We have purified and characterized human Chk2 both from baculovirus-infected insect cells and from either untreated or DNA damage-stressed human HCT116 cells. Chk2 from unstressed human cells is largely monomeric and inactive in phosphorylating its substrate, Cdc25C. It is also unphosphorylated at Thr-68, a site that is the target of the ataxia telangiectasia-mutated protein kinase. After treatment of HCT116 cells with a radiomimetic compound neocarzinostatin, active Chk2 exists as stable Thr-68-phosphorylated dimers as well as interconvertable Thr-68-unphosphorylated monomers and dimers. Interestingly, Chk2 from insect cells behaves by all criteria tested like active Chk2 from neocarzinostatin-treated HCT116 cells. Based on Stokes radius and sedimentation coefficient values, Chk2 monomers and dimers have asymmetric rather than globular shapes. Both Thr-68-phosphorylated and Thr-68-unphosphorylated forms of active Chk2 are capable of phosphorylating Cdc25C. Thus, although phosphorylation of Thr-68 may be required for initial oligomerization and activation of Chk2, it is not needed for maintenance of dimerization or kinase activity.

In cells the DNA damage checkpoint pathway exists to prevent transmission of altered genetic information to progeny. Checkpoint kinase 2 (Chk2) is a component of this pathway, and mutation of this gene has been linked to cancer (1–4). Chk2 is activated by ATM kinase (5–7), and several substrates and mutation of this gene has been linked to cancer (1–4). Phosphorylation of Chk2, which are involved in cell cycle arrest or DNA repair, has been identified (for review, see Ref. 8). Phosphorylation of Chk2 is activated by ATM kinase (5–7), and several substrates and mutation of this gene has been linked to cancer (1–4). Phosphorylation of Chk2, which are involved in cell cycle arrest or DNA repair, has been identified (for review, see Ref. 8). Phosphorylation at Thr-68, a site that is the target of the ataxia telangiectasia-mutated protein kinase. After treatment of HCT116 cells with a radiomimetic compound neocarzinostatin, active Chk2 exists as stable Thr-68-phosphorylated dimers as well as interconvertable Thr-68-unphosphorylated monomers and dimers. Interestingly, Chk2 from insect cells behaves by all criteria tested like active Chk2 from neocarzinostatin-treated HCT116 cells. Based on Stokes radius and sedimentation coefficient values, Chk2 monomers and dimers have asymmetric rather than globular shapes. Both Thr-68-phosphorylated and Thr-68-unphosphorylated forms of active Chk2 are capable of phosphorylating Cdc25C. Thus, although phosphorylation of Thr-68 may be required for initial oligomerization and activation of Chk2, it is not needed for maintenance of dimerization or kinase activity.

The 543-amino acid human Chk2 (hChk2) protein contains three domains including a SQ/TQ cluster domain (SCD) at the N terminus (residues 19–69), a forkhead homology-associated (FHA) domain within the central portion (residues 115–175), and a kinase domain within the C-terminal region (residues 226–486). Direct phosphorylation of Chk2 by ATM at Thr-68 within the SCD is required for activation and is also a likely priming event for subsequent phosphorylation at other hChk2 sites (16–19). A tumor-derived Chk2 mutation within the FHA domain, R145W, renders the protein incapable of being phosphorylated in an ATM-dependent manner, suggesting that the FHA domain is involved in activation of the protein (19, 20). Despite many studies showing that FHA domains are phosphopeptide recognition motifs (21–24), progress in identification of proteins interacting with FHA domains has been limited. It was shown that a phosphorylated receptor-like kinase interacts with the FHA domain of Arabidopsis thaliana phosphatase (25, 26). Another protein, Saccharomyces cerevisiae Rad53, a homolog of human Chk2, which contains two FHA domains, was also shown to interact with phosphorylated Rad9 in a DNA damage-dependent manner (27). Recently, Ahn et al. (28) demonstrated that synthetic hChk2 peptides spanning Thr-68 interact with isolated hChk2 FHA domains in a DNA damage-dependent manner. They reported that the dimers formed are transient in nature during activation and that active Thr-68-phosphorylated hChk2 protein exists as a monomer. In another study Xu et al. (29) showed that both the SCD and the FHA domains are required for formation of Chk2 oligomers, and they concluded that Chk2 phosphorylation is required for stable dimerization. Here we report the isolation of highly purified forms of Chk2 protein and show that Chk2 is an unusually shaped non-globular protein. Chk2 monomers that are unphosphorylated at Thr-68 can be isolated from either untreated or DNA-damaged cells, whereas Thr-68-phosphorylated and unphosphorylated Chk2 dimers form only in DNA-damaged cells. Interestingly, both Thr-68-phosphorylated dimers and unphosphorylated Chk2 existing as interconvertible dimer-monomer forms are similarly active as kinases toward Cdc25C. We propose a model for activation and oligomerization of Chk2 upon DNA damage to cells.

