Structure of a signal transduction regulator, RACK1, from *Arabidopsis thaliana*

HEMAYET ULLAH,¹ ERICA LOUISE SCAPPINI,² ANDREA FLORENCE MOON,³
LATANYA VERONICA WILLIAMS,^{1,4} DAVID LEE ARMSTRONG,²
AND LARS CHRISTIAN PEDERSEN³

¹Department of Biology, Howard University, Washington, DC 20059, USA

²Laboratory of Neurobiology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA

³Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA

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Abstract

The receptor for activated C-kinase 1 (RACK1) is a highly conserved WD40 repeat scaffold protein found in a wide range of eukaryotic species from *Chlamydomonas* to plants and humans. In tissues of higher mammals, RACK1 is ubiquitously expressed and has been implicated in diverse signaling pathways involving neuropathology, cellular stress, protein translation, and developmental processes. RACK1 has established itself as a scaffold protein through physical interaction with a myriad of signaling proteins ranging from kinases, phosphatases, ion channels, membrane receptors, G proteins, IP3 receptor, and with widely conserved structural proteins associated with the ribosome. In the plant *Arabidopsis thaliana*, RACK1A is implicated in diverse developmental and environmental stress pathways. Despite the functional conservation of RACK1-mediated protein–protein interaction-regulated signaling modes, the structural basis of such interactions is largely unknown. Here we present the first crystal structure of a RACK1 protein, RACK1 isoform A from *Arabidopsis thaliana*, at 2.4 Å resolution, as a C-terminal fusion of the maltose binding protein. The structure implicates highly conserved surface residues that could play critical roles in protein–protein interactions and reveals the surface location of proposed post-transcriptionally modified residues. The availability of this structure provides a structural basis for dissecting RACK1-mediated cellular signaling mechanisms in both plants and animals.

Keywords: RACK1; protein signaling; scaffolding protein; WD40; propeller; protein–protein interaction; drought

Scaffold proteins are uniquely poised to integrate signals from multiple pathways. Such proteins generate considerable functional diversity by mediating concomitant and/

or promiscuous interactions with a vast array of protein partners. The receptor for activated C-kinase 1 (RACK1) is a member of the WD40 repeat family of β -propeller proteins. It was discovered through its ability to function as a scaffold protein, stabilizing signaling complexes involving protein kinase C (Ron and Mochly-Rosen 1994; Ron et al. 1994). In metazoans, RACK1 is now postulated to coordinate many different signal transduction pathways, ranging from cell division to ion channel regulation, by interacting with more than 80 different proteins (McCahill et al. 2002; Alfarano et al. 2005;

⁴Present address: Department of Urology, Northwestern University School of Medicine, Chicago, Illinois 60611, USA.

Reprint requests to: Lars C. Pedersen, Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, 111 TW Alexander Drive, MD F3-09, Research Triangle Park, NC 27709, USA; e-mail: pederse2@niehs.nih.gov; fax: (919) 541-7880.

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Sklan et al. 2006). Global control of gene transcription, translation, and ribosome assembly and activation are also emerging as important cellular functions of RACK1 (Ceci et al. 2003; Nilsson et al. 2004). Definitive molecular tests, however, are lacking for most of RACK1's many postulated roles in signal transduction, partly because the structural basis for its interaction with other signaling molecules has not been elucidated.

RACK1 orthologs have also been discovered in primitive eukaryotes, such as *Chlamydomonas* (Schloss 1990), and are highly conserved in plants (van Nocker and Ludwig 2003), which do not express canonical protein kinase C enzymes (Herold et al. 2002). The sequence of human RACK1 is identical to the orthologs in chicken and mouse, and shares 66% sequence identity to RACK1A from *Arabidopsis thaliana*. This high degree of sequence conservation likely correlates to functional conservation between these diverse species.

