Structural Basis for the Interaction between the Potato Virus X Resistance Protein (Rx) and Its Cofactor Ran GTPase-activating Protein 2 (RanGAP2)*

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Plants have evolved a sophisticated innate immune system to defend themselves against pathogens (1). This includes effector-triggered immunity, wherein intracellular disease resistance (R)3 proteins specifically recognize pathogen effector proteins and trigger a strong resistance response, often leading to a localized cell death response known as the hypersensitive response (HR) (2, 3).

The major R proteins are of the nucleotide-binding (NB) and leucine-rich repeat (LRR) type (NB-LRR), defined by their central NB site and a C-terminal LRR domain. They belong to the Nod-like receptor family of intracellular immune receptors in plants and animals (4). The NB region of R proteins is highly conserved and shares structural and functional similarities with the metazoan apoptosis factors Apaf-1 and CED4 (4). The C-terminal LRR domains of R proteins are highly variable and are implicated in determining recognition specificity (5, 6).

Two structurally different classes of NB-LRR proteins exist, encoding N-terminal domains that either share homology with the TIR (Toll/interleukin-1 receptor) cytoplasmic domain (TIR-NB-LRR class) or have a predicted coiled-coil (CC) domain (CC-NB-LRR class) (7). The role of the N-terminal domains of R proteins is still poorly understood. They were originally thought to act in signal initiation, and many TIR domains are independently capable of inducing resistance responses (8, 9). The N-terminal domains of a distinct family of NB-LRR proteins, encoding atypical CC domains with homology to RPW8 (CCR-NB-LRRs), are also able to induce defense responses (10). However, to date, the CC domain of only one canonical CC-NB-LRR, that of MLA10, has been shown to independently induce defense responses (11). Several proteins termed cofactors have been shown to interact with the N termini of CC-NB-LRR proteins, including the following interactions: RIN4 with RPM1 and RPS2, PBS1 with RPS5, BS1 with R2, Pto with Prf, ZED1 with ZAR1, and RanGAP2 (Ran

The potato (Solanum tuberosum) disease resistance protein Rx has a modular arrangement that contains coiled-coil (CC), nucleotide-binding (NB), and leucine-rich repeat (LRR) domains and mediates resistance to potato virus X. The Rx N-terminal CC domain undergoes an intramolecular interaction with the Rx NB-LRR region and an intermolecular interaction with the Rx cofactor RanGAP2 (Ran GTPase-activating protein 2). Here, we report the crystal structure of the Rx CC domain in complex with the Trp-Pro-Pro (WPP) domain of RanGAP2. The structure reveals that the Rx CC domain forms a heterodimer with RanGAP2, in striking contrast to the homodimeric structure of the CC domain of the barley disease resistance protein MLA10. Structure-based mutagenesis identified residues from both the Rx CC domain and the RanGAP2 WPP domain that are crucial for their interaction and function in vitro and in vivo. Our results reveal the molecular mechanism underlying the interaction of Rx with RanGAP2 and identify the distinct surfaces of the Rx CC domain that are involved in intramolecular and intermolecular interactions.

The potato virus X (PVX) coat protein; ITC, isothermal titration calorimetry; StRanGAP, S. tuberosum RanGAP.
GT-pase-activating protein 2) with the potato Rx and Rx-like proteins R2x and Gpa2. RIN4, PBS1, BS1, Pto, and ZED1 also interact with the effectors of their cognate CC-NB-LRR interaction partners (12–18). Thus, these CC-interacting proteins are likely to mediate recognition rather than signaling (7).

Previous studies have suggested that at least two intramolecular interactions play roles in maintaining NB-LRR proteins in an autoinhibited state: interaction between the CC and NB-LRR moieties and between the CC-NB and LRR moieties (19, 20). The only well conserved motif within canonical CC domains is the EDVID motif, known to be involved in binding to the NB-LRR region (20). The crystal structure of the MLA10 CC domain shows that it forms an antiparallel homodimer that mediates the self-association of MLA10, the latter being required for its function (11). However, it is not known whether homodimerization is a general feature of all CC-type R proteins.

