts with constitutively active and dominant-negative rop1At mutants, although the interaction with dominant-negative rop1At is somewhat weaker. This finding is in contrast with the CRIB motif in Cdc42/Rac effectors, which specifically bind the active form of GTPases (Manser et al., 1994; Burbelo et al., 1995). The C-terminal divergent region lacking the GAP and the CRIB domain did not bind either form of Rop1At. Furthermore, GST alone does not interact with any of the MBP protein fused with the full-length RopGAP or any truncated versions, demonstrating that GST does not contribute to the interaction of Rop1At with RopGAPs (data not shown). Our results from yeast two-hybrid interaction assays are consistent with these in vitro binding assays (data not shown).

To confirm that the CRIB motif is responsible for the interaction between CRIB-MBP and rop1At, we examined in vitro binding of constitutively active rop1At to RopGAP1 mutants in which the specific conserved residues within the CRIB motif are mutated (see Fig. 3A). As shown in Figure 3C, the H125Y point mutation in the CRIB motif drastically reduced the Rop-RopGAP interaction, as did a combination of two or three point mutations within the CRIB motif (data not shown). These mutants interact with Rop only somewhat better than the GAP domain lacking the N-terminal region. Western-blot analyses using anti-MBP antibody confirmed that equal amounts of each MBP fusion protein were used in the assay (Fig. 3C). The difference in Rop-binding capacity between the deletion and point mutations may be due to protein instability for the deletion mutant or a requirement for additional sequences within the N-terminal region of RopGAPs. It is important to note that the reduced interaction is tightly correlated with reduced GAP activity in these mutants (see Figs. 2B and 3B). These results suggest that the CRIB motif is required for the Rop-specific stimulation of RopGAP activity through its enhancement of RopGAP binding to Rop.

The CRIB Domain of RopGAPs Binds to the Transitional State of Rop GTPases

How is the CRIB-mediated interaction of RopGAPs with Rop involved in the stimulation of RopGAP activity? Our observation that the CRIB-containing domain of RopGAP1 interacts with both GTP- and GDP-bound forms of Rop1At suggests that this domain might bind to the transitional state of Rop GTPases. This is supported by our two-hybrid interaction assays showing that the CRIB domain interacted with the wild-type Rop1At much more strongly than with the constitutively active rop1At mutant (data not shown). To further test this hypothesis we created a transitional state from GDP-bound Rop1At using aluminum tetrafluoride (Scheffzek et al., 1997; Hoffman et al., 1998). As shown in Figure 5, treatments with aluminum tetrafluoride allowed RopGAP3 and the GAP domain to interact