Recently, it has been reported that RACK1A mediates multiple hormonal and developmental signaling pathways in *A. thaliana* (Chen et al. 2006). Preliminary results indicate that RACK1A plays an important molecular role in regulating diverse environmental stresses including drought response. As opposed to a single gene in metazoans, the *A. thaliana* genome maintains three different RACK1 genes termed RACK1A, RACK1B, and RACK1C. RACK1B and 1C share 87% and 93% sequence identity to RACK1A.

The first member of the WD repeat family of proteins for which a definitive structure has been determined is the β subunit of the mammalian heterotrimeric ($\alpha\beta\gamma$) G proteins (Wall et al. 1995; Lambright et al. 1996; Sondek et al. 1996). RACK1 orthologs exhibit ~25% sequence identity to the β subunit of the G proteins. Due to a lack of any reported RACK1 crystal structure, the RACK1 molecule has been modeled based on the G_β subunit. These models have provided insight into the global fold of RACK1 but lack the fine structural details of diverse loop regions and divergent surface side chain conformations that are likely responsible for differences in binding interactions with protein partners.

To improve our understanding of RACK1 in signaling pathways, we have solved the crystal structure of RACK1A from *A. thaliana* at 2.4 Å resolution. This structure confirms RACK1A to be a seven-bladed β -propeller protein similar to the G_β subunit, while demonstrating differences that may explain why RACK1 does not function as a canonical G_β protein. Two large conserved surface regions have been identified that may serve as potential protein binding points. The surfaces of this molecule may provide a stable platform for multiple simultaneous protein-protein interactions, suggesting a mechanism for the regulation of inherently complex signaling pathways by RACK1. Since the early 1990s

when RACK1 was identified as a G_β subunit homolog in plants and animals, a great deal of functional characterization, mainly in animals, has established RACK1 as a scaffold protein competent to mediate diverse signaling pathways. Apart from generalized phenotypic characterization of the *A. thaliana rack1a* loss-of-function mutants (Chen et al. 2006), precise biological roles of RACK1A in plants have remained largely unexplored. The availability of structural data will aid in elucidating RACK1A-mediated developmental and environmental stress response pathways in plants.

Results and Discussion

Overall structure

Initial attempts to crystallize native *A. thaliana* RACK1A failed to produce crystals. Therefore, we fused RACK1A to the C-terminal end of a mutant form of the maltose binding protein (MBP), producing a protein with different surfaces to promote crystallization. The MBP fusion protein used in this study utilized an identical linker to that used previously for MBP-fusion crystallization experiments (Kobe et al. 1999). In addition, MBP residues Asp82 and Lys83 from one loop and Lys239 from another were mutated to alanines in order to reduce surface entropy and potentially facilitate better crystal lattice contacts (Derewenda 2004). Based on secondary structure prediction, residue Gly4 of RACK1A was used as the starting residue to reduce the length and flexibility of the linker between the MBP protein and RACK1A. The fusion protein crystallizes with only one residue in the linker, Gly4 from RACK1A, not involved in secondary structure in either protein (Fig. 1A). Surprisingly, there are no interactions in the crystal structure between MBP and RACK1A of the same molecule. Residues lining the interface between the two domains are ~5 Å apart. In addition, there are no RACK1A–RACK1A crystal lattice contacts, suggesting a reason why attempts to crystallize RACK1A alone have failed. Because MBP is used only as a vehicle for crystallization, the rest of the discussion will focus on RACK1A.

The structure of RACK1A is a canonical seven-bladed β -propeller similar to the heterotrimeric G protein G_β . This fold was predicted from the presence of WD40 repeats in the sequence (Wall et al. 1995; Sondek et al. 1996; Sondek and Siderovski 2001). Each propeller blade consists of a four-stranded antiparallel β -sheet (Fig. 1B). The blades are arranged in sequential order around a pseudo-sevenfold axis that creates a water accessible channel in the middle of the propeller with a width of ~9 Å. β -strand A of each blade lines the channel of the propeller while β -strand D is located along the outer surface of the protein. Loops connecting β -strand D from

