Supplementary Materials

Structural and functional insights into the p160 Rho-associated coiled-coil-containing protein kinase ROCK*

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* *Running title*: New structural framework for RHO kinase
[#] Current address: Soheila Rezaei Adariani, Department of Neurosurgery, City of Hope Beckman Research Institute and Medical Center, Duarte, CA, USA; Sicai Zhang, Shanghai Institute for Advanced Immunochemical Studies, ShanghaiTech University, 201210 Shanghai, China; Yang Nie, Institute of Future Agriculture, Northwest Agriculture and Forestry University, Yangling, Shaanxi 712100, China.

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59 **Table S1.** X-ray data collection and refinement statistics of ROCK$^{HR1(Se\text{-}Met)}$

Data collection	
Wavelength (Å)	0.979240
Resolution (Å)	37.0-2.2
Number of observed reflections	116242
Number of unique reflections	30449
Completeness (%)	99.4 (98.6)
Redundancy	3.82 (3.78)
R_{merge}(%)	8.3 (55.2)
I/σ(I)	11.57 (2.83)
Space group	C2221
Unit cell	48.26, 87.07, 148.22 90.00° 90.00° 90.00°
Phasing	
Selenium-atom sites	5 (final model 8)
Figure of merit (acentric/centric)	0.69/0.64
Resolution range (Å)	19.92-2.50
Refinement	
Resolution	37.0-2.2
R_{work}/R_{free} (%)	21.8/28.9
Number of reflections in test set	1106
Average B-factor (Å^2)	39.0
Deviations from ideal geometry	
Bond lengths (Å)	0.010
Bond angles (°)	1.14
Ramachandran plot (%)	99.6

Values in parentheses are for the highest resolution shell (2.3-2.2); R_{free} calculated with 5 % of the data that were not used for refinement.

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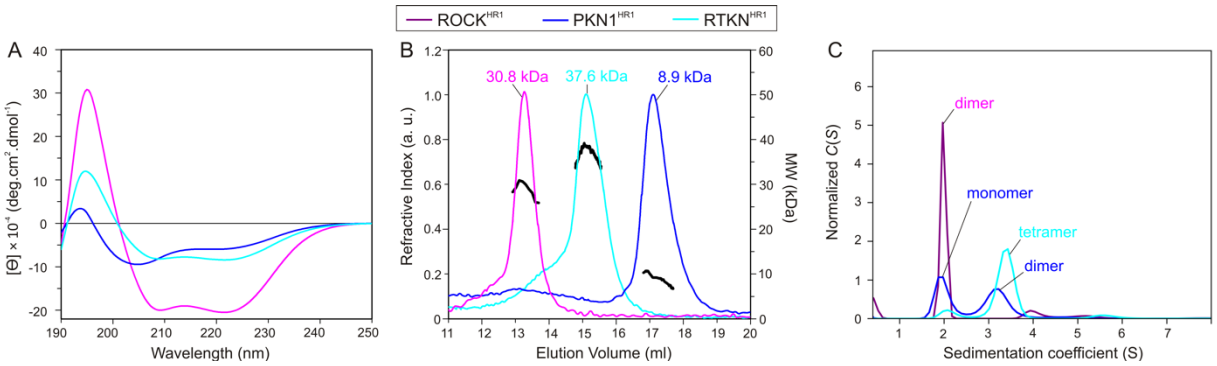

Figure S1. Biophysical properties of the HR1 domains of ROCK, PKN1, and RTKN. (**A**) Far-ultraviolet circular dichroism (Far-UV CD) spectra. The CD data were adjusted for molar ellipticity (Θ), taking into account the concentration and molar mass of the protein samples. Each spectrum is the average of ten replicate scans. The spectra exhibit typical α-helical profiles, displaying a minimum at 208 nm and 222 nm, except for PKN1^{HR1}, which exhibits a minimum at 204 nm instead of 208 nm. (**B**) Size exclusion chromatography-multiangle light scattering (SEC-MALS) analysis. The protein elution profilby refractive index (RI). The horizontal black line indicates the molar mass of the respective proteins. (**C**) Sedimentation velocity (SV) analysis. This is a comparison plot of the diffusion-corrected integral sedimentation coefficient distributions obtained from van Holde-Weischet analyses. PKN1^{HR1} is shown in blue, ROCK^{HR1} in magenta, and RTKN^{HR1} in cyan. All data are summarized in Table 1.