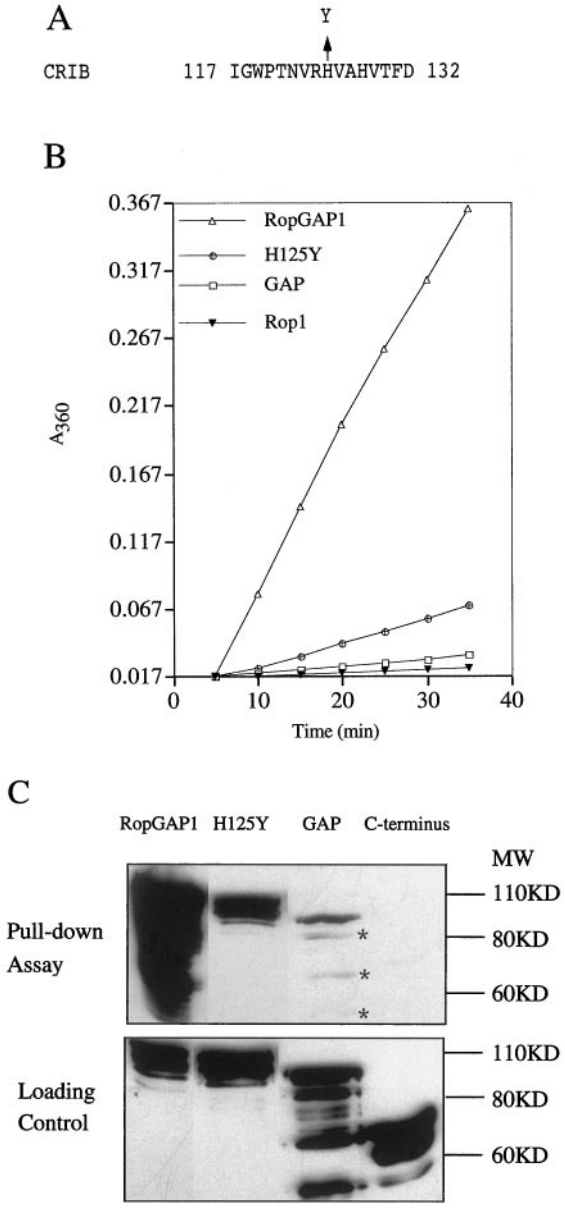

Figure 3. Effects of point mutations within the CRIB motif of RopGAP1 on its GTPase stimulation and interaction with Rop1At. A, The conserved His-125 residue within the CRIB motif was replaced with Tyr by site-directed mutagenesis as described in text. The point mutation (H125Y) was generated from the MBP-RopGAP1 fusion construct (see Fig. 2A). B, For GAP activity assays, 350 nM of MBP-RopGAP1 and mutant fusion proteins was used in each GTP hydrolysis reaction. C, Binding of the RopGAP1(H125Y) mutant with the constitutively active rop1At was compared with the full-length RopGAP1 and its deletion mutant lacking the CRIB domain (see Fig. 2A). In vitro binding and GAP activity assays were performed as described in Figure 2; however, a different protein preparation and 350 nM of MBP fusion proteins were used instead. Western-blot analyses using anti-MBP antibody (bottom) confirmed that equal amounts of each MBP fusion protein were used in the pull-down assays. These analyses also indicate that the GAP domain is partially degraded and that some degraded forms remain capable of interacting with Rop1At. Lane 1, RopGAP1; lane 2, H125Y; lane 3, GAP; lane 4, C terminus.

with the GDP-bound rop1At. This was expected because GAPs are known to bind the transitional state of small GTPases. In a similar manner, the aluminum tetrafluoride treatment dramatically enhanced the interaction of the GDP-bound rop1At with the CRIB domain, indicating that the CRIB domain indeed binds the transitional state of Rop. The addition of an equal molar ratio of the GAP domain reduced the interaction of the CRIB domain with the GDP-bound rop1At (lanes CRIB + GAP), suggesting that the GAP domain associates more strongly with the transitional state of Rop than the CRIB domain does. It is interesting that the CRIB and GAP domains associated with the GDP-bound rop1At synergistically in the absence of aluminum tetrafluoride, implying that the CRIB domain either is able to create a transitional state or stabilize the interaction of the GAP domain with the transitional state.

DISCUSSION

Our in vitro studies clearly indicate that RopGAPs belong to a unique class of Rho GAPs that may act as negative regulators in Rop GTPase signaling. Furthermore, these studies reveal a CRIB motif-dependent novel mechanism for the regulation of Rho GAPs and a role for this mechanism in defining substrate specificity for Rho GAPs. To our knowl-

