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Conformational Change of Human Checkpoint Kinase 1 (Chk1) Induced by DNA Damage*

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Phosphorylation of Chk1 by ataxia telangiectasia-mutated and Rad3-related (ATR) is critical for checkpoint activation upon DNA damage. However, how phosphorylation activates Chk1 remains unclear. Many studies suggest a conformational change model of Chk1 activation in which phosphorylation shifts Chk1 from a closed inactive conformation to an open active conformation during the DNA damage response. However, no structural study has been reported to support this Chk1 activation model. Here we used FRET and bimolecular fluorescence complementary techniques to show that Chk1 indeed maintains a closed conformation in the absence of DNA damage through an intramolecular interaction between a region (residues 31–87) at the N-terminal kinase domain and the distal C terminus. A highly conserved Leu-449 at the C terminus is important for this intramolecular interaction. We further showed that abolishing the intramolecular interaction by a Leu-449 to Arg mutation or inducing ATR-dependent Chk1 phosphorylation by DNA damage disrupts the closed conformation, leading to an open and activated conformation of Chk1. These data provide significant insight into the mechanisms of Chk1 activation during the DNA damage response.

The genome integrity of eukaryotic cells is threatened by various sources of DNA damage arising in the environment (*e.g.* UV light) and/or within the cell (*e.g.* free radical species). Cells have evolved complex networks termed the DNA damage response (DDR)[4] to counter these assaults by inhibiting cell division and repairing damaged DNA (1, 2). Central to the DDR is the Ser/Thr checkpoint kinase Chk1, which plays a key role in responding to a wide range of DNA-damaging agents (3). Activation of Chk1 requires its phosphorylation at two conserved sites, Ser-317 and Ser-345, by the upstream kinase ataxia telangiectasia-mutated and Rad3-related (ATR) (4–6). Activated Chk1 then phosphorylates a number of downstream targets to control the cell cycle transition and facilitate DNA damage repair (3). However, a key question remains: how does phosphorylation lead to Chk1 activation in cells?

A crystal structure of the N-terminal kinase domain of human Chk1 revealed that the catalytic site adopts an active conformation without the need for phosphorylation of a Thr residue at the kinase domain as with cyclin-dependent kinases (7). However, the Chk1 protein displays only a low level of basal activity and does not trigger a checkpoint response under normal growth conditions (5, 6). This suggests that the open conformation of the catalytic site of Chk1 is inhibited in the absence of DNA damage. Studies from several laboratories showed that the C-terminal regulatory domain of Chk1 interacts with its N-terminal kinase domain *in vitro* or *in vivo* (8–13), suggesting that Chk1 may form a "closed" conformation. This intramolecular interaction and the resulting closed conformation make it likely that the C terminus of Chk1 provides a physical hindrance to the open conformation of its catalytic site and restrains its activity under normal growth conditions. ATR-dependent phosphorylation of Chk1 at Ser-317 and Ser-345 (or *Xenopus* Ser-344) correlates with checkpoint activation (4–6, 14–16). The kinase domain of human Chk1, which lacks the C-terminal half, including the two ATR sites, displayed much stronger catalytic activity than the full-length protein *in vitro* (7), suggesting that phosphorylation *per se* is not required for robust catalytic activity of Chk1. In addition, recent studies suggest that Ser-317 phosphorylation is required for phosphorylation at Ser-345 and that maximal phosphorylation at Ser-345 is essential for the full-scale checkpoint activation (6, 8, 9, 17, 18). Further, mutating conserved residues in the kinase-associating domain located at the C terminus of Chk1 results in Chk1 activation without ATR-dependent phosphorylation at Ser-317 or Ser-345 (19–21). These findings imply that ATR-dependent phosphorylation may just function as a trigger to disrupt the closed conformation of Chk1 so that the catalytic site is exposed to downstream substrates (Fig. 1*A*).

Although this conformational change model of Chk1 is compelling, several studies have challenged its validity. First, yeast two-hybrid screening failed to detect a direct interaction between the N and C termini of *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* Chk1 (22, 23). Second, although the conformational change model indicates a negative role of the

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[4] The abbreviations used are: DDR, DNA damage response; ATR, ataxia telangiectasia-mutated and Rad3-related; BiFC, bimolecular fluorescence complementary; CFP, cyan fluorescent protein; CPT, camptothecin.

C-terminal regulatory domain in Chk1 activation, studies from *S. pombe* suggest that the C terminus also positively regulates its activation (24). Third, a recent report suggested an intermolecular but not an intramolecular interaction of human Chk1, which is achieved through the interaction between a short splicing variant of Chk1 and full-length Chk1 (25). These conflicting findings suggest that a thorough investigation of the Chk1 activation mechanism is needed.

We reasoned that the best way to resolve this issue is to study the structural properties of the Chk1 full-length protein. In this study, we used both FRET and BiFC, two widely used techniques that are powerful in delineating protein conformational changes, to examine the Chk1 full-length protein conformation both under normal growth conditions and during DNA damage. Our results confirmed the intramolecular interaction between the N and C termini and a closed conformation of Chk1 under normal growth conditions. We also found that Chk1 does not form an intermolecular complex. We further established that DNA damage or disrupting the intramolecular interaction opens the conformation of Chk1, consistent with its activation during the DDR.

Experimental Procedures

Cell Cultures and Transfection—HEK293T and U2-OS cells, obtained from the ATCC and tested for contamination, were cultured in DMEM with 10% FBS (Thermo Fisher, Grand Island, NY). Cells were transfected with X-tremeGENE HP transfection reagent (Sigma) according to the protocols of the manufacturer.

