Supporting Information

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SI Materials and Methods

Protein Expression and Purification. The *ctkA* (*c*ell *t*ranslocating *k*inase *A*) gene (*jhp940*) was cloned from the genomic DNA of *Helicobacter pylori* J99 strain (ATCC 700824D) into the expression vector pET-21b(+) (Novagen), adding a hexa-histidine-containing eight-residue tag to the C terminus of CtkA. The recombinant protein was overexpressed in *Escherichia coli* Rosetta2 (DE3)pLysS cells using Terrific-Broth culture medium. Protein expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and the cells were incubated for additional 18 h at 15 °C following growth to mid-log phase at 37 °C. The cells were lysed by sonication in a lysis buffer [50 mM Tris-HCl at pH 7.9, 500 mM NaCl, and 10% (vol/vol) glycerol] containing 50 mM imidazole followed by centrifugation to remove cellular debris. The supernatant was applied to an affinity chromatography column of nickel-nitrilotriacetic acid-agrose (Qiagen). The protein was eluted with the lysis buffer containing 500 mM imidazole, and the eluted sample was further purified by ion exchange and size-exclusion chromatography using HiTrap-Q ion exchange and HiLoad 16/60 Superdex-200 columns (GE Healthcare Bio-Sciences), respectively. The mutant variants of CtkA were expressed and purified as the wild type as described above.

Crystallization, X-ray Data Collection, and Structure Determination. Because the full-length CtkA fused with a C-terminal fusion tag did not produce crystals, we grew the crystals of CtkAΔC fused with a C-terminal fusion tag. The crystals were grown by the sitting-drop vapor diffusion method at 22 °C by mixing equal volumes (2 μL each) of the protein solution (at 8 $mg\,mL^{-1}$ concentration in 20 mM Hepes at pH 7.5 and 200 mM NaCl) and the reservoir solution. The apo crystals were grown using a reservoir solution consisting of 100 mM Tris-HCl at pH 8.5, 1.5 M ammonium sulfate (Sigma), and 15% (vol/vol) glycerol.

A mercury-substituted crystal was frozen in the cold nitrogen gas stream at 100 K using a cryoprotectant solution consisting of 100 mM Tris-HCl at pH 8.5, 1.5 M ammonium sulfate, and 20% (vol/vol) glycerol. X-ray diffraction data were collected at 100 K on an Area Detector Systems Corporation Quantum 315 CCD area detector system at the BL-4A experimental station of Pohang Light Source. The raw data were processed using the program suit HKL2000 (1). Table S1 summarizes the data collection statistics.

We solved the crystal structure of CtkA by mercury multi-wavelength anomalous diffraction (MAD) phasing at 2.70-Å resolution (Table S1). Ten mercury sites were located with the program SOLVE (2) and were used to calculate the phases with RESOLVE (3). Subsequent manual model building was done using the program COOT (4). The model was refined with the program REFMAC (5) and PHENIX (6) including the bulk solvent correction. Ten percent of the data were randomly set aside as the test data for the calculation of R_{free} (7).

The crystals of ADP-bound CtkA were grown using a reservoir solution consisting of 100 mM sodium acetate at pH 5.5, 0.2 M sodium thiocyanate, and 20% (vol/vol) PEG 3350, while supplementing the protein solution with 2 mM $MgCl_2$ and 10 mM of ADP. ADP-bound CtkA crystals were cryoprotected using the reservoir solution containing 10% (vol/vol) glycerol. The ADP-bound structure was solved by molecular replacement using the apo model of CtkA with the program PHASER (8). We failed to cocrystallize CtkA with AMP-PNP. Therefore, we soaked the ADP-bound crystals in the cryoprotectant solution used for cryoprotection of the ADP-complex crystals after supplementing it with 2 mM $MgCl_2$ and 10 mM of AMP-PNP. Table S1 summarizes the refinement statistics.