**EXPERIMENTAL PROCEDURES**

Mammalian Cell Lines and Culture Conditions—An HCT116-derived cell line stably expressing N-terminally HA-tagged wild-type hChk2 (HA-Chk2) that was used for many of the experiments herein was kindly provided by Dr. Junjie Chen (Mayo Clinic, Rochester, MN) (20). These cells were cultured in RPMI 1640 medium containing 400 μg/ml of G416 and 10% fetal bovine serum. Where indicated, cells were cultured in the presence of 100 μM neocarzinostatin (CA87497). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
treated with a radiomimetic compound, nocodazolatin (NCS; 500 ng/ml, Kayaku Co, Tokyo, Japan) for 2 h before purification of HA-Chk2 as described below. Alternatively, cells were γ-irradiated with 14 gray (using a 137Cs source) and harvested after 2 h for purification of HA-Chk2.

**Purification of Proteins**—C-terminally FLAG-tagged Chk2 (Chk2-FLAG) wild-type and D347A mutant proteins were immunopurified from recombinant baculovirus-infected Sf9 insect cells described previously (12). Immunopurified Chk2-FLAG was dialyzed against Buffer A containing 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 1 mM DTT, HCT116 derivative cell lines were either untreated or treated with γ irradiation or NCS as indicated to immunopurify HA-tagged Chk2 proteins as follows. Typically, cells in 200 ml of growth media were collected and lysed with Buffer B containing 50 mM HEPES, pH 7.8, 150 mM KCl, 10 mM NaCl, 1 mM EDTA, 1.5 mM MgSO4, 0.1% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride, 60 mM okadaic acid, 240 μM cypermethrin, 1 mM NaF, 100 μM NaVO4, and 20% glycerol. Extracts were pre-cleaned with 400 μl of agarose beads conjugated with protein A (Amersham Biosciences) for 4 h at 4 °C and incubated with 100 μl of agarose-protein A cross-linked with anti-HA antibody for 12 h at 4 °C. The beads were collected and washed with Buffer B containing 20 mM HEPES, pH 7.8, 150 mM KCl, 0.1 mM EDTA, 1.5 mM MgSO4, 0.1% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride, 60 mM okadaic acid, 240 μM cypermethrin, 1 mM NaF, 100 μM NaVO4, and 20% glycerol. Proteins were eluted with 100 μg/ml HA-peptide (SynPep, Dublin, CA) in Buffer B and then dialyzed against Buffer A. Plasmids encoding GST-Cdc25C-(200–325) in 200 μl of phosphate-buffered saline (8 mM Na2HPO4, 1.94 g/l of protein kinase buffer. Mixtures were incubated at 30 °C for 30 min and then added with 1 mM isopropyl-β-D-thiogalactoside for 2.5 h (1 mM final concentration) at 20 °C. The columns were calibrated using global standards of known molecular masses (Bio-Rad). Results from gel filtration analysis were used to calculate the Stokes radius (Rg) using the empirical equation (30),

\[ \log(R_g) = 0.369 \log (M) - 0.254 \]  

(Eq. 1)

where \( M \) is the molecular weight of a polypeptide derived from its amino acid sequence.

**Sucrose Gradient Centrifugation**—Immunaffinity-purified Chk2-FLAG (10 μg) in 200 μl of phosphate-buffered saline (8 mM Na2HPO4, 1.5 mM KH2PO4, 140 mM NaCl, 3 mM KCl, pH 7.5) was layered directly onto a gradient of 5–20% (v/v) sucrose in 35 ml of phosphate-buffered saline. 50 μg each of thyroglobulin (670 kDa, \( s_{20w} = 7.1 \)), γ-globulin (158 kDa, \( s_{20w} = 4.3 \)), chicken ovalbumin (44 kDa, \( s_{20w} = 3.6 \)), and bovine serum albumin (67 kDa, \( s_{20w} = 4.3 \)) were layered together on the gradient. Ultracentrifugation was performed with a Beckman SW 28 rotor at 26,000 rpm for 3 h. Fractions were collected in volumes of 1.75 ml. Each fraction was analyzed by SDS-PAGE followed by silver staining for standard proteins or immunoblotting for Chk2-FLAG using anti-FLAG antibody (Sigma) or anti-phospho-Thr-68 antibody (Cell Signaling Technologies, Beverly, MA). Molecular weight and frictional ratios were calculated from sedimentation coefficients obtained from sucrose gradient centrifugation, and Stokes radii were derived from gel filtration based on Siegel and Monty (31),

\[ M = 6\pi n \eta a / (1 - \eta p) \]  