Plasmid Construction and Mutagenesis—For FRET analysis, we used mTurquoise and SYFP2, the modified versions of cyan fluorescent protein (CFP) and YFP, respectively. SYFP2 and mTurquoise have enhanced brightness and quantum yields and thus are more suitable for FRET than the regular CFP and YFP (26). However, for the purpose of clarity, these are referred to as CFP and YFP in this study. In brief, CFP was engineered at the N terminus of Chk1, and YFP was engineered to the C terminus of the same construct. A linker of two to four Gly residues was added between Chk1 and either CFP or YFP to provide flexibility to these fluorescent proteins. Two steps were used to generate the FRET construct CFP-Chk1-YFP. First, human Chk1 (WT or the L449R mutant) was amplified by PCR using the pCS3 + 6Myc-Chk1 (WT or L449R) (8) as the template. The restriction enzyme digestion sites EcoRI and AgeI were added to the forward (GCCGAATTCATGGCAGTGCCCTTTGTGGAAGACTG) and reverse (GGCACCGGTCCACCACCTCCTGTGGCAGGAAGCCAAACCTTCTG) primers, respectively. The resulting PCR products were digested with EcoRI/AgeI and ligated to the same sites in the pYFP-N1 vector to produce the WT or L449R mutant Chk1-YFP construct. Next, the full-length CFP was amplified using forward (NheI) GGCGCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTC and reverse (HindIII) GGCAAGCTTCACCACCTCCCTTGTACAGCTCGTCCATGCCGAG primers. Then the PCR product was digested and ligated to the same sites of the Chk1-YFP construct to generate the WT or L449R mutant CFP-Chk1-YFP constructs.

To generate BiFC constructs, Chk1 (WT or L449R mutant) was amplified as described above but with different forward (HindIII, GCCAAGCTTATGGCAGTGCCCTTTGTGGAAGACTG) and reverse (KpnI, GCCGGTACCTCCACCTCCTGTGGCAGGAAGCCAAACCTTC) primers. The N-terminal (1–155) and C-terminal (156–239) CFPs were PCR-amplified using the N-CFP forward (NheI, GCCGCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTC), N-CFP reverse (HindIII, GCCAAGCTTTCCACCTCCGGCGGTGATATAGACGTTGTCGC), CFP-C forward (KpnI, GCCGGTACCGACAAGCAGAAGAACGGCATCAAGG), and CFP-C reverse (EcoRI, GCCGAATTCTTACTTGTACAGCTCGTCCATGCCG) primers. Then the PCR products N-CFP, Chk1 (WT or L449R), and CFP-C were ligated into the accepting vector pcDNA3.1(+) to create the pcDNA3.1-N-CFP-Chk1, pcDNA3.1-Chk1-CFP-C, and pcDNA3.1-N-CFP-Chk1 (WT or L449R)-CFP-C constructs.

To generate the double L449R/S345A mutants, the CFP-Chk1-YFP L449R (for FRET) or the N-CFP-Chk1-CFP-C L449R (for BiFC) construct was used to perform mutagenesis using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA). The following primers were used: forward, CAAGGGATCAGCTTT**G**C**T**CAGCCCACATGTCC; reverse, GGACATGTGGGCTG**A**G**C**AAAGCTGATCCCTTG.

To generate deletion mutants of the Chk1 kinase domain, pCS3 + 6Myc-Chk1 (WT) was used as the template to perform PCR reactions with the following primers: 1–87 forward (BglII), GCCAGATCTATGGCAGTGCCCTTTGTGGAAGAC; 1–87 reverse (XbaI), GCGTCTAGATCAACAGTACTCCAGAAATAAATATTG; 88–124 forward, (BglII) GCCAGATCTAGTGGAGGAGAGCTTTTTGACAG; 88–124 reverse, (XbaI) GCGTCTAGATCAAATACCATGCAGATAAACCAC; 125–182 forward, (BglII) GCCAGATCTATTGGAATAACTCAC AGGGATATTAAAC; 125–182 reverse (XbaI), GCGTCTAGATCATCTTCTCTTCAGAAGTTCTGG; 183–235 forward (BglII), GCCAGATCTGAATTTCATGCAGAACCAGTTGATG; 183–235 reverse (XbaI), GCGTCTAGATCAATCGATTTTTTTCCAAGGGTTG; 236–265 forward (BglII), GCCAGATCTTCTGCTCCTCTAGCTCTGCTGCAT; and 236–265 reverse (XbaI), GCGTCTAGATCAGTACCATCTATCTTTTTTGATG. These PCR products then were cloned into the BglII/XbaI site of the pCS3 + 6Myc vector. Further deletion of the N-terminal kinase domain (residues 1–87) was carried out with the QuikChange mutagenesis kit (Thermo Fisher) using the following primers: Δ2–30 forward, AGATCTGGGGAATTCATGACTGAAGAAGCAGTC GCA; Δ2–30 reverse, TGCGACTGCTTCTTCAGTCATGAATTCCCCAGATCT; Δ31–60 forward, CTTGCTGTGAATAGAGTAATGCTAAATCATGAAAAT; Δ31–60 reverse, ATTTTCATGATTTAGCATTACTCTATTCACAGCAAG; Δ61–87 forward, GAGATCTGTATCAATAAAAAGCGTGGAAAGCTGAAG; and Δ61–87 reverse, CTTCAGCTTTCCACGCTTTTTATTGATACAGATCTC. All constructs were confirmed by sequencing.

Immunoblotting, Immunofluorescence, and Antibodies—Immunoblotting was performed as described previously (8, 26, 46). Mouse anti-Chk1 (DCS-310, SC-56291) antibodies were from Santa Cruz Biotechnology (Dallas, TX). Rabbit monoclonal anti-Ser(P)345-Chk1 antibody (133D3, 2348) was from Cell