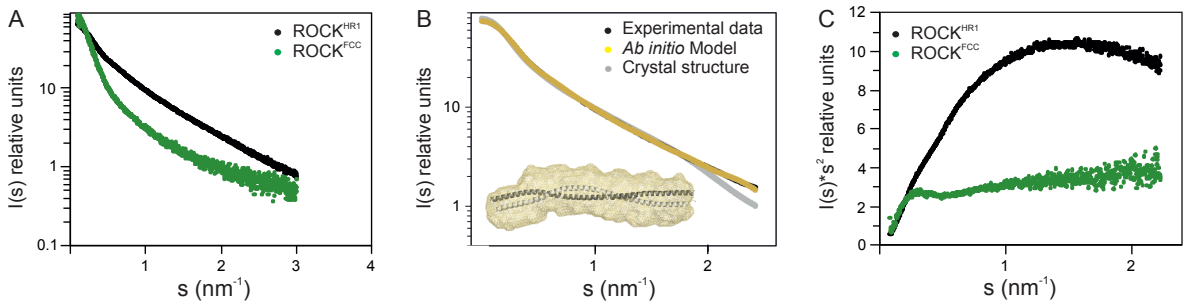

Figure S2. Structural properties of ROCK^{HR1} in solution obtained by small-angle X-ray scattering (SAXS). (**A** The forward scattering intensities were recorded for ROCK^{HR1} (in black) and ROCK^{FCC} (in green). The experimental data are shown as dots. The scattering from *ab initio* models computed by DAMMIF, and the fit from the crystal structure are shown as continuous white lines. The inset shows the ROCK^{HR1} crystal structure superimposed on the averaged ROCK ^{HR1} bead model (in orange), which was calculated using DAMMIF. (**B**) This shows the experimental scattering data, a theoretical scattering pattern calculated from the crystal structure of ROCK^{HR1} using CRYSOL, and a ROCK^{HR1} *ab initio* model calculated from 20 independent DAMMIF runs. The inset shows the SAXS-derived ROCK^{HR1} *ab initio* model superimposed with the ROCK^{HR1} crystal structure. **(C)** Kratky plots of ROCK^{HR1} and ROCK^{FCC}. All data are summarized in Table 1.