The potato (Solanum tuberosum) Rx protein has a typical CC-NB-LRR modular arrangement (see Fig. 1A) and mediates resistance to potato virus X (PVX) through recognition of the PVX coat protein (CP) (21, 22). The Rx CC domain does not independently trigger resistance responses when overexpressed in plants, but its interaction with RanGAP2 is required for Rx function (20). Deletion or mutation of the EDVID motif abolishes the intramolecular interaction between the CC and NB-LRR fragments but does not affect the interaction between the Rx CC domain and its cofactor RanGAP2. In contrast, mutations in the Rx CC domain that disrupt its interaction with RanGAP2 do not affect the intramolecular interaction. These results suggest that the two interactions are mediated by different surfaces (20).

RanGAP2 is required for Rx-mediated resistance responses to PVX (15, 16) and interacts with the Rx CC domain through its N-terminal WPP domain, which is named after a conserved three-residue Trp-Pro-Pro motif that plays a role in nuclear envelope localization (23, 24). Although direct interaction between RanGAP2 and the PVX CP has not been detected, it has been proposed that RanGAP2 might nonetheless interact transiently or indirectly with the CP, bringing it into proximity with the Rx LRR, which would in turn recognize the CP through direct binding or through detecting an alteration of RanGAP2 (7). This is supported by artificial tethering experiments with RanGAP2, the Rx-like protein Gpa2, and its cognate effector RBP-1, which suggested that RanGAP2 might function by facilitating interactions between Rx-like proteins and effector proteins (25).

Here, we report the crystal structure of the Rx CC domain in complex with the RanGAP2 WPP domain at a resolution of 2.1 Å. The structure of the Rx CC domain is strikingly different from that of the previously described MLA10 CC domain. Unlike the homodimeric MLA10 CC domain, the Rx CC domain forms a heterodimer with the RanGAP2 WPP domain. The interfaces between these two molecules are formed primarily by hydrophobic interactions. Using in vitro and in vivo mutational analyses, we experimentally demonstrate a significant role for Rx Trp-90 and RanGAP2 Ala-89 in this interaction. A structural comparison demonstrates that the Rx CC domain possesses distinct surfaces for intramolecular versus intermolecular interactions. These two surfaces are in close proximity, which suggests the potential for these two interactions to mutually affect each other.

### EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—The expression and purification methods used in this study have been described elsewhere in detail (26, 27). In summary, the Rx CC domain (residues 1–122) and the RanGAP2 WPP domain (residues 1–112), as well as mutant versions thereof, were subcloned into pGEX-6P-1 (GE Healthcare) and pET-30a (Novagen), respectively, and coexpressed in *Escherichia coli* BL21(DE3) cells by overnight induction at 16 °C. The proteins were purified by glutathione-Sepharose 4B (GE Healthcare) affinity chromatography. After removal of the GST tag by PreScission protease (GE Healthcare Life Sciences), the proteins were further purified by anion-exchange column (Source 15Q) and gel-filtration (Superdex 200) chromatography. The gel-filtration column had been pre-equilibrated with a solution of 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Fractions corresponding to the CC-WPP complex were pooled and concentrated to ~10 mg/ml for crystallization.

**Isothermal Titration Calorimetry (ITC)**—A direct binding affinity between the Rx CC domain (residues 1–122) and the RanGAP2 WPP domain (residues 1–112) was measured using ITC. The two corresponding DNA fragments were subcloned into pGEX-6P-1 and then expressed and purified separately as described above. Approximately 0.2 mM Rx CC was titrated against 10 μM RanGAP2 WPP using a MicroCal VP-ITC microcalorimeter. Both protein domains were prepared in buffer containing 25 mM HEPES (pH 8.0) and 100 mM NaCl. Data were collected at 30 °C and analyzed using Origin data analysis software (MicroCal). The binding parameters were as follows: *n* = 1.08 ± 0.0063 sites, *K* = 2.51 × 10^7 ± 9.27 × 10^6, Δ*H* = −2619 ± 24.4 cal/mol, and Δ*S* = 25.2 cal/mol/degree.