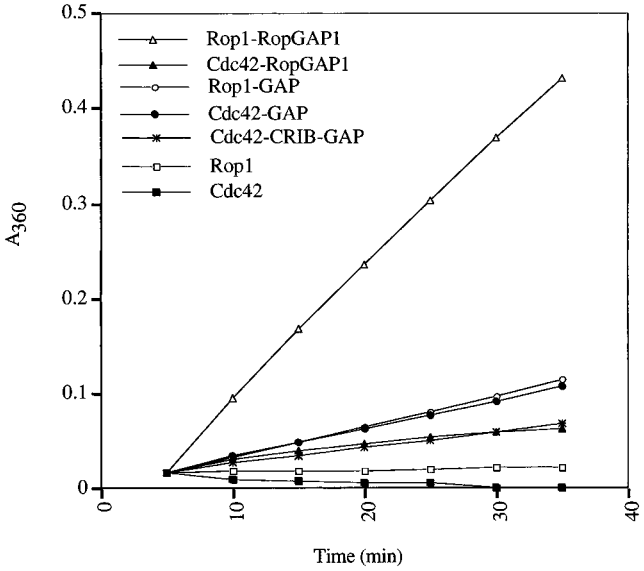

Figure 4. Comparison of GTPase stimulation by RopGAP1 and deletion mutants between Rop1At and Cdc42. GAP activities of RopGAP1 and deletion mutants were compared between Rop1At and Cdc42 using GTP hydrolysis assays. Rop1At and human Cdc42 fused to GST and RopGAP1 and its deletion mutants fused with MBP were isolated and used for GAP activity assays as described in Figure 2.