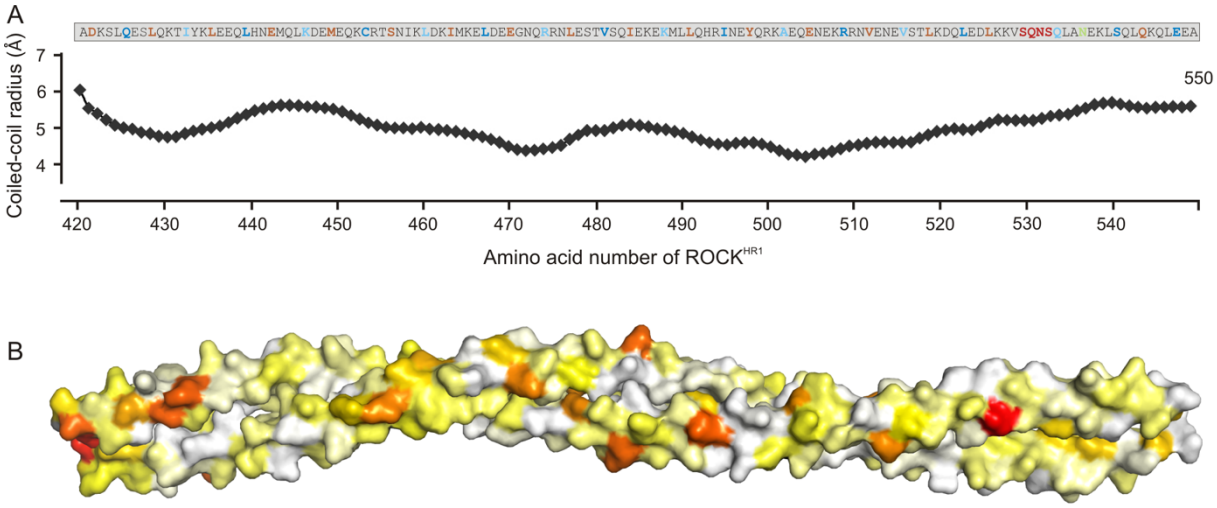

Figure S3. Structural characteristics of ROCK^{HR1} coiled-coil dimer. (**A**) The radius of the coiled-coil is plotted as a function of amino acid number. The amino acid sequence, including the heptad repeats, is shown above. (**B**) The conservation of residues of ROCK^{HR1} is mapped onto the overall surface of the coiled-coil. The color gradient, ranging from white through yellow, orange to red correlates with residue variability, from conserved to most variable.

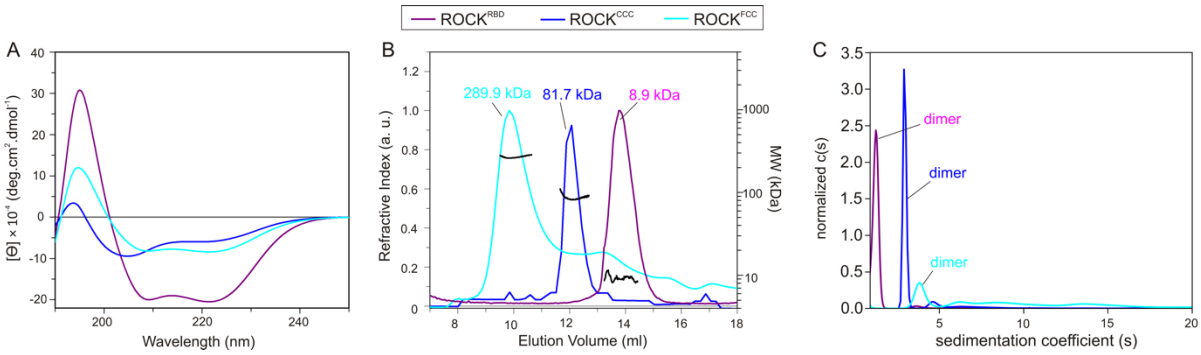

Figure S4. Biophysical properties of ROCK^{RBD}, ROCK^{CCC}, and ROCK^{FCC}. Far-UV CD spectra (**A**), SEC-MALS (**B**), and SV analyses (**C**). Comparison plot of the diffusion-corrected integral sedimentation coefficient distributions obtained from van Holde-Weischet analyses. ROCK^{RBD} is shown in magenta, ROCK^{CCC} in blue, and ROCK^{FCC} in cyan. Analyses were conducted for various fragments of the central amphipathic region of ROCK. All data are summarized in Table 1.