(Eq. 2)

\[ f_p = a/(3M^2/4\pi n^2) \]  

(Eq. 3)

where \( M \) is the molecular mass, \( \eta \) is the viscosity of the medium (value used, 1), \( N \) is Avogadro’s number, \( a \) is the Stokes radius, \( s \) is the sedimentation coefficient, \( n \) is the partial specific volume (value used, 0.725), and \( p \) is the density of the medium (value used, 1). Glutaraldehyde Cross-linking—Typically protein (50 ng) was incubated with 2.5 mM glutaraldehyde in 20 μl of a buffer containing 20 mM HEPES, pH 7.8, 100 mM KCl, 10 mM MgCl2, 20 mg/ml BSA, 1 mM DTT, 60 μM okadaic acid, 240 μM cypermethrin, 1 mM NaF, 100 μM NaVO4, 20% glycerol, and 100 μM ATP supplemented with 1 μCi of (γ-32P)ATP. The reactions were terminated by heating to 95 °C, and the sample loading buffer (0.5 ml of SDS-PAGE sample buffer) was added. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and subsequently identified by immunoblotting. For detection of GST fusion proteins, anti-GST antibody (Sigma) was used. Anti-FLAG, anti-HA, or anti-Chk2 antibody (12) was used for detection of Chk2 proteins. Radiolabeled proteins were visualized with autoradiography and quantitated with PhosphorImager (Amersham Biosciences).

For filter binding assays Chk2 (41 kDa) was incubated with 0–96 μM GST-Cdc25c-(200–256) in 20 μl of protein kinase buffer. Mixtures were incubated at 30 °C for 45 min and then mixed with 500 μl of Stop Buffer (at 4°C) containing 16 mM Na2HPO4, 3 mM KH2PO4, 280 mM NaCl, and 6 mM KCl, pH 7.5. Mixtures were immediately layered onto and filtered through 25-mm nitrocellulose filters (Osmonics Inc, Minnetonka, MN) under vacuum. Filters were washed 6 times with 5 ml of Stop Buffer, dried, and counted by liquid scintillation. The data were fitted to a Michaelis-Menten curve using Kaleidagraph (Synergy Software) to determine the kinetic constants Vmax and Km.

**RESULTS**

**Monomeric and Dimeric Forms of Chk2-FLAG Have Non-globular Shapes and Differ in Their Ability to be Cross-linked and Phosphorylated at Thr-68**—We first analyzed recombinant baculovirus-expressed Chk2 protein tagged with the FLAG epitope at the C terminus (Chk2-FLAG) (12). Chk2-FLAG protein was immunopurified with anti-FLAG antibody cross-linked to agarose beads and then subjected to gel filtration chromatography followed by SDS-PAGE to determine its size (Fig. 1A, top panel). The Chk2-FLAG protein eluted with two peaks (fractions 9 and 12), and according to the elution profile of protein molecular mass standards, the estimated molecular mass of the first (early) eluting peak (peak 9) was ~210 kDa, and that of the second (later) eluting peak (peak 12) was ~114 kDa. Silver staining of fractions 9 and 12 support the likelihood that the relative sizes of the Chk2 protein in the two peak fractions are not the result of the presence of interacting partners co-purifying with Chk2-FLAG (Fig. 1A, bottom panel). The monomeric molecular mass of Chk2-FLAG derived from the amino acid sequence is 61.8 kDa. As shown below, the 210- and 114-kDa forms have molecular masses consistent with dimeric and monomeric Chk2, respectively. The first result supporting this was derived from an experiment where each fraction from gel filtration chromatography was treated with glutaraldehyde before resolution by SDS-PAGE (Fig. 1B). Immunoblot analysis revealed that the 210-kDa form from the gel filtration column was efficiently cross-linked by glutaraldehyde to a form that migrated with an apparent molecular mass of 120–150 kDa by SDS-PAGE, whereas the 114-kDa gel filtration form was inefficiently cross-linked by glutaraldehyde and migrated mainly as a polypeptide of approximate 60 kDa in SDS-PAGE. The second line of evidence came from an experiment in which Chk2-FLAG was subjected to sucrose gradient sedimentation. Chk2-FLAG sediments with a fairly broad distribution between standard markers from 1.9 to 7.1 S (Fig. 1C, bottom panel). These include proteins with monomeric molecular masses of (lysozyme: 14.1 kDa, 1.9 S; ovalbumin: 44 kDa, 3.6 S; bovine serum albumin: 66 kDa, 4.6 S; γ-globulin: 158 kDa, 7.1 S). Although it was not possible using these gradient conditions to discern two discrete sedimenting forms, we were able to
identify the more rapidly sedimenting component by its state of phosphorylation. It was reported that phosphorylation of Thr-68 is concomitant with dimerization of Chk2 (28, 29). Using a phosho-specific anti-Chk2 antibody that detects phosphorylated Thr-68, we found that the peak of Thr-68-phosphorylated Chk2 sedimented as 5.4 ± 0.5 S (Fig. 1C, top panel). It was therefore estimated that the more slowly sedimenting form is 2.9 ± 0.5 S. Additional evidence supporting this came from examining Thr-68 phosphorylation of the two forms isolated from the gel filtration column. The 210-kDa form of Chk2-FLAG was phosphorylated at Thr-68, whereas the 114-kDa form was not phosphorylated at the site (Fig. 1D). By applying the values obtained from gel filtration and sedimentation analysis of the two forms of Chk2-FLAG to Equation 2 (31), our data show that Chk2 FLAG consists of dimers with an estimated molecular mass of 117 ± 12 kDa and monomers with an estimated molecular mass of 52 ± 9 kDa (Table I). The mass of the Chk2-FLAG dimer is fairly in line with the mass derived from the amino acid sequence (123.6 kDa), whereas the mass of the monomer (52 kDa) is somewhat lower than the amino acid sequence-derived mass of 61.8 kDa. This may in part be due to our inability to identify accurately the sucrose gradient peak of the more slowly sedimenting form. Nevertheless by combining the results of gel filtration, cross-linking SDS-PAGE, and sucrose gradient sedimentation, we conclude that Chk2-FLAG exists as Thr-68-phosphorylated dimers and Thr-68-unphosphorylated monomers. We cannot assess whether and how many dimers are doubly or singly phosphorylated at Thr-68. Furthermore, the data below will show that a subset of Chk2 dimers lack phosphorylation at Thr-68.