Figure 1. (A) Ribbon diagram of the MBP-RACK1A fusion protein. The MBP molecule is colored gray and the RACK1A molecule is colored based on the different blades in the β -propeller. (B) Top view of the β -propeller of RACK1A. Each blade of the propeller is made up of four antiparallel β -strands with β -strand A lining the inside of the open pore and β -strand D on the outside of the propeller.

one propeller to β -strand A of the next and β -strand B to C form the top of the propeller, while loops connecting β -strand A to B and C to D line the bottom of the propeller. The sequentially defined WD repeat spans two blades and consists of strand D from one blade and A, B, and C of the following. The last blade therefore consists of β -strands from both the C terminus (β -strands A, B, and C) and N terminus (β -strand D). The WD residues, for which the repeat is named, are located at the end of strand C on the bottom side of the propeller and are conserved in blades 2, 5, and 6. The aspartate residue, of the WD motif, has been substituted with a Lys in blade 1, an asparagine in blades 3 and 4, and a glycine in blade 7. Also conserved within the WD40 repeat is a Asp-His-Ser hydrogen-bonding network that holds the blade together, while connecting it to the adjacent blades (Fig. 2). The major difference between the WD repeats is in the loops connecting the β -strands. It is the structural organization of these loops that provides the distinct features of each member of the WD family and distinguishes

RACK1 interactions from those of other WD proteins (Garcia-Higuera et al. 1996; Sklan et al. 2006).

The high degree of sequence identity among different orthologs of RACK1 suggests conservation of biological functions that evolved prior to separation of the plant and animal kingdoms (Neer et al. 1994; Sklan et al. 2006). RACK1 is proposed to be a scaffolding protein, based on the array of proteins that can form interactions with it (Sklan et al. 2006), yet little is known about the specific interactions between RACK1 and its binding partners. Knowledge of specific residues involved in these interactions is particularly lacking. To better understand what residues may be important in complex formation for functional conservation across the plant/animal kingdoms, conserved residues from Figure 3 were mapped to the surface of the protein, revealing two conserved surface regions (Fig. 4A,B). The first conserved region (region 1) is located on the top rim of the propeller involving side chains from residues Arg36, Lys38, Ser63, His64 from blade 1; Trp83, Asp107 from blade 2; Arg125 from blade 3; and Trp152 from blade 4 (Fig. 4A). The second large conserved surface region (region 2) of RACK1 is located on the bottom of the propeller and is mainly comprised of conserved residues Pro204, Asp205, Tyr230 from blade 5 and Asn246, Tyr248, and Trp249 from blade 6 (Fig. 4B). These regions represent potential protein-protein interaction sites. Earlier mutational studies have implicated blade 5 and 6 as major docking stations for interaction with RACK1 supporting region 2 as a protein-protein interaction site (Chang et al. 2001; Steele et al. 2001; Sengupta et al. 2004).

Figure 2. Ribbon diagram of Asp-His-Ser hydrogen-bonding network responsible for maintaining the integrity of the β -sheet within blade 2 of the β -propeller. His62 lies on the loop connecting blade 1 with blade 2. Trp90 and Asp91 represent the WD repeat of blade 2.

When RACK1 was originally cloned from tobacco and from a rat brain cDNA library, it was reported to be a homolog of the G_{β} subunit owing to high sequence similarity and the presence of WD40 repeats (Ishida et al. 1993; Ron et al. 1994). Crystal structures of the G_{β} subunit and other WD40 repeat proteins revealed that the β -propeller structure is a common feature of WD40 repeat proteins (Wall et al. 1995; Sondek et al. 1996; Sprague et al. 2000; Pickles et al. 2002). The crystal structure of the bovine G_{β} subunit has been widely used as a template to build homology models of RACK1 protein. Although WD repeat proteins, including RACK1 (Sondek and Siderovski 2001; Steele et al. 2001), can be