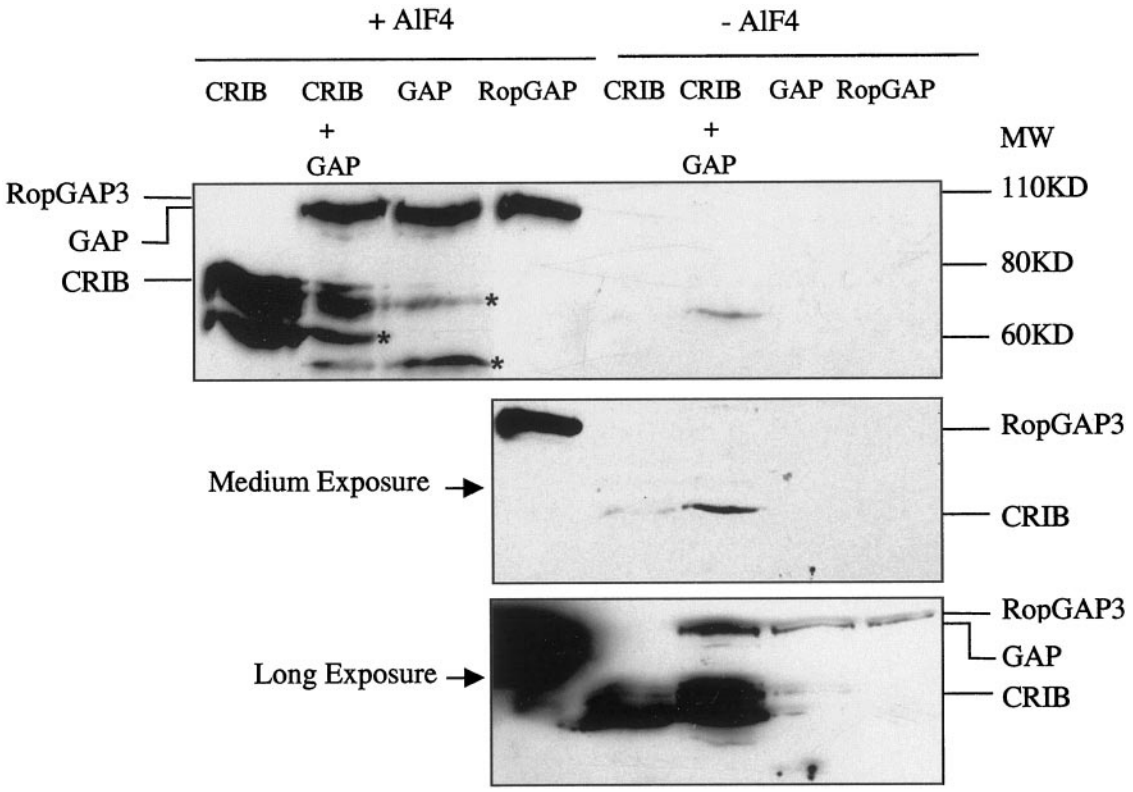

Figure 5. Effects of aluminum tetrafluoride on the interaction between Rop and RopGAP. To determine the interaction of RopGAP and its deletion mutants with the transitional state of Rop, the dominant negative rop1At mutant was used for in vitro binding experiments in the presence ($+AlF_4$) or absence ($-AFl_4$) of 20 mM aluminum tetrafluoride. The binding assays were as described in Figure 3C except for the addition of AlF_4 to the binding buffer. All fusion proteins except for RopGAP3 fused with MBP are described in Figure 2. Near full-length (residues 16–388) RopGAP3 was used in this assay for comparison with the CRIB domain, which is derived from RopGAP3 (see Fig. 2). Bands marked with an asterisk are most likely degraded forms of the GAP domain. Similar products were observed when the interaction was performed using the constitutively active rop1At mutants (see Figs. 2C and 3C). Because signals for the CRIB domain treated with AlF_4 were higher by several orders of magnitudes than the signals for the reaction without AlF_4, three different exposures of the film were necessary to compare these signals. A short exposure is shown in the top, and medium and long exposures are shown in the middle and bottom, respectively. Only lane 4 through lane 8 are shown for the longer exposure. The long exposure also detected an extremely weak signal for the GAP domain and RopGAP3 in the absence of AlF_4.

edge, this report is the first to demonstrate a role for CRIB motifs in the regulation of GAPs.

RopGAPs Belong to a Novel Family of Rho GAPs Containing a CRIB Motif

All five Arabidopsis RopGAPs share a unique structural characteristic that is critical for the Rop-specific regulation of GAP activity, i.e. the presence of a CRIB motif near the GAP domain in their N-terminal region. RopGAPs with similar structural features are also found in other plant species such as lotus (Borg et al., 1999), rice, and maize (EST databases). Many Rho GAPs possess multiple signaling domains besides the GAP domain (see "Introduction"). However, RopGAPs are the only GAPs known to contain a CRIB motif.

Cdc42/Rac effectors such as Ste20, $p65^{PAK}$, $p120^{ACK}$, and WASP are the only other group of proteins known to contain CRIB motifs, which mediate the specific interaction of effectors with Cdc42/Rac GTPases in a GTP-dependent manner (Burbelo et al., 1995; Symons et al., 1996; Tapon and Hall, 1997). The CRIB motif in these Cdc42/Rac effectors binds to the effector domain of the GTP-bound Cdc42 or Rac. The Rho GAP domain also binds to the effector domain of GTPases in a GTP-dependent manner, and thus mammalian CRIB motifs inhibit GAP activity by competing with the GAP domain for the effector-binding site (Zhang et al., 1997). We found that the CRIB domain of RopGAPs similarly inhibits the RopGAP-stimulated GTP hydrolysis by Cdc42 (Fig. 4). Thus a possible explanation for the presence of the CRIB motif in RopGAPs is that the CRIB-containing N-terminal region functions as a Rop effector. Another explanation is that the CRIB motif has a role in RopGAP regulation. Although our current studies could not rule out a possible effector functioning for RopGAP through the CRIB motif, our results provide concrete evidence that the CRIB motif acts as a pos-

itive regulator of GAP activity in RopGAPs as discussed below.

The CRIB Motif Defines the Specificity of RopGAPs by Regulating the Activity of the GAP Catalytic Domain

We have shown that RopGAPs preferentially stimulate GTPase activity on the Rop subgroup of Rho GTPases, but only weakly promote GTP hydrolysis catalyzed by Cdc42. These results indicate that RopGAPs are Rop-specific GAPs, similar to several mammalian Rho GAPs, e.g. *n*-chimearin specifically stimulates GTPase activity of Rac, but not Cdc42 or Rho, and p50rhoGAP preferentially activates Cdc42 and TC10 (Lancaster et al., 1994; Leung et al., 1998; Neudauer et al., 1998). The molecular and structural basis for member-specific activation of GTPase for these Cdc42 and Rac GAPs is unknown (Lancaster et al., 1994).