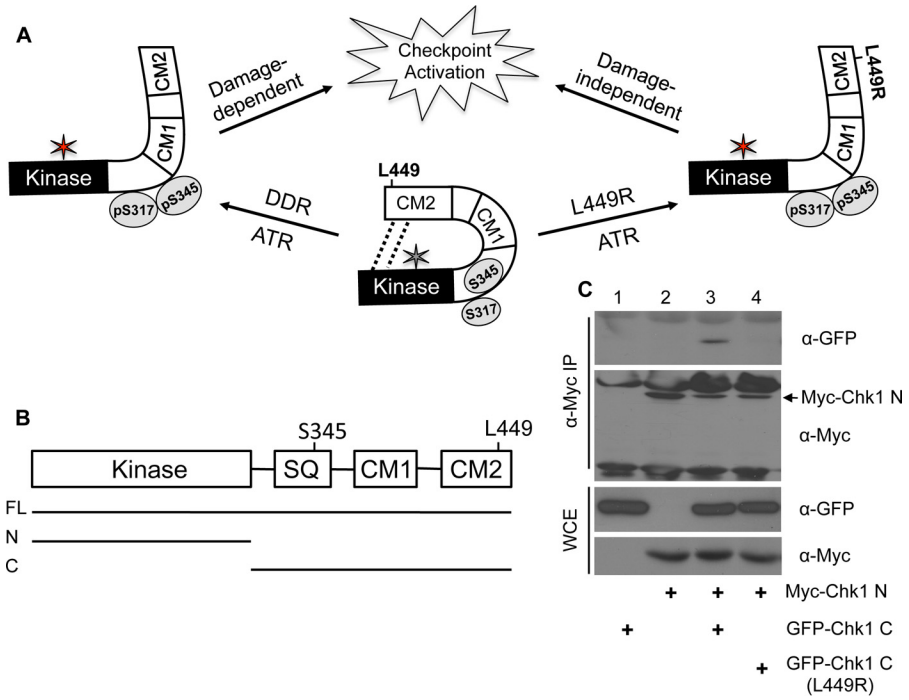

FIGURE 1. **Intramolecular interaction of Chk1.** *A*, model of the conformational change of Chk1 during DDR. The catalytic site, indicated by *stars*, is buried within the protein conformation under normal growth conditions. This closed conformation restrains the activity of Chk1, which is indicated by the *gray star*. DNA damage-induced phosphorylation or mutating Leu-449 to Arg disrupts the closed conformation, leading to the exposure of the catalytic site and subsequent activation of downstream checkpoint signaling. The *red stars* indicates an open active conformation of the catalytic site. *B*, schematic of human Chk1. *SQ*, Ser/Gln cluster; *CM*, conserved motif; *FL*, full-length (476 amino acids); *N*, amino terminus (1–264); *C*, carboxyl terminus (265–476). *C*, HEK293T cells were transfected with the Myc-Chk1 N terminus in the presence or absence of GFP-Chk1 C or GFP-Chk1 C L449R for 48 h, immunoprecipitated (*IP*) with anti-Myc antibodies, and immunoblotted with anti-GFP antibodies. The same membrane was stripped and reprobed with anti-Myc antibodies. The *arrow* indicates the Myc-Chk1 N band, which is just below the band representing the IgG heavy chain. Whole cell extracts (*WCE*) were examined to monitor protein expression levels in cells.

Signaling Technology (Danvers, MA). Mouse anti-Myc (9E10) and rabbit anti-GFP antibodies were described previously (8, 27, 28).

For immunofluorescence, U2-OS cells grown on glass coverslips were transfected with either pcDNA3.1-N-CFP-Chk1, pcDNA3.1-Chk1-C, pcDNA3.1-N-CFP-Chk1 plus pcDNA3.1-Chk1-C, pcDNA3.1-N-CFP-Chk1-CFP-C WT or L449R, or CFP-Chk1 constructs for 48 h at 37 °C. Cells were fixed with 1 ml of PBS containing 3.75% formaldehyde and 0.2 M sucrose for 10 min at room temperature and then washed three times (10 min each) with PBS containing 0.1 M glycine. Cells were permeabilized and blocked with PBS containing 2% normal goat serum (or 10% FBS) and 0.4% Triton X-100 for 20 min at room temperature, washed three times with washing buffer (PBS containing 0.2% Triton X-100 and 0.1% BSA), and then visualized by fluorescence microscopy.

For live cell imaging, U2-OS cells were transfected with pcDNA3.1-N-CFP-Chk1-CFP-C WT or CFP-Chk1 for 48 h at 37 °C, treated with 100 nM CPT for 2 h, washed, and cultured in drug-free medium for an additional 12 h before imaging by fluorescence microscopy.

FRET Analysis—FRET analysis was performed as described previously with modifications (29). HEK293T cells were transfected with either a positive control CFP-YFP or various Chk1 constructs (CFP-Chk1, Chk1-YFP, CFP-Chk1-YFP WT, or CFP-Chk1-YFP L449R) for 48 h. Cells were suspended in PBS, and an equal number of cells was placed in a cuvette for FRET analysis with a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon). FRET was determined by monitoring the sensitized emission of YFP at 535 nm upon exciting CFP. Samples were excited at 425 nm (2-nm slit), and emission spectra were collected (5-nm slit). The spectrum obtained from non-transfected cells was used as the background. Additionally, the spectrum obtained from cells expressing Chk1-YFP was subtracted from spectra obtained from cells expressing any YFP species. All curves were normalized to the maxima for CFP emission (474 nm). All experiments were repeated three times in duplicate.

Results

Leu-449 and the 31–87 Amino Acid Region of the N-terminal Kinase Are Important for the Intramolecular Interaction of Chk1—Several studies have revealed that the N-terminal kinase domain of Chk1 co-immunoprecipitated with the C terminus *in vitro* or *in vivo* (8–13). These data suggest that Chk1 may form a closed conformation in cells under normal growth conditions (Fig. 1*A*). To further confirm this model, we performed experiments to map regions or residues critical for the intramolecular interaction of Chk1. We recently reported that two residues (Gly-448 and Leu-449) at the distal C terminus of Chk1 play important roles in Chk1 activation because mutating either one bypasses the requirement of DNA damage for inducing ATR-dependent phosphorylation of Chk1 and checkpoint activation (8). These results imply that Gly-448 and Leu-449 may be critical in forming the closed conformation of Chk1. To test this idea, we asked whether mutating these two residues