### TABLE 1

| Data collection and refinement statistics for the Rx CC-RanGAP2 WPP complex | Native | Selenium |
|---|---|---|
| **Data collection** | | |
| Space group | *P*2₁ | *P*2₁ |
| Cell dimensions | | |
| a, b, c (Å) | 74.487, 90.194, 87.674 | 74.085, 91.142, 87.762 |
| Resolution (Å) | 2.18 (2.26) | 3.15 (3.23) |
| *R*merge (%) | 5.3 (38.7) | 8.6 (23.9) |
| I/σ(I) | 27.7 (2.88) | 27.9 (3.59) |
| Redundancy | 3.6 (3.0) | 6.8 (4.3) |
| **Refinement** | | |
| Resolution (Å) | 28.65–2.10 | |
| Completeness (%) | 98.3 | |
| No. of reflections | 65.379 | |
| Rmerge/Rfree (%) | 24.8/27.2 | |
| No. of atoms | 7464 | |
| Protein | 7290 | |
| Water | 174 | |
| r.m.s.d. | | |
| Bond lengths (Å) | 0.007 | |
| Bond angles | 1.050° | |
| Ramachandran plot | Most favored regions (%) | 98.98 | |
| Additionally allowed regions (%) | 1.02 | |
| Outliers (%) | 0.00 | |
| PDB ID | 4M70 | |

* r.m.s.d., root mean square deviation; PDB, Protein Data Bank.
Crystallization and Data Collection—Crystallization for the CC-WPP complex was determined from a sparse matrix screen (Hampton Research). For crystallization, the protein solution was supplemented with trypsin to a final concentration of 10–20 μg/ml prior to screening, which was found to strikingly improve the diffraction of the CC-WPP complex crystals. Screening was performed using hanging drop vapor diffusion by combining 1 μl of protein solution with an equal volume of buffer. Native crystals of the CC-WPP complex were grown in 39% (v/v) Tacsimate (pH 7.0). Selenomethionine-substituted protein was produced using previously established methods (26, 27) and was crystallized similarly as described above. The crystals grew to their maximal size within 3 days.

The single-wavelength anomalous diffraction data sets for native and selenomethionine-substituted crystals were collected at the Shanghai Synchrotron Radiation Facility (SSRF) to resolutions of 2.1 and 3.1 Å, respectively. Both crystals belong to space group P2_1.

Structure Determination and Refinement—The crystal structure of the Rx CC-RanGAP2 WPP complex was determined by single-wavelength anomalous diffraction. Model building and structure refinement were performed using Coot and PHENIX (28, 29), respectively. The structure was refined to a resolution of 2.1 Å with an R factor of 24.8% and R_free of 27.2% and was deposited in the Protein Data Bank (ID 4M70). Statistics for data collection and refinement are summarized in Table 1.

Mutant Protein/Protein Interaction Assays—Mutant versions of the GST-tagged Rx CC domain were expressed together with the untagged wild-type RanGAP2 WPP domain as described above. Likewise, GST-tagged RanGAP2 WPP mutant derivatives were expressed in E. coli together with the untagged wild-type Rx CC domain. The proteins were purified by glutathione-Sepharose 4B affinity chromatography. Protein/protein interactions were determined by subjecting the eluted proteins to SDS-PAGE and visualization by Coomassie Blue staining.

Transient Protein Expression and Analysis in Vivo—Constructs based on the pBin61 binary expression vector encoding Rx CC-HA (Rx residues 1–144), Rx NB-LRR-Myc (Rx residues 139–937), and S. tuberosum RanGAP2-FLAG have been described previously (5). The HA-tagged Rx CC W90D and
FLAG-tagged RanGAP2 A89D mutants were generated by extension overlap PCR.

Binary vectors were transformed into Agrobacterium tumefaciens strain C58C1 carrying the virulence plasmid pCH32, and agro-expression was performed as described previously (20) at $A_{600} = 0.2$, with the exception of PVX-GFP (30), which was transformed into A. tumefaciens strain GV3101 carrying the plasmid pSoup and diluted to $A_{600} = 0.001$ (31). Co-immunoprecipitations and immunoblotting were carried out as described previously (20). For experiments assessing virus replication, GFP fluorescence was monitored 5 days after Agrobacterium infiltration using a handheld UV lamp. Each experiment was repeated at least three times with similar results.