The disparity between values obtained from gel filtration analysis, SDS-PAGE, and sucrose gradient sedimentation strongly indicates that Chk2-FLAG dimers and monomers are asymmetric with non-globular shapes. This was confirmed by calculating the frictional coefficient of these two forms (dimer, 1.54; monomer 1.59), where a typical globular protein would have a frictional coefficient of ~1.2 (32).

It is noteworthy that even though the insect cells had not been subjected experimentally to DNA-damaging treatments, a fraction of the baculovirally derived Chk2-FLAG was phosphorylated at Thr-68. The process of baculoviral infection may trigger a damage response program resulting in phosphorylation of the expressed recombinant Chk2-FLAG protein.

**Table I**

| Amino acid sequence<sup>a</sup> | Dimer | Monomer |
|-----------------------------|-------|---------|
| 123.6 kDa                   | 52.0 kDa | 9.0 kDa |
| Gel filtration<sup>b</sup> | 210 ± 6 kDa | 114 ± 5 kDa |
| Sedimentation coefficient<sup>c</sup> | 5.4 ± 0.5 S | 2.9 ± 0.5 S |
| Sedimentation data<sup>d</sup> | 117 ± 12 kDa | 52 ± 9 kDa |
| Frictional ratio<sup>e</sup> | 1.54 | 1.59 |

<sup>a</sup> FLAG tag was considered in the calculations.

<sup>b</sup> See “Experimental Procedures” for the computational details.

<sup>c</sup> By applying the values obtained from gel filtration and sedimentation analysis of the two forms of Chk2-FLAG to Equation 2 (31), our data show that Chk2 FLAG consists of dimers with an estimated molecular mass of 117 ± 12 kDa and monomers with an estimated molecular mass of 52 ± 9 kDa (Table I). The mass of the Chk2-FLAG dimer is fairly in line with the mass derived from the amino acid sequence (123.6 kDa), whereas the mass of the monomer (52 kDa) is somewhat lower than the amino acid sequence-derived mass of 61.8 kDa. This may in part be due to our inability to identify accurately the sucrose gradient peak of the more slowly sedimenting form. Nevertheless by combining the results of gel filtration, cross-linking SDS-PAGE, and sucrose gradient sedimentation, we conclude that Chk2-FLAG exists as Thr-68-phosphorylated dimers and Thr-68-unphosphorylated monomers. We cannot assess whether and how many dimers are doubly or singly phosphorylated at Thr-68. Furthermore, the data below will show that a subset of Chk2 dimers lack phosphorylation at Thr-68.