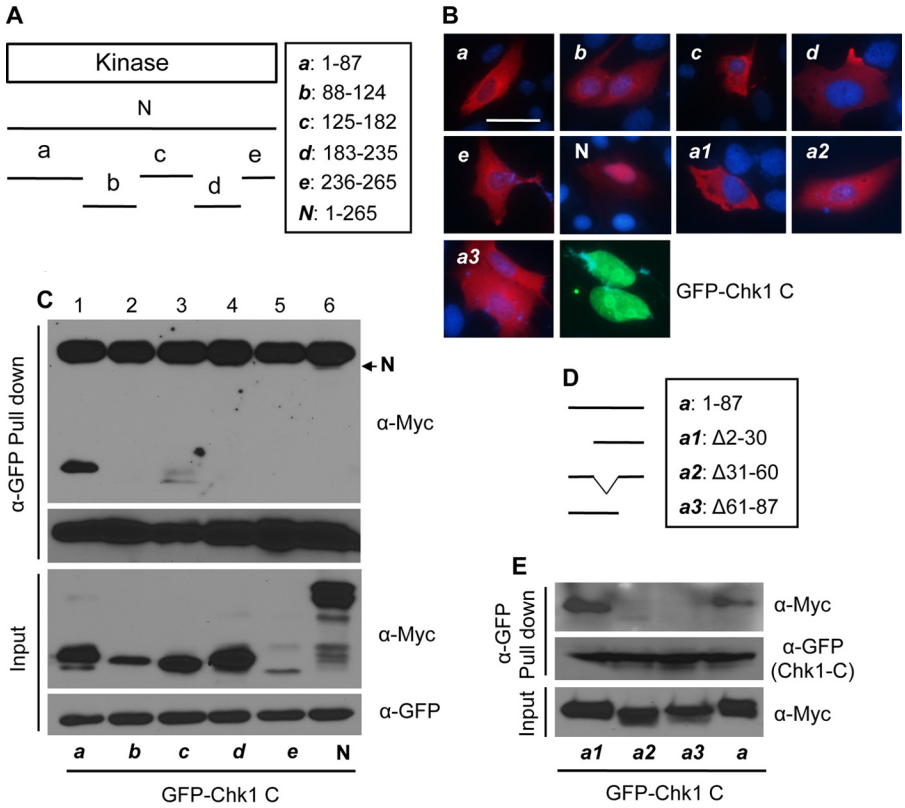

FIGURE 2. **The N-terminal region of the kinase domain interacts with the Chk1 C terminus.** *A*, design of deletion mutants (*a*–*e*) of the N-terminal Chk1 kinase domain (*N*). *Numbers* represent amino acid residues. *B*, representative images of cellular localization of Chk1 fragments and deletion mutants expressed in human osteosarcoma U2-OS cells. Except for GFP-Chk1 C being visualized in *green*, all other Chk1 mutants were stained with the mouse anti-Myc (M2) antibody followed by goat anti-mouse Alexa Fluor 594 secondary antibodies (*red*). The nuclei were stained by DAPI in *blue*. *Letters* represent corresponding Chk1 deletion mutants. *Scale bar* = 10 μm. *C*, Myc-Chk1 fragment mutants targeting different regions of the kinase domain and the GFP-Chk1 C terminus were transfected into HEK293T cells for 48 h. Cells then were collected and lysed. GFP-Chk1 C was immunoprecipitated with anti-GFP antibodies and captured on agarose beads. Cell lysates containing equal amounts of Myc-Chk1 fragment mutants (*a*–*e* and *N*) were added to the anti-GFP beads and incubated at 4 °C for 2 h. Beads were collected by centrifugation and washed three times with lysis buffer, boiled for 5 min with 1× SDS sample buffer, run on SDS-PAGE gels, and probed with anti-Myc antibodies. The same membrane was stripped and reprobed with anti-GFP antibodies. Protein input was also examined. The *arrow* indicates Myc-Chk1 N just below the band representing the IgG heavy chain. *D*, diagram of deletion mutants of the first 87 amino acids of the Chk1 kinase domain as described under "Experimental Procedures." *a1*, *a2*, and *a3* had deletion of residues 2–30, 31–60, and 61–87 of the Chk1 N-terminal kinase domain, respectively. *E*, pulldown experiments carried out in the same manner as in *C*.

(*e.g.* the L449R mutant) would abolish the ability of the C terminus to interact with the N-terminal kinase domain. Thus, we overexpressed the truncated Myc-tagged Chk1 N-terminal kinase domain (Myc-Chk1 N) with GFP-tagged Chk1 C terminus (GFP-Chk1 C or GFP-Chk1 C L449R) (Fig. 1*B*) in HEK293T cells, immunoprecipitated the Myc-Chk1 N proteins with anti-Myc antibodies, and examined the presence of GFP-Chk1 C with anti-GFP antibodies. The results show that Myc-Chk1 N interacted with GFP-Chk1 C (Fig. 1*C*, *lane 3*), as reported previously (8–13). However, we could not detect GFP-Chk1 C L449R in the anti-Myc IP (Fig. 1*C*, *lane 4*), indicating the importance of the Leu-449 residue in the interaction between the N-terminal kinase domain and the C-terminal regulatory domain of Chk1.

To further confirm this intramolecular interaction, we next asked which region of the kinase domain of Chk1 is involved in its interaction with the C terminus. The human Chk1 N-terminal kinase domain consists of 265 residues. We generated five Myc-tagged short fragments targeting different regions of the Chk1 kinase domain (Fig. 2*A*). The Chk1 C terminus mainly resided in the nucleus, whereas the N-terminal kinase was located in both the nucleus and the cytoplasm (Fig. 2*B*), similar to our previous observations (27). However, the N-terminal kinase domain fragments of Chk1 were mainly expressed in the cytoplasm (Fig. 2*B*), likely because of the lack of a functional nuclear localization signal. Therefore, we took an indirect approach to assess the ability of these N-terminal kinase domain fragments to interact with the C terminus. To do so, we overexpressed these fragments and GFP-Chk1 C fusion proteins in HEK293T cells separately. GFP-Chk1 C fusion proteins were then immunoprecipitated with anti-GFP antibodies and used to pull down those small fragments of the Chk1 kinase domain from the cell lysates. Under these particular experimental conditions, the band representing the Myc-Chk1 N ran almost identical to the heavy chain of rabbit IgG. Nonetheless, we repeatedly observed a band running just below the IgG heavy chain (Fig. 2*C*, *lane 6*), confirming the interaction between the N and C termini of Chk1. The middle region (residues 125–183) of the Chk1 kinase domain showed a weak interaction with the C terminus; however, the strongest interaction was observed for the fragment covering the first 87 residues of Chk1 (Fig. 2*C*, *lanes 1* and *3*). To further narrow down the region (residue 1–87) required for the intramolecular interaction, we generated three additional mutants with deletions of