**RESULTS**

Biochemical Characterization of the CC/WPP Interaction—On the basis of previous deletion analyses of the Rx CC domain, which identified the minimal region of the Rx CC domain required for function (20), we selected residues 1–122 of Rx (hereafter referred to as Rx CC) for purification and crystallization. As the full-length RanGAP2 protein was prone to degradation (Fig. 1B), we used a mapping strategy to identify a minimal Rx CC domain-binding region in the RanGAP2 WPP domain. We tested the interaction of the following fragments with Rx CC: RanGAP2 residues 1–124, 1–112, 1–90, and 1–75.

The GST-tagged RanGAP2 fragments were individually coexpressed with untagged Rx CC in E. coli, and the proteins were purified on glutathione-Sepharose 4B resin. As shown in Fig. 1B, the two longer RanGAP2 fragments exhibited a similar interaction activity with Rx CC, whereas the two shorter fragments did not interact with Rx CC. This is consistent with previous reports showing that RanGAP2 residues 1–112 are capable of interacting physically and functional in planta with Rx (32). Therefore, RanGAP2 residues 1–112 (hereafter referred to as RanGAP2 WPP) were chosen as the fragment to co-crystallize with Rx CC.

To verify the interaction of Rx CC with RanGAP2 WPP in vitro, we expressed the two proteins in E. coli, purified the proteins to homogeneity, and then examined their interaction using a gel-filtration assay. In agreement with our coexpression data, Rx CC and RanGAP2 WPP formed a stable complex in solution as demonstrated by co-migration of the two proteins (Fig. 1C). To further characterize the interaction between Rx CC and RanGAP2 WPP, we measured their binding affinity using ITC. The ITC results showed that Rx CC interacted strongly with RanGAP2 WPP, with a dissociation constant of 0.039 μM (Fig. 1D).

Crystal Structure of the Rx CC Domain—Despite intensive crystallization attempts, we were unable to crystallize the isolated Rx CC domain. However, we successfully co-crystallized Rx CC with RanGAP2 WPP. The final atomic model of the crystal structure of the Rx CC-RanGAP2 WPP complex contained residues 1–112 of the Rx CC domain. No electron density was observed corresponding to residues 39–50 of the Rx CC domain presumably because they were disordered in solution.

The structure of Rx CC bound by RanGAP2 WPP consists of four α-helices forming a four-helix bundle fold. The four helices from the N terminus to the C terminus are designated α1, α2, α3, and α4, with short loops linking α1–α2 and α3–α4 (Fig. 2A). The overall structure of Rx CC is stabilized by extensive hydrophobic interactions formed by residues located on the interior surfaces of the four helices. The conserved EDVID motif is exposed on the surface, available for protein/protein interactions.

Crystal Structure of the St RanGAP2 WPP Domain—Within the CC-WPP co-crystal, the final atomic model contained residues 16–102 of the WPP domain. The residues not seen in the crystal were likely cleaved by proteases or were not resolved due to their disorder in solution. The WPP domain is primarily helical and contains three short α-helices forming a helix-loop-helix structure. These helices were designated αA, αB, and αC to differentiate them from the Rx CC helices. We named the long loop between αA and αB loop D (Fig. 2A). The conserved WPP motif (residues 18–20) is localized at the N-terminal side of αA and forms an extended loop. A structure-based sequence alignment shows that the amino acids involved in the formation of the three-helix bundle in RanGAP2 WPP are generally conserved between St RanGAP2 and St RanGAP1 (Fig. 2B).

Overall Structure and Interface of the Rx CC-RanGAP2 WPP Complex—The interaction between Rx CC and RanGAP2 WPP resulted in the formation of a 1:1 complex, burying a surface area of 1102.4 Å². Rx CC helix α4 packs against the adjacent RanGAP2 WPP helices αA and αC. RanGAP2 WPP loop D makes contacts with Rx CC helices α3 and α4, further strengthening the interactions of the complex (Fig. 4, A–C). The interfaces between the two protein molecules are formed primarily
The RanGAP2 WPP domain forms a three-helix bundle. A, ribbon representation of the crystal structure of the RanGAP2 WPP domain. Helices αA, αB, and αC and the long loop linking αA and αB (loop D) are indicated. N and C represent the N and C termini, respectively. B, structure-based sequence alignment of the WPP domains from potato RanGAP2 and its homolog RanGAP1. Identical amino acids are indicated.