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**Recombinant HA-Chk2 Purified from Mammalian Cells after DNA Damage and Chk2-FLAG Purified from Insect Cells Are Competent to Phosphorylate Cdc25C**—To analyze Chk2 in human cells before and after DNA damage, we used HCT116 colorectal tumor-derived cells that were engineered to express wild-type Chk2 proteins tagged with the HA epitope at the N terminus (20). In these cells HA-tagged Chk2 is expressed at comparable levels to those of the endogenous Chk2 protein (20). Cells were treated or not with NCS (500 ng/ml), a compound that has been previously used as a radiomimetic drug to study the damage response pathway (29, 30). As shown in Fig. 2A, treatment of cells with NCS led to hyperphosphorylation of HA-Chk2 as evidenced by an upward gel mobility shift as early as 20 min after treatment. This was also accompanied by accumulation of p53 in a dose- and time-dependent manner (data not shown). The mobility shift was due to phosphorylation since treatment of the protein with calf intestinal phosphatase at 50 °C caused the protein to migrate as in untreated cells (Fig. 2B). The mobility shift was not detectable when the phosphatase was incubated with Chk2 at 37 °C. We were not able to detect the mobility shift of Chk2 when treated with protein phosphatase I, suggesting that some phosphorylated...
residues responsible for the mobility shift are protected from this phosphatase either by intra- or intermolecular interaction. HA-Chk2 protein was purified from untreated or NCS-treated or γ-irradiated HCT116 cells and incubated with a truncated version of GST-tagged Cdc25C that spans the Chk2 phosphorylation site (33). As expected, only after these DNA-damaging treatments was HA-Chk2 capable of phosphorylating GST Cdc25 (Fig 2C). The kinase activities of HA-Chk2 purified from HCT cells after treatment of NCS and Chk2-FLAG purified from insect cells were essentially identical, further supporting our conclusions that baculovirus-infected cells express active forms of Chk2 (Fig 2D). As expected HA-Chk2 protein purified from NCS-treated but not untreated HCT116 cells was phosphorylated at Thr-68 (Fig 2E). These results support previous studies showing that phosphorylation of Thr-68 is required for activation of Chk2 during DNA damage response pathway (16–19). 

**Monomer and Dimer Forms of HA-Chk2 Proteins Can Be Isolated from Mammalian Cells after DNA Damage—Chk2-FLAG from insect cells can form dimers, and the specific activity of Chk2-FLAG is comparable with that of HA-Chk2 purified from HCT cells after treatment of NCS and Chk2-FLAG purified from insect cells were essentially identical, further supporting our conclusions that baculovirus-infected cells express active forms of Chk2 (Fig 2D). As expected HA-Chk2 protein purified from NCS-treated but not untreated HCT116 cells was phosphorylated at Thr-68 (Fig 2E). These results support previous studies showing that phosphorylation of Thr-68 is required for activation of Chk2 during DNA damage response pathway (16–19).**

![Image](https://example.com/image.png)
absent in the corresponding protein isolated from untreated cells. When the corresponding gel filtration column fractions of both forms of HA-Chk2 were subjected to chemical cross-linking with glutaraldehyde, only the HA-Chk2 heavier fractions (peak at fraction 10) from NCS-treated cells could be efficiently cross-linked, consistent with data from baculovirally expressed Chk2-FLAG (compare Fig. 1B and Fig. 3B, left panel). Furthermore, no cross-linked forms were discerned when we compared the equivalent fractions of Chk2 isolated from unstimulated cells (Fig. 3B, right panel). Representative fractions containing either form were immunoprecipitated with anti-HA antibody cross-linked to protein A-agarose beads. As shown in Fig. 3C, the dimeric form (fraction 10) of HA-Chk2 from NCS treated cells was phosphorylated at Thr-68, whereas the monomeric form (fraction 13) was only very weakly phosphorylated at that site. Thus, human Chk2 proteins isolated either from baculovirally infected insect cells or from DNA-damaged human cells consists of monomers that are not phosphorylated at Thr-68 and dimers that are phosphorylated at that site.