In Vitro Kinase Assay. In vitro kinase labeling reactions were carried out in 20 μL reaction buffer consisting of 20 mM Hepes, pH 7.4, 2 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 0.20 μM ATP, and 0.033 μM [$\gamma - {}^{32}P$] ATP (5 $\mu Ci/\mu l$). Purified CtkA or its mutant variants were added to start the reaction, which lasted for 10 min at 30 °C and was stopped by the addition of sodium dodecyl sulfate loading solution. The products were analyzed by electrophoresis in 15% denaturing polyacrylamide gels, and the dried gels were exposed to X-ray films (Agfa) for 24 h.

Cell Culture, Transfection, and Western Blotting. Human gastric epithelial cancer cell (AGS cell) (ATCC CRL 1739), HeLa cells, and Thp1 cells were grown in RPMI medium 1640 (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 1 mmol/L sodium pyruvate (Gibco), and 100 units/mL penicillin-streptomycin (Gibco). Transfection of the cells with the plasmid p3XFLAG-CMV-10 containing CtkA or its mutants was performed using the LipofectAMINE 2000 (Invitrogen).

The protein levels were analyzed by Western blotting. The primary antibodies used for Western blotting were anti-FLAG (Cell Signaling Technologies), anti-phospho-Ser276 p65 (Cell Signaling Technologies), anti-p65 (Cell Signaling Technologies), antitopoisomerase I (Cell Signaling Technologies), and anti-β-actin (Abcam).

Cellular Translocation Analysis. To monitor intracellular translocation of CtkA, we treated HeLa cells with EGFP-full-length CtkA and EGFP-CtkAΔC (5 and 10 μM) for 2 h at 37 °C. After incubation, cells were washed three times with PBS, and EGFP fluorescence was detected by FACS analysis (FACS Calibur, BD Biosciences). For confocal microscopy, HeLa cells were treated with 10 μM of EGFP-CtkA proteins for 2 h at 37 °C and then washed with PBS. The cells were fixed with 4% paraformaldehyde containing 0.2% Triton X-100. The cellular translocation of the EGFP-fused CtkA proteins was visualized using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

Electrophoretic Mobility Shift Assay. The double-stranded NF-κB consensus oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with the Redivue adenosine 5′-[γ-^{32}P] triphosphate triethylammonium salt (GE Healthcare). Nuclear extracts (10 μg total protein) of the AGS cells, in which the full-length wild-type and D155Q/D179Q double mutant CtkA were expressed, were incubated with 1 × Tris buffered saline and ^{32}P-labeled NF-κB probe for 20 min at 37 °C and then DNA–protein complexes were resolved on a 6% nondenaturing polyacrylamide gel at 100 V in 0.5 × Tris/Borate/EDTA buffer. The gel was exposed to a BAS-MS2040 plate (Fujifilm) for 30 min, and signals were detected using a BAS-2500 Phosphor Imager (Fujifilm).

Secreted Alkaline Phosphatase (SEAP) Reporter Assay. For the SEAP assay, a reporter plasmid carrying the NF-κB promoter (pNF-κB-SEAP; 1.0 μg; Clontech), the pGAL plasmid (1.0 μg), and the p3XFLAG-CMV-10 vector containing the full-length wild-type CtkA or its mutants (CtkAΔC, D155Q, D179Q, or D155Q/D179Q; 1.0 μg) were cotransfected to AGS cells. After 48 h of cotransfection, the culture medium was collected for the SEAP

assay, which was performed using the Great EscAPe SEAP Fluorescence Detection Kit (Clontech).

Enzyme-Linked Immunosorbent Assay. Thp1 cells were seeded in six-well tissue culture plates at a density of approximately 1 million/mL. To differentiate them into the adherent macrophage-like cell state, Thp1 cells were treated with 5 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma). After 48 h of PMA treatment, the PMA-containing medium was aspirated, and differentiated cells were washed with RPMI medium 1640. These cells were later treated with full-length CtkA, CtkAΔC, and full-length D155Q/D179Q double mutant, respectively, at 0.1, 0.5, and 1.0 μg/mL concentrations. The proteins were treated with 20 μg/mL of polymyxin B (Sigma) to circumvent the effects of any possible endotoxin contamination. After treatment, cells were kept in RPMI medium 1640 supplemented with 100 units/mL penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Culture supernatants were collected after 48 h and stored at −80 °C until analyzed. The induction of TNF-α was assessed using human TNF-α colorimetric ELISA Kit (Thermo Scientific).

1. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
2. Terwilliger TC, Berendzen J (1999) Automated MAD and MIR structure solution. *Acta Crystallogr D* 55:849–861.
3. Terwilliger TC (2000) Maximum-likelihood density modification. *Acta Crystallogr D* 66:213–221.
4. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D* 60:2126–2132.
5. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* 53:240–255.
6. Adams PD, et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D* 66:849–861.
7. Brünger AT (1992) The free R-value: A novel statistical quantity for assessing the accuracy of crystal structures. *Nature* 355:472–474.
8. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser crystallographic software. *J Appl Cryst* 40:658–674.

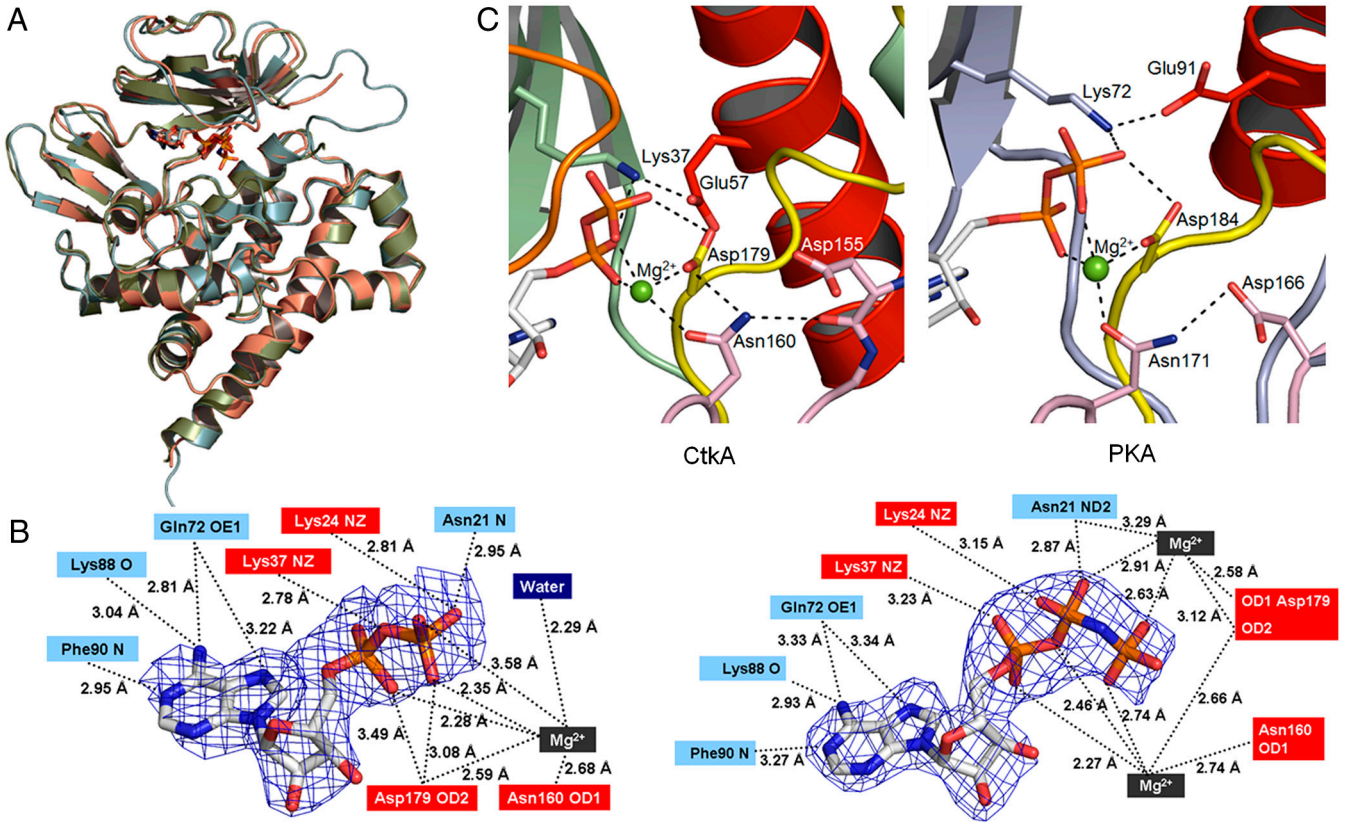

Fig. S1. Ligand binding in the active site of CtkA. (*A*) Superposition of apo (green), ADP-bound (cyan), and AMP-PNP-bound (pink) structures of CtkA. The bound ADP and AMP-PNP molecules are shown in sticks. (*B*) Binding modes of the bound ligands [ADP (*Left*) and AMP-PNP (*Right*)]. Invariantly conserved residues are boxed in red, and *Fo–Fc* electron density maps of the bound ligands are contoured at 2.5σ. (*C*) Catalytically important residues of CtkA and PKA are shown in sticks, and αC helix is colored in red for both proteins.