A comparison of the structures of RACK1A and G β reveals some possible reasons for the functional differences of these proteins (Fig. 5). Based on structural alignments with bovine G β , the overall root-mean-squared deviation for 278 structurally conserved C α atoms is 1.5 Å, with an overall sequence identity of 23% (PDB ID code 1GP2). The greatest structural deviations between the two proteins are a long helix on the N terminus of G β (residues 1–44) and a loop insertion

Figure 4. Surface diagrams of conserved regions within the RACK1 proteins. (A) Conserved region 1. Conserved residues are colored according to the blade to which they belong based on the ribbon diagram in Figure 1B. Residues Arg36, Trp83, Arg125, Trp152, and Lys214 line the upper rim of the β -propeller pore. (B) Conserved region 2. Tyr230 and Tyr248 on RACK1A are proposed phosphorylation sites. Residue Pro204 is located on the lower rim of the pore of the β -propeller. Molecular surfaces were created using PyMOL (DeLano Scientific).

in plant RACK1A between propeller blades 6 and 7 that is unique to the majority of plant RACK1 proteins (Fig. 3). Despite the remaining similarities between the two proteins, it is unlikely that RACK1 binds the G_γ subunit in the same fashion as does G_β . G_β and G_γ are tightly associated with the N termini of both proteins engaged in a coiled-coil interaction. RACK1A lacks this helix as well as Asp258 of G_β within the propeller that forms the interactions with Arg22 of G_β and Arg27 of G_γ , properly positioning the coiled coil with respect to the propeller domain. In addition, the hydrophobic cleft that exists in G_β , between β -sheets 1 and 7 that accommodates Phe61 of G_γ , is occupied in RACK1A by larger side chains, so that penetration by a phenylalanine side chains is unlikely. The current position of Tyr248 in the RACK1A structure would also cause significant steric clashes with the G_γ subunit.

The G_α subunit alone also does not bind RACK1. However, the $G_{\beta\gamma}$ dimer and the $G_{\alpha\beta\gamma}$ trimer are both capable of forming complexes with RACK1, with the affinity for the $G_{\alpha\beta\gamma}$ trimer being threefold higher than for the $G_{\beta\gamma}$ dimer (Dell et al. 2002). Dell et al. (2002) also demonstrated that a GST–RACK1 C-terminal truncation consisting of RACK1 residues 1–207 was required for

trimeric G protein binding whereas residues 207–317 would not bind the G protein complex. This suggests that the conserved region 1 of RACK1 may be involved in trimeric G protein binding. So, although RACK1 is not a canonical substitute for the G_β subunit, it can form interactions with the entire trimeric complex.

Despite the structural differences between RACK1A and G_β , there is evidence to suggest that RACK1 homologs in some species might retain G_β -like function. Zeller et al. (2007) recently established Asc1, a yeast homolog of RACK1, as a bona fide G_β subunit since Asc1 has been shown to interact directly with the G_α subunit in a guanine nucleotide-dependent manner. In addition, the Asc1 ortholog in *Cryptococcus neoformans*, Gib2, has been identified as a G_β -like subunit that functions as a positive regulator of cAMP, as opposed to a negative regulator in yeast where it regulates melanization and capsule formation associated with virulence (Palmer et al. 2006). This G_β -like function occurs in the absence of interaction with a G_γ subunit. These proteins are thus described as “non-canonical G_β subunits.” However, the structural basis of the reported interaction of RACK1 homologs in yeast and in *Cryptococcus* with the G_α subunit remains to be identified.