**Chk2-FLAG from Insect Cell Associates Specifically with Activated Forms of HA-Chk2 from Human Cells**—Having established that DNA damage to cells yields Chk2 protein that can form cross-linkable phosphorylated dimers, we were concerned that we were not able to observe a stable interaction between HA-Chk2 and endogenous Chk2 in HCT116 cells after treatment with NCS even though the levels of the two forms of Chk2 are roughly comparable (Fig. 4A). To show an interaction between the endogenous Chk2 protein and the HA-tagged version it would be necessary to immunoprecipitate the putative complex with the HA antibody and demonstrate the presence of the untagged endogenous protein in the complex. In fact, when such an immunoprecipitate was performed and gel lanes were greatly overloaded before transfer and immunoblotting with anti-Chk2 antibody, we were still unable to detect any endogenous Chk2 in the anti-HA antibody immunoprecipitate. We estimate that the level of sensitivity of our immunodetection reaction is such that as little as 2% of the endogenous Chk2 protein could be detected in co-immunoprecipitates (Fig. 4B). Therefore, the possibility that the anti-HA antibody interferes with the dimerization of HA-Chk2 and endogenous Chk2 protein was considered. The epitope tag on baculovirally expressed Chk2-FLAG is at the C terminus, however, and so extracts of insect cells expressing Chk2-FLAG were incubated with extracts of HCT116 cells expressing HA-Chk2 that had either been treated or untreated with NCS. Immunoprecipitation of such mixtures with anti-FLAG antibody revealed that the active forms of HA-Chk2 isolated from NCS-treated cells could indeed form stable complexes with Chk2-FLAG, whereas HA-Chk2 from untreated cells could not (Fig. 4C). This supports the possibility that the anti-HA antibody prevents the interaction of HA-Chk2 with endogenous Chk2. To show that the interaction between the two tagged forms of Chk2 was direct, we then incubated Chk2-FLAG that had been purified from insect cells with either HA-Chk2 purified from NCS-treated or untreated HCT116 cells and performed immunoprecipitation with anti-HA antibody (Fig. 4D). Here again we were able to demonstrate that the HA-Chk2 from DNA damaged but not untreated cells can form a stable complex with the Chk2-FLAG. Thus, stable oligomers of Chk2 can be formed in vitro provided the protein is activated presumably as a result of phosphorylation at Thr-68.

**Fig. 4.** Activated Chk2 proteins oligomers can form in vitro. A, lysates of NCS-treated HCT116 cells expressing HA-Chk2 were either directly sampled (Cell lysate) or pre-cleared with agarose beads conjugated with protein A (Pre-Cleared). HA-Chk2 was immunoprecipitated from pre-cleared cell lysate with anti-HA antibody cross-linked to protein A-agarose beads (IP), and the supernatant was saved from the immunoprecipitate (Sup). Aliquots of each step (equivalent to 200 μg of total cell lysate) were resolved by SDS-PAGE followed by immunoblotting with either anti-HA antibody (top panel) or anti-Chk2 antibody (bottom panel). In the bottom panel the position of HA-tagged (HA-Chk2) and endogenous Chk2 (Endo-Chk2) are indicated. Although both HA-Chk2 and endogenous Chk2 can be detected in the lysate with or without pre-clearing, the supernatant from the anti-HA immunoprecipitate contains only the endogenous Chk2, and the immunoprecipitate contains only HA-Chk2. B, HA-Chk2 (120 ng) purified from HCT116 cells with or without NCS treatment was subjected to SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody along with Chk2-FLAG (2.5–10 ng), that was also immunoblotted with anti-Chk2 antibody. C, lysates of untreated (−) or NCS treated (+) HCT116 cells were mixed with lysates of either uninfected (mock) or Chk2-FLAG baculovirus-infected (Chk2-FLAG sf9 insect cells. Mixtures were immunoprecipitated with anti-FLAG antibody cross-linked to agarose (right two panels) and subjected to SDS and immunoblotting with anti-HA antibody. The left two panels show HCT116 cell lysates (far left panel) and sf9 lysates (second from left panel) at 2% of the amount immunoprecipitated that were subjected to SDS-PAGE and immunoblotted with anti-HA and anti-FLAG antibodies, respectively. Protein complexes of HA-Chk2 and Chk2-FLAG were immunoprecipitated with anti-FLAG antibody. D, purified HA-Chk2 from untreated (−) or NCS treated (+) cells and Chk2-FLAG were mixed together, subjected to immunoprecipitation, and analyzed by immunoblotting with anti HA antibody or anti-FLAG antibody as indicated. The position of the IgG polypeptide in these immunoprecipitates is indicated.
Unexpectedly, however, all fractions from the NCS-treated cells were active rather than solely the heavier dimeric form of Chk2. When we compared equivalent amounts of the peak monomer and dimer fractions from Chk2 purified from NCS-treated cells they displayed fairly similar kinase activities, with the dimeric form somewhat more active (Fig. 5B). We then compared the activities of dimers and monomers purified from baculovirally expressed Chk2-FLAG using the peak fractions 9 (dimer form) and 12 (monomer form) and again observed that both forms were active in phosphorylating the substrate (Fig. 5C). To determine the kinetic constants of both forms of Chk2 kinase, filter binding assays were performed using a 100–2300 μM molar excess of substrates over Chk2 proteins. As shown in Table II, the $V_{max}$ of the dimer form was greater than that of the monomer form by a factor of ~2-fold, whereas their $K_m$ values were essentially similar. Thus, a monomer form of Chk2 protein is active as a protein kinase in a DNA damage-dependent manner even when lacking Thr-68 phosphorylation.