35872 JOURNAL OF BIOLOGICAL CHEMISTRY

The Rx CC/RanGAP2 WPP interfaces can be subdivided into two major binding regions. In the first region, the side chains of Rx Ser-86, Trp-90, Phe-93, and Phe-94 form a protruding hydrophobic patch (Fig. 4C). RanGAP2 Leu-17, Pro-20, Thr-24, Leu-28, Val-85, Tyr-88, and Ala-89 in this interacting region form a complementary hydrophobic surface groove (Fig. 4B). Together, these residues undergo an extensive network of hydrophobic interactions. In the second binding region, loop D of RanGAP2 WPP forms a hydrophobic convex patch on the surface (Fig. 4, B and D). Two residues of RanGAP2 in this patch, Ile-41 and Phe-42, make hydrophobic contacts with a cavity on the surface of Rx CC formed by Rx Val-61, Thr-64, Ala-99, and Cys-102 (Fig. 4, C and D). In addition to these two main binding regions, Rx Glu-101 makes a salt bond with RanGAP2 Lys-101, and Rx Gln-98 makes a hydrogen bond with RanGAP2 Asn-35 (Fig. 4D). The side chains of the Rx CC EDVID motif form a negatively charged region on the surface, which is distinct from the RanGAP2 WPP interfaces. This observation is consistent with previous results that the EDVID motif is not required for the interaction with RanGAP2 (20).

Structure-guided Mutagenesis of Residues Involved in the Interaction between Rx CC and RanGAP2 WPP—To validate observations from the crystal structure of the CC-WPP complex, we made the following substitutions in Rx CC: V61D, T64D, S86D, W90D, E91Q, F93D, F94D, Q98G, and A99D. The mutant V61D and T64D proteins for Rx CC had very low expression levels in E. coli (data not shown). Other mutants had expression levels similar to the wild-type protein, and only the W90D mutant demonstrated impaired binding to RanGAP2 WPP (Fig. 5A).

We also made the following substitutions in RanGAP2 WPP: L17D, P20D, T24D, E31Q, N35G, I41D, F42D, V85D, A89D, and K101A. Using the same GST pulldown assay, we found that the A89D mutation abolished the RanGAP2 WPP interaction with Rx CC (Fig. 5B). We were unable to successfully produce the mutant L17D and P20D proteins for RanGAP2 WPP due to very low expression levels (data were not shown).

Structurally Informed Rx CC and RanGAP2 Mutational Analysis in Vivo—The two mutations that significantly compromised the in vitro interaction, W90D of Rx and A89D of WPP, were incorporated into the Rx CC-HA and full-length RanGAP2-FLAG binary vector constructs for expression in planta (20). Previously, it has been shown that the Rx protein can be separated into two functional fragments, CC and NB-LRR, which undergo a physical interaction. When coexpressed in planta, these fragments reconstitute a functional protein that can induce cell death (HR) or virus resistance in the presence of the PVX CP (19, 20). As shown in Fig. 6A, coexpression of Rx CC and Rx NB-LRR plus the CP in Nicotiana benthamiana leaves induced an HR, and the CC W90D mutant showed...
an activity in this assay similar to wild-type Rx CC in this assay (upper panel). However, the HR is often induced as a result even of a relatively weak, but sustained induction of a defense response. We therefore tested whether the W90D mutation compromised the ability of reconstituted Rx to control virus accumulation, a more sensitive assay of Rx function (20). When

wild-type Rx CC and NB-LRR fragments were coexpressed with PVX expressing GFP, HR lesions were induced at the sites of PVX infection, and very little green fluorescence was observed (Fig. 6A, middle and lower panels). However, when Rx CC W90D was coexpressed with NB-LRR and PVX-GFP, Rx function was considerably diminished, as observed by the reduced incidence of HR lesions and increased GFP fluorescence in the infiltrated area (Fig. 6A, middle and lower panels). These results indicate that although the W90D mutation did not completely abolish Rx function, it did result in significant functional impairment in vivo. We were unable to evaluate the functional relevance of the RanGAP2 A89D mutation in this assay, as native N. benthamiana RanGAP2 is sufficient for Rx function, and it is not possible to completely knock down the expression of N. benthamiana RanGAP2 (15, 16).