RACK1 ribosome interactions

RACK1 has been shown to regulate translation initiation by recruiting PKC to the ribosome (Ceci et al. 2003; Nilsson et al. 2004). Despite the lack of any known PKC ortholog in plants, a recent genome-wide screen for *A. thaliana* RACK1A interacting proteins has identified at least four ribosomal proteins (data not shown). Based on cryo-EM studies, a model of *Saccharomyces cerevisiae* RACK1 (derived from G_β) binding to the 80S ribosomal subunit has been created. This model places RACK1 on the head region

Figure 5. Superposition of the trimeric $G_{\alpha\beta\gamma}$ protein complex with RACK1A. G_α is colored green; G_β , gray; and G_γ , yellow (PDB ID code 1GP2). RACK1A is colored blue.

of the 40S ribosomal subunit near the mRNA exit channel (Sengupta et al. 2004). In addition, the authors demonstrated that mutations of *S. cerevisiae* RACK1 at Arg38 and Lys40 (Arg36 and Lys38 of RACK1A, in conserved region 1) disrupt the RACK1 interaction with the 40S subunit (Sengupta et al. 2004). These results are consistent with the crystal structure of RACK1A. A superposition of our RACK1A structure onto the RACK1-40S ribosome model (PDB ID code 1ARI) positions RACK1A so that the top rim of RACK1A is in contact with the ribosome, exposing the bottom rim to solvent (Fig. 6). Thus, the conserved region 1 may lie against the ribosome, exposing conserved region 2 to the solvent. This arrangement would allow RACK1 to associate with both the ribosome and other proteins simultaneously.

Proposed post-translational modification of RACK1A

Tyrosine phosphorylation and protein sumoylation have been implicated in regulation of RACK1 function (Chang et al. 2001; Yang and Gregoire 2006). Src-tyrosine kinase-mediated RACK1 phosphorylation has been implicated in regulating cell migration and cell cycle checkpoints in human colon cancer cells (Chang et al. 2001; Doan and Huttenlocher 2007; Mamidipudi et al. 2007). RACK1 has also been found to be a binding partner and inhibitor of Src tyrosine kinase (Chang et al. 1998, 2001). Mutagenesis work by this group identified Tyr228 and

Tyr246 as potential phosphorylation sites and suggested a correlation between enhanced tyrosine phosphorylation of RACK1 and binding of RACK1 to Src.

Though no bona fide tyrosine kinase has been identified in plants, tyrosine phosphorylation and dephosphorylation have been documented to regulate growth and development in seeds, hypocotyls, and roots in *A. thaliana* (Reyes et al. 2006; Hussain et al. 2007; Yemets et al. 2008). It is hypothesized that tyrosine phosphorylation in plants is carried out by dual-specificity serine/threonine/tyrosine (STY) protein kinases (Rudrabhatla et al. 2006). In addition, tyrosine-specific protein kinase-like kinases (TKLs) have also been reported as possible tyrosine kinases (Miranda-Saavedra and Barton 2007). Interestingly, the two proposed tyrosine phosphorylation sites of human RACK1 are conserved in *A. thaliana* RACK1A (Tyr230 and Tyr248). These residues are located on the bottom side of the propeller in conserved region 2 (Figs. 4B, 6). Tyr230 is located at the start of β -strand D of blade 5 on the surface of the protein, but with the acceptor hydroxyl tucked back into the protein so that it is only slightly accessible. This suggests that some rearrangement might be necessary prior to phosphorylation. However, the side chain of Tyr248 is located at the end of the loop connecting β -strands A and B of blade 6 and is fully exposed to the solvent, with the putative phosphate acceptor hydroxyl pointing away from the protein, easily accessible for modification.

SUMO-1 (Small Ubiquitin-like Modifier) is an ubiquitin-related protein that is covalently conjugated to a diverse assortment of substrate proteins and regulates cellular functions by mediating protein-protein interactions and subcellular compartmentalization, as well as effecting protein stability (Salinas et al. 2004). It has been suggested that the underlying principle of sumoylation is to alter inter- or intramolecular interactions of the modified substrate (Geiss-Friedlander and Melchior 2007). At the molecular level, these functions can be controlled by creating intra-molecular conformational change or by creating or blocking the binding interface (Meulmeester and Melchior 2008). Metazoan RACK1 has a proposed “phospho-sumoyl” switch which is putatively involved in transcriptional repression (Yang and Gregoire 2006). The human RACK1 sequence, 270-LKQEVIST-277, has the consensus LK-x-E/D-x-x-SP found in many transcriptional factors. It has been suggested that phosphorylation of the serine enhances sumoylation of the lysine residue (Yang and Gregoire 2006). In plants, sumoylation is implicated in stress hormone ABA-mediated signaling pathways, attenuating ABA response (Lois et al. 2003). Kurepa and colleagues provided evidence for the potential involvement of sumoylation substrates in mediating elevated temperature stress responses (Kurepa et al. 2003). Miura and colleagues have extensively