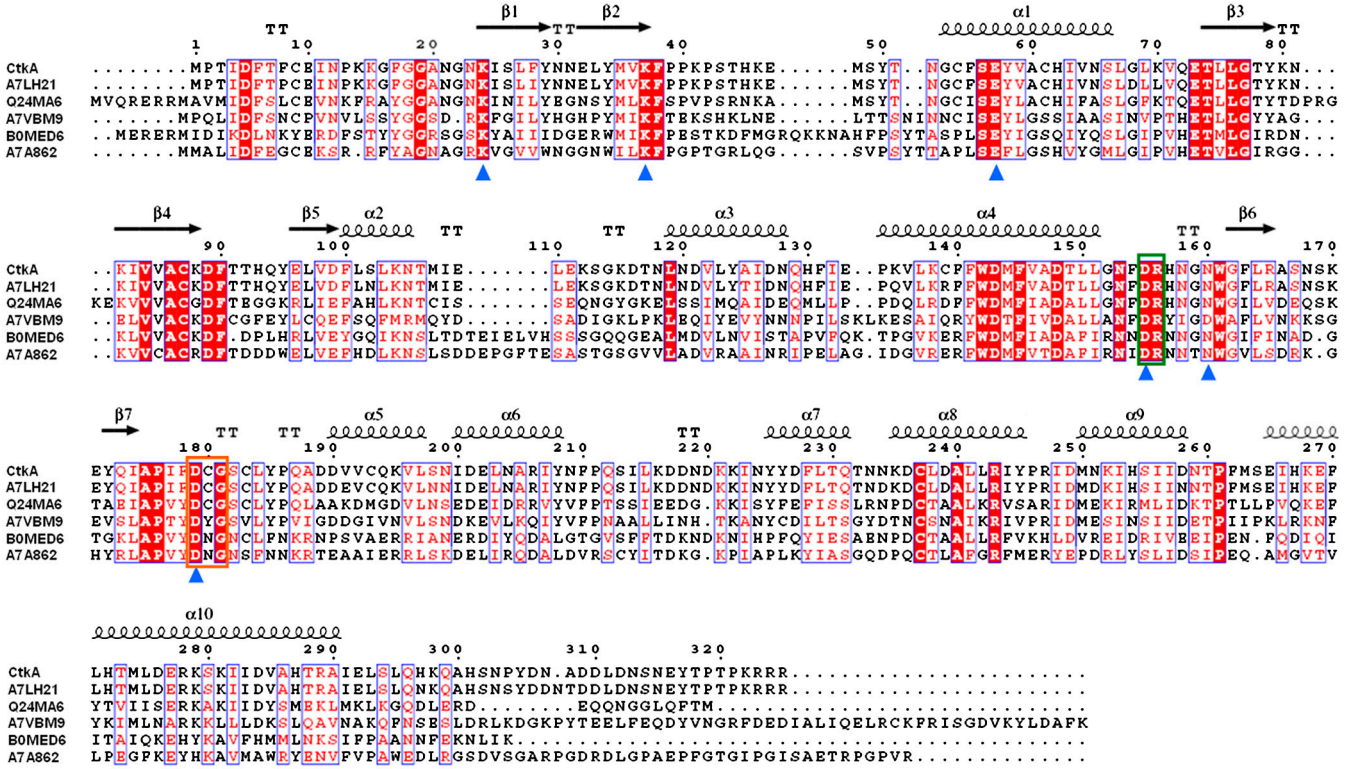

Fig. S2. Sequence alignment of CtkA and its homologs. SWISS-PROT accession codes and the bacterial origins of CtkA homologs in this figure are as follows: A7LH21, *Helicobacter cetorum*; Q24MA6, *Desulfitobacterium hafniense* Y51; B8G0K5, *Desulfitobacterium hafniense* DCB-2/DSM 10664; C0CGZ0, *Blautia hydrogenotrophica* DSM 10507; C0C7E9, *Subdoligranulum variabile* DSM 15176; C2KXV7, *Oribacterium sinus* F0268; A7VBM9, *Clostridium* sp. L2-50; C1UBY9, *Eggerthella lenta* DSM 2243; B0MED6, *Anaerostipes caccae* DSM 14662; B9Y733, *Holdemania filiformis* DSM 12042; A7A862, *Bifidobacterium adolescentis* L2-32; A7VBN1, *Clostridium* sp. L2-50. Conserved residues are enclosed in blue boxes, and the highly conserved DR and DxG motifs are boxed in green and orange, respectively. The secondary structures of CtkA are indicated above the sequence and TTs denote tight β-turns. The alignment figure was made using the program ClustalX (1) and ESPript (2).

1. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882.
2. Gouet P, Courcelle E, Stuart DI, Métoz F (1999) ESPript: Analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15:305–308.

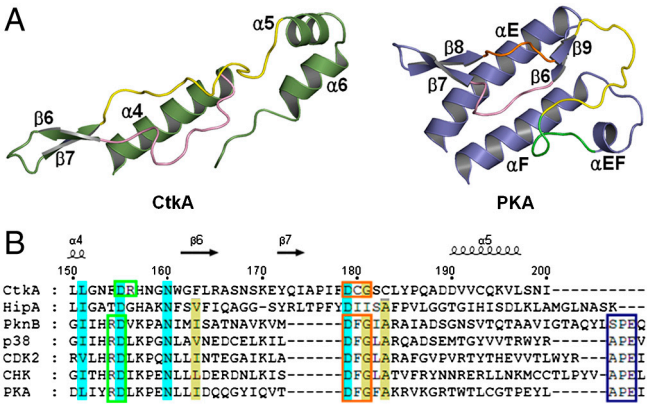

Fig. S3. The conformation of CtkA activation loop. (*A*, *Left*) The catalytic loop and the activation loop of CtkA. The catalytic loop (residues 152–162) and the activation loop (residues 175–189) are colored in pink and yellow, respectively. (*Right*) The catalytic loop and the activation segment of PKA. The catalytic loop (residues 166–171), the Mg^{2+} binding loop (residues 183–186), the activation loop (residues 189–201), and the $P+1$ loop (residues 202–206) are colored in pink, orange, yellow, and green, respectively. (*B*) Alignment of the activation segment sequences of proteins kinases. Highly conserved RD (DR in CtkA), DFG (DxG in CtkA), and APE motifs (missing in CtkA) are boxed in green, orange, and blue, respectively.