To confirm that the loss of function of Rx CC W90D is due to compromised interaction with RanGAP2 in planta, we performed co-immunoprecipitation assays with full-length RanGAP2 expressed with either wild-type Rx CC or the W90D mutant. These analyses showed that although RanGAP2 interacted robustly with the wild-type Rx CC domain in planta, the W90D mutant showed a dramatically reduced ability to interact with RanGAP2 (Fig. 6B). Likewise, the Rx CC domain was able to robustly co-immunoprecipitate wild-type RanGAP2, but the RanGAP2 A89D mutant showed very little binding to Rx CC (Fig. 6B). Finally, expression of Rx CC W90D with RanGAP2 A89D resulted in a complete loss of interaction in planta (Fig. 6B). To demonstrate that the Rx W90D mutation specifically alters the interaction with RanGAP2, we tested the ability of wild-type and mutant Rx CC to interact with the Rx NB-LRR fragment, as the interaction between CC and NB-LRR involves

![Figure 5](image-url)  
**FIGURE 5.** Mutagenesis of residues involved in the interaction between the Rx CC and RanGAP2 WPP domains. A, effects of substitutions in Rx CC on interaction with RanGAP2 WPP. Each GST-tagged Rx CC mutant protein was coexpressed with untagged RanGAP2 WPP and purified on glutathione-Sepharose 4B resin. The eluted proteins were visualized by SDS-PAGE and Coomassie Blue staining. MW, molecular weight; WT, wild-type. B, effects of substitutions in RanGAP2 WPP on interaction with Rx CC.

![Figure 6](image-url)  
**FIGURE 6.** Functional analysis of Rx and RanGAP2 point mutants in vivo. A, impact of the W90D mutation on Rx-mediated resistance. The Rx NB-LRR fragment was coexpressed in N. benthamiana leaves by Agrobacterium-mediated transient expression together with the wild-type (WT) or the W90D mutant Rx CC domain in the presence of either the PVX CP (upper panel) or PVX-GFP (middle and lower panels). Leaves co-infiltrated with the CP were photographed after 3 days under white light (upper panel), and those co-infiltrated with PVX-GFP were photographed after 6 days under white light (middle panel) or UV light (lower panel). B, impact of point mutations on CC/RanGAP2 binding. Rx CC-HA (WT or W90D) was transiently expressed in N. benthamiana leaves along with RanGAP2-FLAG (WT or A89D). Protein extracts were subjected to anti-HA and anti-FLAG immunoprecipitation (IP), followed by immunoblotting (IB) as indicated. C, NB-LRR binding by the Rx CC W90D mutant. Rx CC-HA (WT or W90D) was agro-expressed in N. benthamiana leaves together with NB-LRR-Myc. Protein extracts were subjected to anti-HA and anti-Myc immunoprecipitation, followed by immunoblotting as indicated.
different regions of Rx CC than are required for the interaction with RanGAP2 (20). As shown in Fig. 6C, both the wild-type Rx CC domain and the W90D mutant were able to co-immuno-precipitate the Rx NB-LRR fragment. This result further demonstrates the physical distinction between the residues mediating the interaction of the CC domain with the rest of the Rx molecule versus its interaction with RanGAP2.