Figure 6. Ribbon diagram of the model of RACK1 binding to the 80S ribosome complex, based on the cryo-EM structure (Sengupta et al. 2004). Shown are the H39 and H40 of rRNA from the 40S subunit and RACK1A. To make this figure, RACK1A was superimposed on the model of RACK1 created from G_{β} (PDB ID code 1TRJ). Residues colored red, orange, and yellow are conserved residues from conserved site 1 of RACK1A. The purple tyrosine residues are the sites of putative Src phosphorylation at the second conserved site. The pink lysine is residue 273, the proposed sumoylation site. This figure suggests conserved region 1 could interact with the ribosomal RNA and conserved region 2 could recruit Src to the ribosome.

reviewed the role of sumoylation in plant growth and development (Miura et al. 2007). In *A. thaliana* RACK1A, there are two predicted sumoylation sites, Lys273 and Lys 277. These sites are located at the end of β -strand D of blade 6, similar to the predicted site for metazoan RACK1. For RACK1A this marks the beginning of a long loop that is not present in other nonplant RACK1 proteins. The presence of Lys273 and Lys277 on an exposed loop is consistent with predicted sumoylation sites (Bernier-Villamor et al. 2002; Macauley et al. 2006). However, RACK1A lacks the consensus Ser required for phosphorylation.

Homo-/heterodimerization of RACK1

WD40 repeat-mediated protein–protein interactions are well documented. Evidence is accumulating for the homodimeric interactions of the WD40 repeat containing RACK1 proteins (Thornton et al. 2004; Liu et al. 2007). Dimerization events are frequently employed in transducing signals from the cell surface to the nucleus (Klemm et al. 1998). Phosphorylation of mammalian RACK1 at Ser146 promotes dimerization and leads to degradation of HIF-1 α , a component of HIF-1, the master regulator of transcriptional response to changes in oxygen levels. Dephosphorylation of Ser146 by calcineurin impairs RACK1 dimerization (Liu et al. 2007). Ser146 is located in one of two loop regions within the propeller domain containing insertion differences between mammalian RACK1 and plant RACK1A (Fig. 3). This loop lacks a serine in RACK1A, instead containing a two-residue insert, GlyGly, which would alter the surface structure of RACK1A compared to mammalian RACK1. In the crystal structure of MBP–RACK1A, there are no RACK1A–RACK1A interactions. The GlyGly insert is located on the opposite face of the propeller from the MBP, suggesting that, if RACK1A were to form a symmetrical homodimer, this interaction could still occur in the fusion protein. Likewise, in the crystal, only the sides of blades 1 and 7 are occluded by the presence of the MBP, suggesting that many other permutations of non-symmetrical homodimers might be able to form. However, we cannot rule out the possibility of homo- or heterodimerization under specific physiological environments such as water stress conditions in plants. Specially, homo- and heterodimerization between different family members of a protein family can generate considerable functional diversity. In this regard, as opposed to metazoan RACK1 genes, the plant RACK1 protein family has more than one family member, including three members in *A. thaliana*. As can be seen in Figure 7, the *A. thaliana* *rack1a* loss-of-function mutants show significant resistance to water stress conditions, limiting water loss through the guard cells. Presence of this conditional phenotype