Our studies provide convincing evidence that the CRIB motif plays an essential role for the Rop-specific stimulation of GTP hydrolysis by RopGAPs because deletion of the CRIB motif or mutations of conserved residues within this motif abolishes or dramatically reduces Rop-specific stimulation of GTPase activity by RopGAPs. For the reasons summarized below we believe that the elimination or dramatic reduction of Rop-specific GAP activity in these mutants is not due to instability of the GAP domain or disruption of its active three-dimensional structure. First, western-blot analyses show that all of the mutant GAP proteins used for GAP assays, except for the CRIB domain deletion mutant, did not show significant degradation. Although the deletion mutant showed some degradation, it could not have accounted for the drastic reduction of GAP activity. Second, the CRIB deletion mutant had a greater GAP activity on Cdc42 than the full-length RopGAPs, demonstrating that this mutant retains the GAP catalytic activity. Third, compared with the point mutations within the CRIB motif, the removal of the N-terminal region containing this motif caused similar effects on GAP activity, although the effect was somewhat weaker. This suggests that the three-dimensional structure of the GAP domain in RopGAPs is independent of the N-terminal region. That the modification of RopGAP activity by a CRIB-dependent mechanism does not involve altering the structure of the GAP catalytic domain is consistent with the fact that the CRIB domain directly binds different forms of Rop GTPases to alter the interaction of Rop with RopGAPs as discussed below. Because the CRIB-containing domain has no GTPase-stimulating activity on its own we conclude that the CRIB motif functions to regulate the activity of the GAP catalytic domain. It is possible that additional sequences in the N-terminal region of RopGAPs are also involved in the regulation of the GAP activity.

CRIB Motif Allows High-Affinity Binding of RopGAPs to Rop and Facilitates the Stabilization of the Transitional State of Rop GTPases

How does the CRIB motif mediate the Rop-specific activation of RopGAPs? One role for the CRIB motif in RopGAPs is to enhance their affinity for the GTP-bound form of Rop, as suggested by our results showing that CRIB deletion and point mutations dramatically reduce the interaction of RopGAPs with the GTP-bound Rop (see Figs. 2C and 3C). The reduced interaction of various RopGAP mutants with the active Rop is tightly associated with their reduced GAP activity on Rop, suggesting that the CRIB-mediated RopGAP-Rop interaction may be critical for the regulation of RopGAP activity. Furthermore, with the aid of the CRIB motif, RopGAPs would have a much greater capacity to compete with Rop effectors for binding to the Rop effector domain. It is known that Rho GAPs and effectors bind the effector domain of Rho GTPases, and that Rho effectors are known to inhibit Rho GTPase activity (Manser et al., 1994; Zhang et al., 1997). Thus the CRIB motif in RopGAPs is expected to be critical for an effective in vivo de-activation of Rop.

How can the CRIB motif-dependent interaction of RopGAPs with Rop contribute to the regulation of RopGAP activity? Our results strongly support the hypothesis that the CRIB domain regulates GTPase activity of RopGAPs via its interaction with the transitional state of Rop GTPase during GTP hydrolysis. This was first hinted by two observations: (a) That the CRIB domain interacts much more strongly with wild-type Rop1At, which likely exists in different (GTP- or GDP-bound and transitional) forms, than with constitutively active or dominant-negative rop1At mutant; and (b) that the CRIB domain interacts with constitutively active and dominant-negative rop1At mutants, whereas the GAP domain specifically interacts with the constitutively active rop1At. More importantly, we showed that the binding of the CRIB domain to the GDP-bound Rop is dramatically enhanced by aluminum fluoride. Aluminum fluoride is known to enhance the binding of the GAP catalytic domain to GDP-bound Rho GTPases by mimicking the GTPase transitional state (Vincent et al., 1998). Based on the crystal structure of the complex of the aluminum fluoride-mimicked GTPase transitional state and the GAP domain, it is proposed that Rho GAPs stimulates GTPase activity through stabilizing the transitional state of GTPases (Vincent et al., 1998). The interaction of the CRIB domain with the Rop transitional state is further supported by the ability of the GAP domain to compete with the CRIB domain for their interaction with the GDP-bound Rop in the presence of aluminum fluoride (Fig. 5). It is interesting that the CRIB and GAP domains of RopGAPs bind the GDP-bound rop1At synergistically in the absence of aluminum fluoride. These results imply that the CRIB domain promotes the formation of the