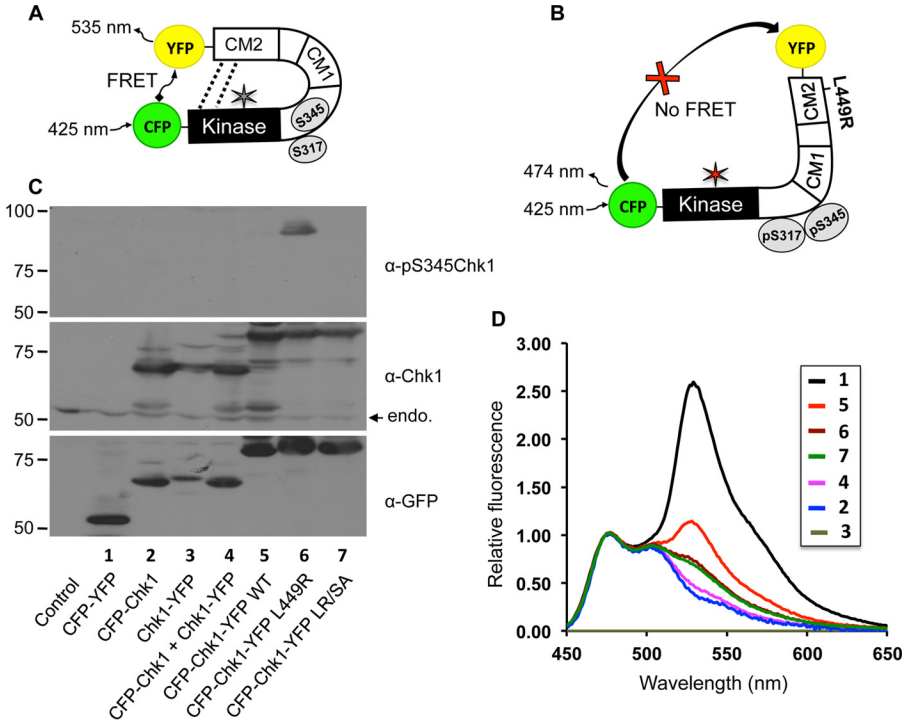

FIGURE 3. **FRET analysis of Chk1.** *A* and *B*, CFP and YFP were attached to the N and C termini of human Chk1, respectively. For Chk1 WT, we assumed that the closed conformation of Chk1 would bring the two fluorophores together to produce a FRET signal (*A*), whereas the L449R mutant would lose this FRET signal because of its open conformation (*B*). *C*, HEK293T cells were transfected with various Chk1 constructs for 48 h and immunoblotted with the indicated antibodies. Only CFP-Chk1-YFP L449R showed phosphorylation at the Ser-345 site. The arrow in the anti-Chk1 blot indicates endogenous (endo) Chk1 protein. *D*, parallel samples from *C* were collected in PBS, and FRET was measured as described under "Experimental Procedures." *Numbers* correspond to samples in *C*. The sensitized emission peak at around 535 nm is indicative of FRET.

residues 2–30, 31–60, and 61–87, respectively (Fig. 2*D*). Because these three fragments were also located in the cytoplasm (Fig. 2*B*), we performed similar pulldown experiments and observed that deleting residues 31–60 or 61–87 abolished the interaction between the Chk1 N and C termini (Fig. 2*E*). Together, these data suggest that the Leu-449 residue and region 31–87 in the N-terminal kinase domain are critical for the intramolecular interaction of Chk1.

FRET Analysis of the Chk1 Conformational Change—Several studies from yeast and human cells argued that the intramolecular interaction-based conformational change model of Chk1 has yet to be solidified (22–25). To resolve this issue, we used structural approaches to investigate the conformational change model of Chk1. To do so, we turned to the non-radioactive FRET technique, a powerful tool to study protein conformational changes as well as protein-protein interactions (30). The distance between the donor (here, CFP) and the acceptor (YFP) is critical for FRET analyses (usually within 100 Å) so that a small change will cause large alterations in the FRET signal (31). Chk1 constructs were engineered to have CFP at the N terminus and YFP at the C terminus (CFP-Chk1-YFP). Biochemical data showed that the distal C-terminal end (*e.g.* Leu-449) interacts with the distal N terminus (residues 30–87) (Figs. 1 and 2), indicating that both ends might be sufficiently close to each other for FRET to occur. Also, the kinase domain alone measures ~65 Å across (7). Therefore, an open conformation could have a distance of more than 100 Å between the N and C termini of Chk1, making it feasible to detect changes in the FRET signal. We hypothesized that the closed inactive conformation of the Chk1 WT should produce a FRET signal because the two chromophores are sufficiently close to each other (Fig. 3*A*), whereas the open active conformation of Chk1 (*e.g.* the L449R mutant) would lose this signal because of the increased distance between the two chromophores (Fig. 3*B*).

To this end, we generated various fluorescent protein constructs, transfected them into HEK293T cells for 48 h, and confirmed their expression by immunoblotting (Fig. 3*C*). As expected, based on the behavior of the Chk1-L449R mutant (8), the CFP-Chk1-YFP L449R mutant was phosphorylated at the ATR site in the absence of DNA damage (Fig. 3*C*), suggesting an open Chk1 conformation and checkpoint activation. We then measured FRET in parallel samples using methods described previously (29). The positive control (CFP-YFP), a fusion of the two proteins, produced a robust FRET signal (at an emission wavelength of 535 nm) as predicted (Fig. 3*D*, *line 1*). Interestingly, the CFP-Chk1-YFP WT construct also elicited a significant FRET signal (Fig. 3*D*, *line 5*). Most importantly, the FRET signal was substantially reduced in the CFP-Chk1-YFP L449R mutant compared with the WT (Fig. 3*D*, *lines 5* and *6*). The remaining FRET signal for the L449R mutant might emanate from a small portion of the mutant protein that had not yet been fully opened.