Figure 7. *rack1a* mutant plants are resistant to water stress. (A) WT *Arabidopsis* and *rack1a* mutant plants were grown with normal water regimen for 3 wk, and then water was withheld for 12 d. Compared to the WT plants, reduced wilting was observed for *rack1a* mutant plants during water stress. (B) Water loss in detached leaves from 4-wk-old plants placed on lab bench for 12 h. (C) Comparison of rates of water loss from detached leaves of the WT and *rack1a* mutant plants. Water loss is expressed as the proportion of initial fresh weight. Values are means $\pm$ SE from 10 leaves for each of three independent experiments.

indicates a physiological and/or structural basis of RACK1A function in *A. thaliana*. In addition, the availability of more than one family member certainly could provide considerable opportunity for dimerization-mediated regulation of different pathways.

Scaffolding proteins, such as RACK1, are uniquely poised to integrate signals from multiple pathways by bringing interacting signaling components into close proximity (Hall and Lefkowitz 2002; Dard and Peter 2006). The repertoire of RACK1 interacting proteins is growing steadily and includes factors that mediate vital cellular processes. The deduced molecular structure of RACK1A will be helpful in exploring its promiscuous and dynamic capacity to interact with multiple proteins, facilitating the integration of diverse signal transduction pathways. This structure lays the foundation for future work involving surface mutagenesis and structural characterization of protein complexes to obtain greater detail of these interactions.

Materials and Methods

Cloning, expression, and purification

The MBP–RACK1A fusion protein was created using pMAL-c2x (New England Biolabs) as the starting vector. The amino acid sequence of MBP contained the mutation E359A and was truncated at Asn367. The linker region encodes three alanine residues and contained a NotI site for cloning. In addition, residues Asp82, Lys83, and Lys239 of MBP were also mutated to alanines. RACK1A containing residues Gly4–Tyr327 from *A. thaliana* was cloned into this vector using the NotI and BamHI restriction sites. The MBP–RACK1A fusion protein was expressed in BL21(DE3)CodonPlusRIL cells (Stratagene). Cells were grown at 37°C in LB media containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol, shaking at 275 rpm. When the OD₆₀₀ reached 1.0, the temperature was decreased to 18°C, IPTG was added to a final concentration of 500 µM, and cells were allowed to shake overnight at 275 rpm. Cells were pelleted by centrifugation, resuspended in the sonication buffer (25 mM Tris pH 7.5 and 500 mM NaCl), and sonicated three times for 20 sec each. The insoluble fraction was pelleted by centrifugation and the soluble fraction was loaded in batch onto amylose resin (New England Biolabs). Samples were placed on a rocker at 4°C for 1 h under gentle agitation. Resin was then washed with sonication buffer, followed by elution from the resin in 25 mM Tris pH 7.6, 500 mM NaCl, 1 mM DTT, and 40 mM maltose. Eluted protein was concentrated to 10 mg/mL then loaded onto a 16/60 Superdex200 size exclusion column (GE Healthcare) pre-equilibrated in the same buffer. The purified protein was then dialyzed against 25 mM Tris pH 7.6, 100 mM NaCl, 1 mM DTT, and 5 mM maltose and concentrated to 16.7 mg/mL for crystallization trials.