transitional state, which is then stabilized by the GAP domain, or facilitates the GAP-mediated stabilization of the transitional state. Thus it is conceivable that the CRIB motif could regulate the GAP activity via either of these two mechanisms. Resolution of the three-dimensional structure of RopGAPs and elucidation of the mechanism for the CRIB-Rop interaction should further our understanding of the mechanism by which the CRIB motif regulates RopGAP activity.

MATERIALS AND METHODS

Materials and Chemicals

The yeast two-hybrid screen system including an Arabidopsis seedling cDNA library constructed in the prey vector pACT, the bait vector pAS2, and the yeast strain Y190 was obtained from The Ohio State University Arabidopsis Biological Resources Center (Columbus). Oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). DNA sequencing was performed on an automated sequencer (model 373A, ABI, Foster City, CA). All chemicals, unless specified, were purchased from Sigma (St. Louis).

Construction of Constitutively Active and Dominant-Negative Rop1At Mutants

Dominant *rop1At* mutant genes were generated by site-directed mutagenesis as described previously (Li et al., 1999). In brief, a *Nco*I site was created at the translation initiation codon of the Rop1At coding sequence (Li et al., 1998) using PCR. The modified coding sequence was then cloned into pSelect for site-directed mutagenesis to create constitutively active (G15V) and dominant negative (D121A) mutations, respectively. An isoprenylation-defective mutation (C188S) was subsequently introduced into each of the dominant mutants by PCR. These rop1At mutants were used for the two-hybrid screen or in vitro binding assays described below.

Two-Hybrid Screen

The constitutively active rop1At mutant (G15V/C188S) was fused into the C terminus of GAL4 DNA-binding domain encoded in pAS2. The resulting construct pDP1S was used as a "bait" to screen the Arabidopsis seedling library (Kim et al., 1997). The yeast strain Y190 containing pDP1S was transformed with the library plasmid DNA (Ausubel et al., 1998). The transformants were selected on a yeast drop-out (-His-Trp-Leu) synthetic dextrose medium supplemented with 100 mM 3-aminotriazole. After incubation at 30°C for 1 to 2 weeks, the plates were used for the β-galactosidase filter assay to detect positive clones (Breeden and Nasmyth, 1985). The plasmids from putative positive clones were first rescued in *Escherichia coli* and then reintroduced into the yeast strain containing pDP1S to confirm the interaction. The confirmed clones were then sequenced, and the sequences were used for the Blast search of the GenBank or the Arabidopsis database.

Site-Directed Mutagenesis of RopGAP1

To create point mutations on RopGAP1, we used PCR-based site-directed mutagenesis. The MalE primer (5′-ggtcgtcagactgtcgatgaagcc) and a mutant primer containing H125Y mutation (5′-gatacgtagcgcacgttacctttga) were used to amplify a mutant *RopGAP1* fragments from pMAlc2-RopGAP1. This mutation also creates a *Sna*B1 site in the fragment. The PCR fragment was subcloned into *Bam*HI (a vector cloning site) and PmlI (compatible with the *Sna*B1 site) to replace the corresponding wild-type fragment in pMAlc2-RopGAP1. Other mutations including H125YH128R, P120G&H125Y, P120G&H125Y&T130G, and P120G&H125Y&H128R were similarly generated. The effect of these mutations on RopGAP activity and interaction of Rop is very similar to that of the single site mutation (H125Y) and thus is not described in this report.