We also considered the possibility that differential interaction between dimeric or monomeric forms of Chk2 and other regions of Cdc25C might affect the kinase activity of Chk2. To test this, the ability of both forms of Chk2-FLAG to phosphorylate GST-tagged full-length Cdc25C (GST-Cdc25C(WT)) was measured. GST-Cdc25C(WT) was in a wild-type conformation, as determined by measuring its phosphatase activity as described in Morris and Divita (34) (data not shown). As shown in Fig. 5D, GST-Cdc25C(WT) was phosphorylated to similar extents by the two forms of Chk2-FLAG. Note that GST-Cdc25C(WT) was far less well phosphorylated by either form than was GST-Cdc25C(C200–256), leading to the requirement for greater quantities of Chk2 protein. Thus, due to limited availability of dimeric forms of HA-Chk2, we were not able to compare kinase activities of both forms of active HA-Chk2 proteins with GST-Cdc25C(WT). Nevertheless, based on the fact that two sources of Chk2 from insect and DNA-damaged human cells were similar in all other assays, it is likely that the same result would be observed.

**Activated Chk2 Proteins Can Oligomerize Independently of Thr-68 Phosphorylation**—Because both forms of baculovirally expressed Chk2-FLAG were active yet differed in phosphorylation at least at the Thr-68 position, we were interested in whether they exist as stable entities or in dynamic equilibrium with each other. To this end we first subjected immunopurified Chk2-FLAG to Superdex 200 gel filtration chromatography, and elution fractions were collected in 500-μl aliquots (Fig. 6A). The majority of dimeric Chk2-FLAG eluted in fractions shown in lanes 2 and 3, whereas monomer Chk2-FLAG was in fractions shown in lanes 5 and 6. Again, only the dimer form was phosphorylated at Thr-68. The peak fractions (lanes 2 and 6) were then used to decrease the possibility of cross-contamination, each was subjected to a second round of gel filtration, and the collected fractions were again analyzed by immunoblotting (Fig. 6B). Interestingly each of these fractions appeared to produce both monomers and dimers, although in each case the starting material (monomer or dimer) was predominant (i.e. ~70%) and the other form was more minor (~30%). Even though the initial dimer fraction contained material eluting as monomer, the only fractions that reacted with the anti-phospho Thr-68 antibody were those spanning the

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**FIG. 5.** Protein kinase activities of monomer and oligomer forms of Chk2. **A**, each fraction (25 μl) from the Superdex 200 gel filtration experiment shown in Fig. 3A was incubated with 500 ng of GST-Cdc25C-C(200–256) in protein kinase buffer containing [γ-32P]ATP. The reaction mixtures were subjected to SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. The positions of autophosphorylated HA-Chk2 and substrate GST-Cdc25C are indicated. **B**, HA-Chk2 (1 ng) purified after NCS treatment was incubated with 0.6, 1, 2, and 4 μg of GST-Cdc25C-C(200–256). Reaction mixtures were separated by SDS-PAGE and transferred to nitrocellulose following autoradiography or immunoblotting (IB) with anti-HA antibody as indicated. 5% of reaction mixtures were analyzed in the same way and immunoblotted with anti-GST antibody. **C**, Chk2-FLAG (40 ng) from fractions 9 or 12 in Fig. 1A was incubated with 50, 100, 250, and 500 ng of GST-Cdc25C-C(200–256). Reaction mixtures were analyzed as described above. Anti-FLAG antibody was used for detection of Chk2-FLAG. **D**, Chk2-FLAG (80 ng) protein was incubated with 0.3, 0.6, 1.5, and 3 μg of GST-Cdc25C(WT). They were analyzed as described above.

**Table II**

| Form   | $V_{max}$ (pmol/min/μM) | $K_m$ (μM) | $V_{max}/K_m$ (μM) |
|--------|------------------------|------------|-------------------|
| Dimer  | 14.5 ± 1.1              | 30 ± 5     | 0.48              |
| Monomer| 7.5 ± 0.6               | 23 ± 5     | 0.33              |