Crystallization, data collection, and refinement

Crystals of MBP–RACK1A formed using the sitting-drop vapor diffusion technique, mixing 1 µL of protein solution with 1 µL of well solution consisting of 100 mM HEPES pH 7.5 and 20% w/v Polyethylene glycol 10,000. To obtain cryo-conditions, 0.66 µL of ethylene glycol was added to the crystallization drop, and the ethylene glycol was allowed to diffuse across the drop for approximately 1 min. A crystal measuring 0.1 mm × 0.05 mm × 0.02 mm was captured in a loop and flash-frozen in liquid nitrogen. The crystal was placed in a stream of nitrogen gas cooled to −180°C. Diffraction data were collected on a Rigaku 007HF generator equipped with VariMaxHF mirrors and a Saturn92CCD detector. Original diffraction was observed to 3.6 Å. To improve the resolution, the crystal was briefly thawed for 8 sec at room temperature, followed by refreezing in the nitrogen gas stream. A data set was collected to 2.4 Å resolution. Data were processed using HKL2000 (Otwinowski and Minor 1997). Molrep from the CCP4 suite was used for molecular replacement to obtain phases (Collaborative Computational Project 1994; Vagin and Teplyakov 1997). The position of the MBP molecule was obtained using residues 1–370 of molecule A from the Protein Data Bank coordinates 1HSJ (Liu et al. 2001). Clear density was visible at this point for the β-propeller domain of RACK1A. RACK1A was positioned by molecular replacement using residues 101–408 of platelet-activating factor acetylhydrolase IB β subunit from coordinates 1VYH (Tarricone et al. 2004). This resulted in correct positioning of the model into the density, but rotated by one WD domain

with respect to the sequence alignment. This was corrected by a manual superposition of the starting coordinates of RACK1 onto the molecular replacement solution using O (Jones et al. 1991). The final structure was obtained by iterative cycles of model building and refinement in O (Jones et al. 1991) and CNS (Brunger et al. 1998). Geometry of the structure was assessed using Molprobit (Lovell et al. 2003). Data statistics are shown in Table 1. All structural figures were created using Molscrip (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

Plants, growth conditions, and stress treatments

A. thaliana ecotype Col-0 and mutant RACK1A plants were grown in 4-inch round pots filled with a mixture of metromix 510 soil and vermiculite (3:1) in a growth chamber at 22°C, with light intensity of 100 µmol m^{−2} s^{−1} and 70% relative humidity under long-day conditions (16-h light/8-h dark cycle). The isolation and characterization of RACK1A plants have been described previously (Chen et al. 2006). To induce water stress, wild-type (WT) and mutant plants were grown with sufficient water for 4 wk, and then water was withheld for 12 d to induce stress conditions.

Water loss measurements

Rosette leaves of mutant and WT plants growing under normal conditions for 4 wk were detached and weighed immediately on

Table 1. Crystallographic data statistics

Data set	MBP–RACK1A
Unit cell	$a = 57.8 \text{ \AA}$, $b = 63.3 \text{ \AA}$, $c = 70.1 \text{ \AA}$, $\alpha = 70.4^\circ$, $\beta = 88.4^\circ$, $\gamma = 73.8^\circ$
Space group	<i>P</i> 1
Resolution (Å)	50.0–2.4
No. of observations	142,843
Unique reflections	34,464
$R_{\text{sym}}(\%)$ (last shell)	8.1 (45.2)
$I/\sigma I$ (last shell)	12.4 (2.1)
Mosaicity	0.83–1.2
Completeness (%) (last shell)	98.8 (95.5)
Refinement statistics	
$R_{\text{cryst}}(\%)^a$	21.0
$R_{\text{free}}(\%)^b$	25.1
# of waters	271
B-factors (average)	
Protein	50.1
Water	50.1
Maltose	38.7
RMSD from ideal	
Bond length (Å)	0.007
Bond angle (°)	1.3
Dihedral angle (°)	23.6
Improper angle (°)	0.81
Ramachandran statistics ^c	
Residues in:	
Favored (98%) regions (%)	93.4
Allowed (>99.8%) regions (%)	99.1

^a $R_{\text{cryst}} = \sum ||F_o| - |F_c|| / \sum |F_o|$ calculated from working data set.

^b R_{free} was calculated from 5% of data randomly chosen not to be included in refinement.

^cRamachandran results were determined by MolProbit.

a piece of weighing paper, then placed on a laboratory bench and weighed at designated time intervals. Three replicates were done for each line. The percentage of loss of fresh weight was calculated based on the initial weight of the plants.

Protein Data Bank deposition

The coordinates have been deposited in the PDB with ID code 3DM0.

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