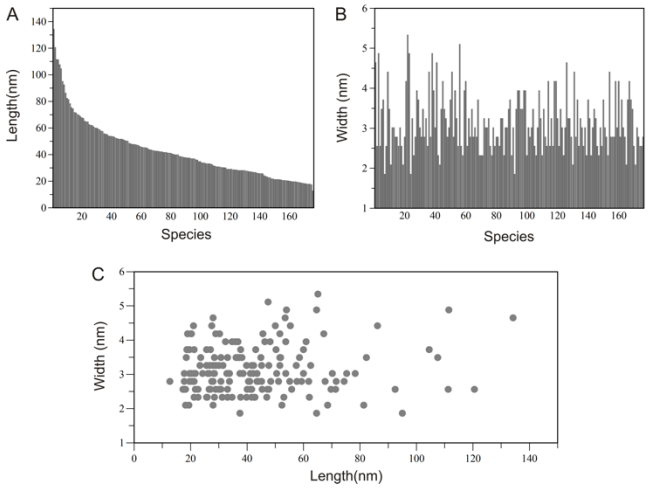

Figure S5. Length and width variations of ROCK^{FCC} molecules. A quantitative analysis of negatively stained ROCK^{FCC} molecules reveals an average length of 42.6 ± 21.7 nm (**A**) and an average width of 3.1 ± 0.7 nm (**B**). The lengths of the rods are ordered from longest to shortest. (**C**) Length-width relationship for all particles.

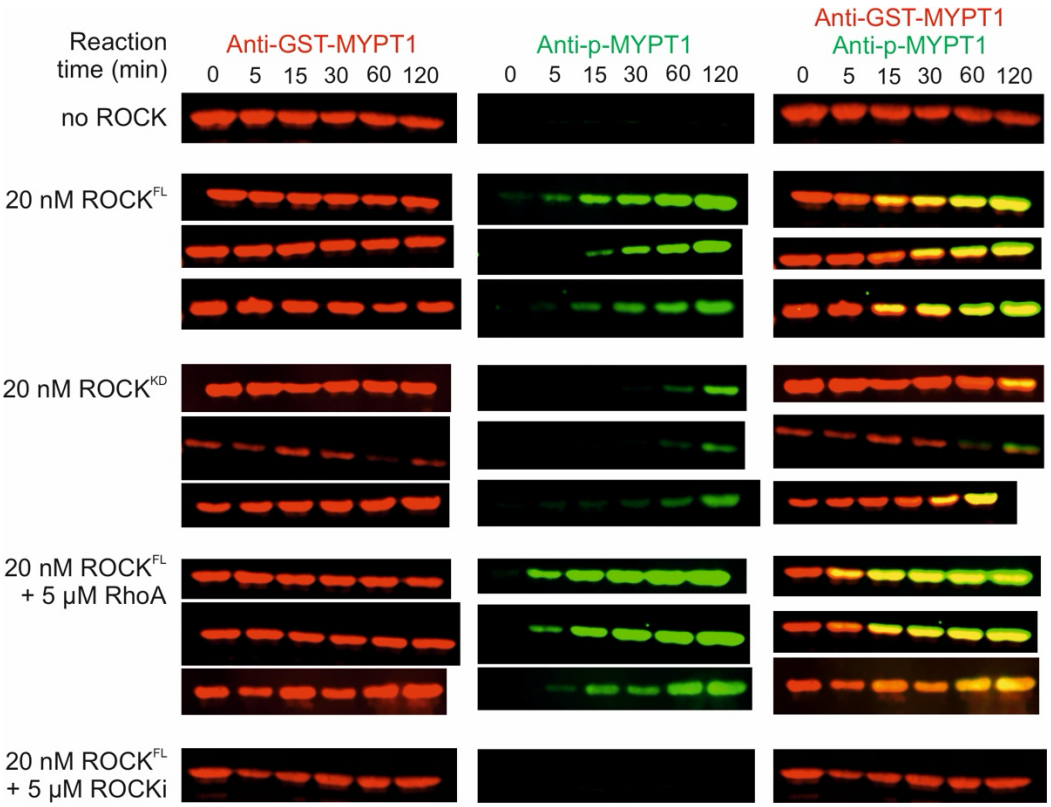

Figure S6. The phosphorylation of MYPT1 by ROCK^{FL} in both the absence and presence of GppNHp-bound RHOA was examined at various time intervals and at a temperature of 25°C. Proteins were purified from the insect cells and *E. coli*. The samples were analyzed by Western blotting using antibodies against GST-MYPT1 and p-MYPT1. As controls, the experiments were performed in the absence of ROCK^{FL}, and with ROCK^{KD}, and in the presence of the ROCK inhibitor Y-27632, all under the same conditions.

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