Fusion Protein Preparations

The constitutively active mutant, dominant-negative mutant, or wild-type *Rop1At* gene was cloned in frame with the GST gene in pGEX-KG using *Nco*I and *Sst*I sites (Guan and Dixon, 1991). GST-Cdc42 fusion is a gift from Y. Zheng (Li et al., 1997). GST fusion proteins were purified using glutathione-agarose beads. In brief, *E. coli* cells were lysed by sonication in a HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer [50 mM HEPES, pH 7.5, 150 mM $NaCl_2$, 1 mM EDTA, 1 mM 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 200 mM phenylmethylsulfonyl fluoride, 0.1% (w/v) β-mercaptoethanol, and 0.1% (w/v) Triton-100]. Following centrifugation (10,000*g*, 10 min, 4°C), the supernatants of lysates were mixed with glutathione-agarose beads, which were washed five times with 10 mL of HEPES buffer. Fusion proteins were eluted with 10 mM glutathione solution in the HEPES buffer. Protein concentrations were measured by using Dot Metric protein detection kit (Geno Technology, Inc., St. Louis). Purified proteins were routinely stored in 40% (w/v) glycerol at −20°C.

The full-length RopGAP1 coding sequence and various deletion mutants (see Fig. 2A) were fused in frame with the MBP gene in pMAL-c2 vector (New England Biolab, Beverly, MA). MBP fusion proteins were expressed in *E. coli* and purified using a similar procedure described above for the GST fusion proteins except that the amylose resin (50%, v/v) was used instead of glutathione-agarose beads. The MBP fusion proteins were eluted with a 10 mM maltose solution in the HEPES buffer.

GTPase Activity Assay by Spectroscopy

GTPase activity assays were performed by using Enzcheck TM Phosphate Assay Kit (E-6646, Molecular Probes, Eugene, OR) to monitor the rate of the phosphate release

from GTP bound to the GST-Rop1At fusion protein. For these assays, we used a previously described protocol with minor modification (Li et al., 1997). In brief, 1 nmol of purified GST-Rop1At fusion protein in a volume of 15 μL was mixed in an 1-mL crystal cuvette with 10 μL of 0.2 mM GTP, 0.2 mL of 2-amino-6-mercapto-7-methylpurine ribonucleoside, 10 μL (1 unit) of purine nucleotide phosphorylase, and 0.78 mL of HEPES buffer (pH 7.5). The cuvette was immediately placed in the spectrophotometer (AT UNICAM UV/VIS Spectrometer UV4) to monitor A_{360}. When the multiple turnover reached an equilibrium, 5 μL of 1 M $MgCl_2$ solution containing MBP-RopGAP fusion proteins were added to initiate the single turnover reaction. The A_{360} was recorded every 5 min. The data were standardized to the same starting point.

In Vitro Protein-Protein Interaction Assays

For in vitro binding assays, approximately 10 μg of GST-Rop1At fusion proteins in the glutathione-agarose beads were mixed with a binding buffer {50 mM Tris [tris(hydroxymethyl)-aminomethane], pH 7.5, 10 mM $MgCl_2$, 1 mM 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 10 mg/mL bovine serum albumin, and 5 mM EDTA} containing 3 mM GTP or GDP. Following a 30-min incubation with shaking at 30°C, the beads were aliquoted into four parts. Each part was mixed with 2 μg of each of the four different GAP-MBP fusions (Fig. 2A) in the binding buffer and incubated at 4°C for 2 h. The beads were washed with the binding buffer for four times. The proteins associated with the agarose beads were resuspended in 10 μL of SDS-PAGE loading buffer, separated on a 10% (w/v) PAGE-SDS gel by electrophoresis, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The MBP-fusion proteins were then detected using a polyclonal antibody against MBP (New England Biolab) and the BM Chemiluminescence Western Blot Kit (Boehringer Mannheim, Basel).

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