The L449R mutant of Chk1 is constitutively phosphorylated at the ATR site, Ser-345 (Fig. 3*C*). Based on our model (Fig. 1*A*), phosphorylation of Chk1 at ATR sites triggers the release of the closed conformation. Therefore, it remains possible that the open conformation of the L449R mutant could be due to its phosphorylation but not through the disruption of the intramo-

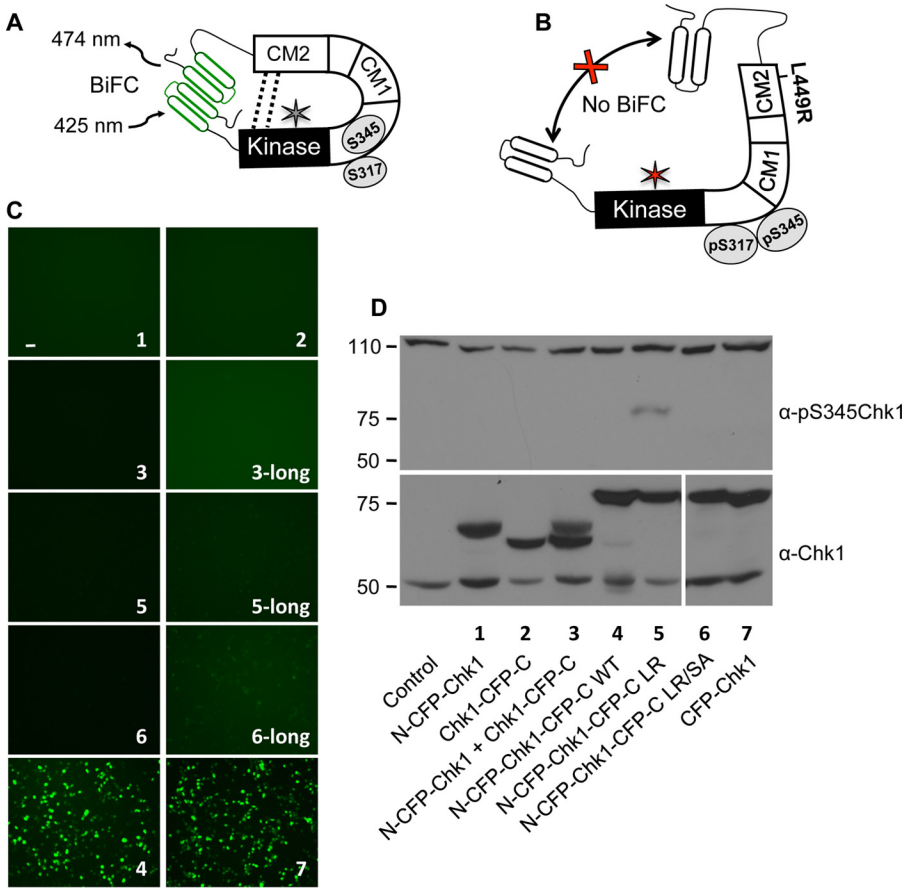

FIGURE 4. **BiFC analysis of Chk1.** N-terminal CFP (designated N-CFP, residues 1–155) and C-terminal CFP (designated CFP-C, residues 156–239) were attached to the N and C termini of human Chk1, respectively. For N-CFP-Chk1-CFP-C WT, reconstitution of fluorescence is expected because the split CFP halves would be brought together through the intramolecular interaction between its N and C termini of Chk1 (*A*). In contrast, the open conformations of Chk1, such as those caused by the L449R mutation, would fail to produce fluorescence because the ends of the split fluorophores are not close enough to each other (*B*). *C*, U2-OS cells grown on glass covers were transfected for 48 h with vectors expressing N-CFP-Chk1 (*1*), Chk1-CFP-C (*2*), a 1:1 ratio of N-CFP-Chk1 and Chk1-CFP-C (*3*), N-CFP-Chk1-CFP-C WT (*4*), N-CFP-Chk1-CFP-C L449R (*5*), the N-CFP-Chk1-CFP-C L449R/S345A double mutant (designated the LR/SA mutant, *6*), or a positive control (CFP-Chk1, *7*). Cells were fixed and visualized by fluorescence microscopy. Representative images are shown from two independent experiments. Images with a five times longer exposure period were also taken for samples *3*, *5*, and *6*. *Scale bar* = 10 μm. *D*, parallel samples were examined for protein expression from *C*. Note again the phosphorylation at Ser-345 of only the N-CFP-Chk1-CFP-C L449R mutant.

lecular interaction by the L449R mutation. To address this issue, we generated the L449R/S345A double mutant (designated the LR/SA mutant in Fig. 3C, *lane 7*) and asked whether blocking Chk1 phosphorylation at ATR sites would affect the FRET signal of the L449R mutant. As expected, mutating Ser-345 to Ala completely abolished the phospho-signal of the Chk1 L449R mutant (Fig. 3*C*, the phospho-Chk1 blot in *lanes 6* and *7*). Remarkably, the double mutant (LR/SA) showed nearly the identical FRET signal as the L449R mutant (Fig. 3*D*, *lanes 6* and *7*). These results suggest that phosphorylation of Chk1 at the ATR site is no longer required for the L449R mutant to open the closed conformation. Together, these data are consistent with our hypothesis (Fig. 3, *A* and *B*), suggesting that Chk1 adopts a closed conformation in the absence of DNA damage and that abolishing the intramolecular interaction disrupts this closed conformation.

BiFC Analysis of the Chk1 Conformational Change—Because a crystal structure of the full-length Chk1 is not yet available, we decided to confirm the FRET results by another approach. We chose the BiFC technique, another useful tool to study protein-protein interactions and protein conformational changes. BiFC operates on the principle that fluorescence will be reconstituted when two halves of a split fluorophore (*e.g.* CFP) are brought in close proximity through a protein-protein interaction or via a closed conformation within the same protein (32). The distance requirement for BiFC is much less stringent than that for FRET (32). Also, the individual split fluorophore does not produce fluorescence when expressed in cells, reducing the background signal of BiFC compared with FRET.