**DISCUSSION**

To understand the function of the Rx CC domain, we determined its three-dimensional crystal structure in complex with the potato RanGAP2 WPP domain at 2.1 Å resolution. The structure of the Rx CC domain is strikingly different from that of the MLA10 CC domain (Fig. 7A) both with respect to the overall tertiary structure and with respect to the formation of homodimers. There is a strong relationship between the CC-dependent self-association of MLA and its ability to induce cell death (11). Structural superimposition of the Rx CC and MLA10 CC monomers (Fig. 7A) revealed structural equivalence between helices α1 and α2 of Rx CC and the long helix α1 of MLA10 CC, as well as between Rx CC helices α3 and α4 and MLA10 CC helices α2a and α2b. The short loops linking Rx CC helices α1-α2 and α3-α4 allow them to fold back and pack against each other, in contrast to the more extended structure of MLA10 CC. Our results show that, at least when in complex with RanGAP2, Rx CC does not form a homodimer, consistent with a lack of reported self-association of Rx. Determining which, if either, of these CC structures is more common among CC-NB-LRR proteins will require analysis of additional CC domains.

The crystal structure of the CC-WPP complex also revealed the composition of the interfaces between the two molecules. This, together with the mutational analysis, led us to conclude that this interaction is mediated primarily by hydrophobic interactions. The WPP motif (residues 18–20 of RanGAP2) forms an extended loop to the N terminus of RanGAP2 WPP helix αA, and Phe-18 and Pro-19 are not involved in the interaction with Rx CC (Figs. 4D and 7B). This observation supports a previous report showing that a form of RanGAP2 in which the WPP residues had been mutated to AAP was still able to activate Rx (15). This motif is exposed on the surface, supporting its proposed involvement in binding to nuclear envelope-localized proteins (33). Most of the residues in the Rx CC domain involved in the interaction with RanGAP2, particularly Ser-86, Trp-90, Phe-93, Phe-94, Asn-98, Ala-99, Glu-101, and Cys-102, show no discernible conservation with the CC domains of other NB-LRR proteins (20). This lack of conservation may explain
why RanGAP2 binds only to Rx-like CC domains (15). It may also explain why other CC domain-binding proteins identified to date show little apparent structural similarity (7). This is in agreement with the idea that this variability may allow CC-NB-LRR proteins to co-opt a variety of recognition cofactors, which in turn could increase recognition potential.

Rx interacts much more weakly (if at all) with full-length RanGAP1 in planta compared with RanGAP2 (15, 34). However, almost all of the residues participating in the interaction with Rx CC are conserved between St RanGAP2 and St RanGAP1 (Fig. 3B). The mechanism underlying this binding specificity remains unclear, although because both RanGAP1 and RanGAP2 interact with the Rx CC domain in yeast (34), it is tempting to speculate that additional plant proteins may influence this interaction. At the same time, we do not rule out the possibility that other parts of the RanGAP proteins influence binding to Rx.

Previous studies suggested that the EDVID motif plays an important role in CC-NB-LRR function by physically linking the CC and NB-LRR regions (7, 20). Structural superposition of the Rx CC and MLA10 CC domains shows that the side chains of the EDVID motif of the two molecules have the same configuration, further supporting the notion that R proteins share common features in CC/NB-LRR interactions (20). The mechanistic importance for this interaction is not yet clear. It has been suggested that the role of the CC domain interaction with recognition cofactors could be to recruit effectors and to bring them into proximity with the LRR domain, which ultimately confers recognition specificity (7, 25). Alternatively, this interaction could serve as a sensor that translates information from the N terminus to the rest of the protein or vice versa. For example, the inability of the NB domain to bind ATP or the LRR domain disrupts the CC/NB-LRR interaction, as does the activation of the Rx protein (19, 20). It is also of interest to note that the two interaction interfaces of the CC domain are in close proximity to each other (Fig. 7B). The CC/RanGAP1 interface involves Val-61 and Thr-64 of Rx, which form part of the extended EDVID motif (LecxVRELAYDAEDVID, where the underlined consensus residues correspond to Val-61 and Thr-64 of Rx). These residues are highly conserved and are also important in the CC/NB-LRR interaction (20). As such, it is plausible that molecular events involving RanGAP2 could affect the rest of the Rx protein through the CC/NB-LRR interface or vice versa. For example, it is interesting to speculate that perception of the PVX CP by RanGAP2 could cause it to undergo a conformational change, which would in turn be translated to the rest of the protein via the EDVID motif, causing Rx to switch to an active state. Taking together previous findings and the results from this study, we propose that the transfer of information regarding recognition may be a common role for the EDVID motif among CC-NB-LRR proteins.

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