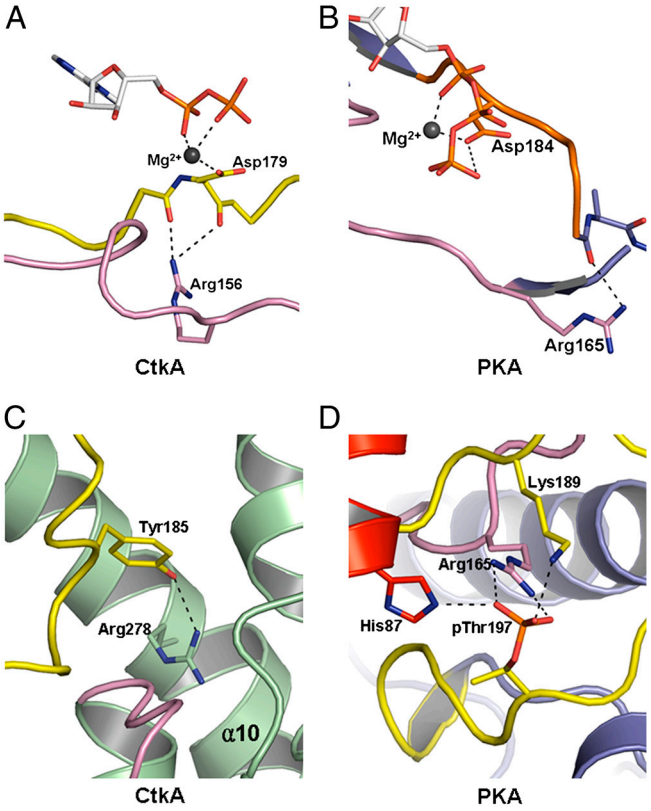

Fig. S4. Stabilization of activation segments in CtkA and PkA. (*A*) DR and DxG motifs of CtkA. The catalytic loop and the activation loop are colored in pink and yellow, respectively. (*B*) RD and DFG motifs of PKA. The catalytic loop and the Mg^{2+} binding loop are colored in pink and orange, respectively. (*C*) Stabilizing interactions of activation loop (yellow) in CtkA. (*D*) Stabilizing interactions of activation loop (yellow) in PKA.

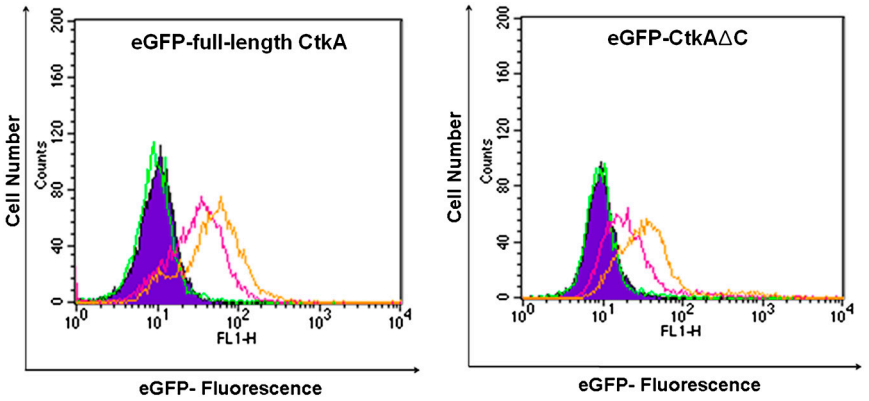

Fig. S5. Fluorescence intensity detected by flow cytometer after treatment of HeLa cells with EGFP-fused CtkA for 2 h. Cells treated with 5 μM (magenta) or 10 μM (orange) of EGFP-fused full-length CtkA (*Left*), or 10 μM of EGFP-fused CtkAΔC (*Right*) showed significant intracellular translocation, whereas 5 μM of EGFP alone (green) did not show intracellular fluorescence.

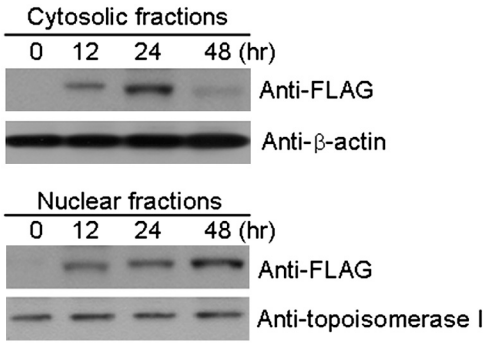

Fig. S6. Nuclear translocation of transiently expressed CtkA. Lanes correspond to the Western blot results of the cytosolic (*Upper*) and nuclear (*Lower*) fractions of the AGS cells transfected with the plasmid p3XFLAG-CMV-10 containing the full-length wild-type ctkA gene, after 0, 12, 24, and 48 h of transfection, respectively.