To implement this approach, we attached the N-terminal half (residues 1–155, termed N-CFP) and the C-terminal half (residues 156–239, termed CFP-C) of CFP to the N and C termini of Chk1 (WT or the L449R mutant), respectively. Control vectors with only N-CFP or the CFP-C attached to Chk1 were also generated. Similarly, we expected that BiFC would only occur with N-CFP-Chk1-CFP-C WT (Fig. 4*A*) but not for the L449R mutant (Fig. 4*B*). The results showed that overexpressing N-CFP-Chk1 or Chk1-CFP-C alone did not produce any fluorescence (Fig. 4*C*, *1* and *2*), consistent with the idea that the split fluorophore of CFP cannot produce fluorescence. Interestingly, overexpressing N-CFP-Chk1 and Chk1-CFP-C simultaneously also failed to reconstitute fluorescence (Fig. 4*C*, *3* and *3* long exposure *(3-long)*). Similarly, simultaneous expression of CFP-Chk1 with Chk1-YFP failed to produce a FRET signal as

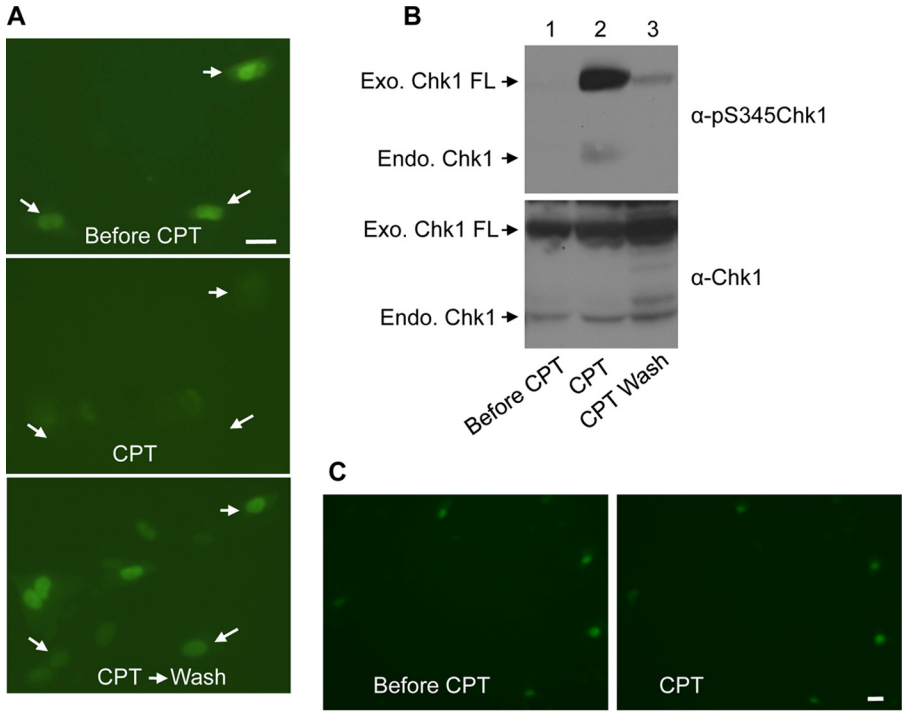

FIGURE 5. **DNA damage-induced loss of BiFC.** *A*, U2-OS cells were transfected with the vector for N-CFP-Chk1-CFP-C WT for 48 h, treated with 100 nM CPT for 2 h, washed, and then cultured in drug-free medium for an additional 12 h. Live cell images before, during, and after CPT treatment were observed under a fluorescence microscope, with representative images shown here. *Scale bar* = 10 μm. *Arrows* indicate three cells with fluorescence changes during CPT treatment. *B*, protein expression from parallel samples in *A* was examined. Exogenous (*Exo*) Chk1 indicates N-CFP-Chk1-CFP-C WT proteins, whereas endo indicates endogenous Chk1 proteins. *C*, U2-OS cells were transfected with the positive control CFP-Chk1 for 48 h, treated with 100 nM CPT for 2 h, and then images (before and after CPT treatment) were taken under a fluorescence microscope. *Scale bar* = 10 μm.

well (Fig. 3*D*, *line 4*). These results suggest that Chk1 does not form intermolecular interactions.

However, when we overexpressed the N-CFP-Chk1-CFP-C WT, fluorescence was readily detected at a level similar to that of the positive CFP-Chk1 control (Fig. 4*C*, *4* and *7*). In contrast, more than 95% of fluorescence was lost when we mutated Leu-449 to Arg in Chk1 (Fig. 4*C*, *5*). Again, phosphorylation of Chk1 at the Ser-345 site was only detected for the L449R mutant, and mutating the Ser-345 to Ala abolished the phospho-Chk1 signal (Fig. 4*D*, *lanes 5* and *6*). Most importantly, neither the L449R mutant nor the double mutant (LR/SA) failed to reconstitute fluorescence (Fig. 4*C*, *5* and *6*). When increasing the exposure time 5-fold compared with that for the WT sample, we detected scattered fluorescence for the single L449R or the double LR/SA mutant in a limited number of cells (Fig. 4*C*, *5-long* and *6-long*). These are similar to the FRET results, reinforcing the idea that ATR-dependent phosphorylation is not required for the conformational change of Chk1 when Leu-449 is mutated to Arg. These results suggest that the L449R mutation is sufficient to open the closed conformation of Chk1.

DNA Damage Reduces the BiFC Signal of Chk1—Recent studies suggest that DNA damage-induced phosphorylation of Chk1 at Ser-345 by ATR functions as a trigger to relieve the intramolecular interaction (7–9, 21), which then allows the already open catalytic site of Chk1 to phosphorylate downstream substrate proteins (Fig. 1*A*). If this model is correct, then DNA damage treatment should reduce or diminish fluorescence of the N-CFP-Chk1-CFP-C WT construct (Fig. 4*C*, *4*) because of Ser-345 phosphorylation and subsequent conformational opening in a manner similar to that exhibited by the L449R mutant (Fig. 4*B*).

To test this hypothesis, we overexpressed the N-CFP-Chk1-CFP-C WT construct in U2-OS cells and treated these cells with camptothecin (CPT), a DNA-damaging agent that inhibits topoisomerase 1 and induces robust Chk1 phosphorylation by ATR (6, 15, 33). We reported previously that a persistent DNA-damaging treatment, *e.g.* 500 nM CPT for longer than 4 h, induced degradation of Chk1 (10, 34). Therefore, to avoid such Chk1 degradation, we treated cells with a relatively low concentration of CPT (100 nM) for only 2 h, conditions that will not induce degradation of either endogenous or exogenous Chk1 proteins (Fig. 5*B*, *lanes 1* and *2*). Immunoblotting with phospho-specific anti-Ser(P)-345 Chk1 antibodies demonstrated robust phosphorylation of both endogenous and exogenous Chk1 proteins by CPT treatment (Fig. 5*B*, *lanes 1* and *2*). Accordingly, the fluorescence of transfected cells was significantly reduced (Fig. 5*A*, comparing three cells indicated by *arrows* before and during CPT treatment). This reduction of fluorescence is reminiscent of the effect of the Chk1-CFP-C L449R mutant (Fig. 4*C*), indicating that phosphorylation may have disrupted the closed conformation of Chk1. In contrast, the same CPT treatment did not significantly reduce the fluorescence of the CFP-Chk1 control (Fig. 5*C*), suggesting that such treatment does not induce Chk1 degradation or abolish the fluorophore *per se*. Interestingly, after washing off CPT and allowing the cells to recover from the DNA damage for ~12 h, the fluorescence recovered (Fig. 5*A*). This recovery could be due to dephosphorylation followed by fluorescence reconstitu-

tion of the CFP-Chk1-CFP-C WT proteins or synthesis of new CFP-Chk1-CFP-C WT proteins or both. Nevertheless, these data collectively suggest that Chk1 undergoes conformational changes in response to DNA damage.

Discussion

As a critical DDR protein, Chk1 regulates both the DNA damage response and normal DNA replication (3). In the absence of DNA damage, Chk1 interacts with DNA replication machinery proteins, including MCM3 and Treslin (35–37). Such interactions allow Chk1 to employ its basal catalytic activity to phosphorylate these DNA replication proteins, which then ensures smooth DNA replication under normal growth conditions (35, 36). However, this Chk1 basal activity does not suffice to handle DNA damage or replicative stress. It requires the full engagement of Chk1 to counter these deleterious assaults to protect genome integrity and promote cell survival. A key step for full activation of Chk1 in the presence of DNA damage is through ATR-dependent phosphorylation at the Ser-317 and Ser-345 residues, especially the latter (6, 8, 9, 17, 18). Phosphorylation of these two residues *per se* does not increase the catalytic activity of Chk1 (3); rather, phosphorylation has been suggested to function as a trigger to relieve an intramolecular interaction that blocks the access of substrates to the catalytic site of Chk1 (7–9, 21). A crystal structure of the kinase domain of Chk1 revealed that its catalytic site adopts an open conformation (7). However, Chk1 does not exhibit its full activity in the absence of DNA damage (5, 6), indicating the existence of an inhibitory mechanism that blocks its maximal activity. Because the C-terminal domain of Chk1 interacts with the N-terminal kinase domain (8–13) and the N-terminal kinase domain alone elicited stronger catalytic activity than the full-length protein *in vitro* (7), it is highly likely that the intramolecular interaction provides a physical hindrance to the catalytic site under normal growth conditions. However, so far, only indirect evidence supported this supposition, and several studies have come to disparate conclusions about the Chk1 activation model (22–25).

To better understand the molecular mechanism of Chk1 activation, we used structural approaches to probe its conformational changes before and after DNA damage. In this study, we used FRET and BiFC, two powerful methods, to provide compelling evidence to support the intramolecular interaction and the conformational change model of Chk1. Our data indicate that, in the absence of DNA damage, the N terminal (the 31–87 amino acid region in particular) and the C-terminal ends of Chk1 (especially Leu-449) are in close enough proximity (within 100 Å of each other) to allow the detection of FRET and fluorescence reconstitution, indicating a closed conformation for Chk1. In contrast, disruption of this intramolecular interaction either by mutating Leu-449, a residue critical for the intramolecular interaction, or by DNA damage-induced phosphorylation, led to a significant reduction in FRET and an almost complete loss of fluorescence reconstitution in BiFC, respectively. These findings suggest an open conformation for both the Chk1 L449R mutant and the Chk1 WT in its phosphorylated form. Although a more precise description of the activation process will require a crystal structure determination of the full-length Chk1 WT and the L449R mutant, the combination of biochemical, pharmacological, and structural evidence presented here strongly support the conformational change model of Chk1 during the DNA damage response.

This study opens the door for developing novel strategies to modulate the activity of Chk1 in cancer therapy. We recently reported that constant activation of Chk1 can bypasses the requirement for DNA-damaging agents (such as radiotherapy or chemotherapeutic drugs) and eventually induce cancer cell death (3, 8). Unlike a conventional strategy that relies on the combination of a Chk1 inhibitor with either radiotherapy or chemotherapy, this new strategy (*i.e.* constant activation of Chk1 in cancer cells) has the potential to reduce toxic side effects of anticancer therapies (3). We hope that new tools, such as the FRET and the BiFC Chk1 constructs, presented here could allow the development of small molecules that can open the closed conformation of Chk1 in the absence of DNA damage. Such small molecules then could constitutively activate the Chk1 signaling pathway in the absence of toxic chemotherapy or radiotherapy and eventually trigger the self-destructive mechanisms of cancer cells. Given that Chk1 is overexpressed in a wide range of human tumors (38–45) and that its expression often positively correlates with tumor grade and disease recurrence (42, 44, 46), it is tempting to speculate that tumor cells rely much more heavily on Chk1 for growth or survival than normal cells do. Therefore, this unique strategy (*i.e.* activating Chk1 in the absence of DNA damage) may show specific toxicity toward tumors, especially those high-grade ones, while having much less impact on normal cells. Clearly such projects will require further independent investigation in the near future.

Author Contributions—Y. Z. designed the experiments and wrote the manuscript. X. H., J. T., J. W., J. Z., P. K., and P. P performed the experiments. K. P. and X. Y. provided critical insights into the project and edited the paper. J. W. contributed substantially to the study but could not be contacted to approve the final version of the manuscript.

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