Table S1. Data collection, phasing, and refinement statistics

A. *Data collection and phasing*

Space group: C2; Unit cell parameters: $a = 256.45$ Å, $b = 56.67$ Å, $c = 47.53$ Å, $\beta = 92.71°$ (for peak data)				
Dataset	Hg λ1 (peak)	Hg λ2 (inflection 1)	Hg λ3 (inflection 2)	Hg λ4 (remote)
X-ray wavelength (Å)	1.0064	1.0084	1.0087	0.9918
Resolution range (Å)	20–2.70	20–2.70	20–2.70	20–2.70
Total/unique reflections	155,846/19,050	155,516/18,821	154,571/18,938	153,281/18,843
Completeness (%)	99.8 (96.5)*	99.8 (97.0)*	99.9 (99.1)*	99.6 (94.3)*
R_{merge} (%)†	9.4 (31.1)†	9.6 (28.2)†	9.5 (26.6)†	9.8 (36.2)†
R_{iso} (%)‡		6.9 (9.2)‡	9.8 (11.7)‡	11.6 (13.6)‡
f'/f'' (e-)	−13.57/10.07	−16.66/10.10	−16.94/10.12	−10.00/9.81
Figure of merit§ for MAD phasing: 0.43/0.73 for 20–2.70 Å data (before/after density modification)				

B. *Refinement*

Dataset	Apo	ADP complex	AMP-PNP complex
Space group	C2	P1	P1
a, *b*, *c* (Å)	255.20, 56.98, 48.67	58.83, 69.56, 75.64	60.12, 74.83, 75.83
α, β, γ (°)	90, 93.28, 90	105.99, 108.82, 92.05	78.85, 71.34, 85.44
X-ray wavelength (Å)	1.0000	1.0000	1.0000
Resolution range (Å)	20–2.00	20–2.50	20–2.90
Total/unique reflections	255,518/47,319	118,812/36,499	99,850/27,093
Completeness (%)	100.0 (100.0)¶	97.9 (95.2)¶	98.4 (94.1)¶
R_{merge} (%)¶	8.2 (35.8)¶	7.1 (24.4)¶	13.9 (36.0)¶
R_{work}/R_{free}‖ (%)	19.4/23.0	17.2/26.1	18.9/28.0
No. of protein atoms/mean B-factor (Å^2)	4,490/42.6	9,390/35.6	9,090/62.9
No. of solvent atoms/mean B-factor (Å^2)	326/43.0	309/28.8	59/58.4
No. of ligand atoms/mean B-factor (Å^2)	-	112/27.4	127/50.5
rms deviation from ideal geometry			
Bond lengths (Å)	0.007	0.008	0.009
Bond angles (°)	0.99	1.21	1.27
Ramachandran plot			
Favored region (%)	97.4	95.5	92.8
Allowed region (%)	2.6	4.5	7.2
Outlier region (%)	0.0	0.0	0.0

*Completeness for $I/\sigma(I) > 0$, with the numbers in parentheses for the high-resolution shell (2.75–2.70 Å).

†$R_{merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections, and $\sum_i$ is the sum over i measurements of reflection h. Numbers in parentheses reflect statistics for the last shell (2.75–2.70 Å).

‡$R_{iso} = \sum ||F_{PH}| - |F_P|| / \sum |F_P|$, where F_{PH} and F_P are the derivative (λ2, λ3 or λ4) and native (λ1) structure factors, respectively. Numbers in parentheses are for the last shell (2.75–2.70 Å).

§Figure of merit $= \langle |\sum P(\alpha) e^{i\alpha} / \sum P(\alpha)| \rangle$, where α is the phase angle and $P(\alpha)$ is the phase probability distribution.

¶Values in parentheses refer to the highest resolution shells (2.07–2.00 Å, 2.54–2.50 Å, 2.95–2.90 Å for apo, ADP complex, and AMP-PNP complex, respectively).

‖$R = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$, where R_{free} is calculated for a randomly chosen 10% of reflections, which were not used for structure refinement, and R_{work} is calculated for the remaining reflections.