**Chk2 from DNA-damaged Cells Can Phosphorylate Cdc25C without Detectable Phosphorylation at Thr-68**—Because Thr-68 phosphorylation is required for activation of Chk2 from cells sustaining DNA damage (16–19), we expected that only the Thr-68-phosphorylated form of Chk2 would display the ability to phosphorylate its substrate. We compared the kinase activities of the gel filtration fractions from either untreated or NCS-treated HCT116 cells using a GST-tagged fragment of Cdc25C (residues 200–256) that had been shown to be an excellent substrate for Chk2 (5, 16–19) (Fig. 5A). Overall the Chk2 protein in the fractions from the NCS-treated cells was far more active as a kinase than that from untreated cells.
dimerization we subjected a baculovirally derived kinase-inactive mutant Chk2 to ionizing radiation, unknown sensors recognize DNA breaks and signal to ATM, which phosphorylates Thr-68 in the SCD domain of inactive monomeric Chk2. Thr-68-phosphorylated monomers form dimers, allowing phosphorylation at other sites by ATM or unknown kinases. The sequence of dimerization and secondary phosphorylation events is not defined. Dimerization facilitates autophosphorylation and activation of the kinase domain. Multiple phosphorylation events may induce conformational change and increase susceptibility of phospho-Thr-68 to phosphatase. Active forms of Chk2 devoid of phosphorylation at Thr-68 can form unstable dimers. Dynamic equilibrium between monomers and dimers makes it uncertain which forms of Chk2 are catalytically competent.

Finally, the kinase activities of re-equilibrated dimers and monomers of Chk2-FLAG obtained after the second round of gel filtration were compared (Fig. 6F). All of the forms were active in phosphorylating GST-Cdc25C, although those derived from Thr-68-phosphorylated dimers and monomers, and again only the former contained Thr-68 phosphorylated protein (Fig. 6C). When the extent of Thr-68 phosphorylation of wild-type and mutant dimers was compared, 25 ng of wild-type dimers and 50 ng of mutant dimers were phosphorylated to a similar level. This shows that at least in the mutant form Chk2 dimers can consist of populations that contain and lack phosphorylation at Thr-68 (Fig. 6D).

Thr-68 phosphorylation-independent oligomerization in vitro was then tested using Chk2-FLAG and HA-Chk2. The Thr-68 phosphorylation-negative form (monomer fraction) of active HA-Chk2 or inactive HA-Chk2 was purified from NCS-treated or -untreated cells, respectively, by gel filtration as shown in Fig. 3A. We then incubated these HA-Chk2 proteins with monomer or dimer peak fractions of wild-type or mutant Chk2-FLAG, respectively. The Thr-68 phosphorylation-negative HA-Chk2 purified after NCS treatment could interact stably with both forms of wild-type or mutant Chk2-FLAG (Fig. 6E). The amount of active HA-Chk2 proteins pulled down by Chk2-FLAG was comparable in all cases. This is probably due to the fact that an excess amount of Chk2-FLAG proteins was used against HA-Chk2 proteins. By contrast, Thr-68-unphosphorylated HA-Chk2 purified from untreated cells failed to form stable complexes with any form of Chk2-FLAG. Taken together, the results suggest that Chk2 can form dimers independently of Thr-68 phosphorylation but only if the protein is activated after DNA damage. They also imply that Thr-68-phosphorylated Chk2 can interact with Chk2 devoid of Thr-68 phosphorylation. Because of the results obtained in Fig. 6B showing a dynamic equilibrium between monomers and dimers, we cannot determine with certainty from these experiments which of the Thr-68-unphosphorylated forms can oligomerize. Nevertheless, our data provide several lines of evidence that Chk2 oligomerization can occur without detectable phosphorylation at Thr-68.

Fig. 6. Active forms of Chk2 can oligomerize and phosphorylate Cdc25C independently of Thr-68 phosphorylation. A, immunofinity-purified Chk2-FLAG (125 μg) was subjected to 24 ml of Superdex 200 gel filtration chromatography. Elution fractions (500 μl each) were collected, and aliquots (20 μl) were subjected to SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Thr-68 antibody (α-Thr68-P) as indicated. B, the dimeric form Chk2 (fraction in lane 2 in A; 200 ng) and the monomeric form of Chk2-FLAG (fraction shown in lane 6 of panel A; 200 ng) were subjected to 2.4 ml of Superdex 200 gel filtration chromatography. Elution fractions (50 μl) were collected and analyzed as in A. C, immunofinity-purified mutant Chk2-FLAG (D347A) (80 μg) was subjected to 24 ml of Superdex 200 gel filtration chromatography. Fractions were analyzed as in A. D, 25 and 50 ng of both dimeric (fraction in lane 2 of panel A) and monomeric (fraction in lane 6 of panel A) forms of Chk2-FLAG were subjected to SDS-PAGE and analyzed by immunoblotting with either anti-FLAG or anti-phospho-Thr-68 antibody along with 6, 12, 25, and 50 ng of either dimeric (lane 2 of panel C) or monomeric (lane 6 of panel C) forms of mutant Chk2 (D347A). E, Thr-68-unphosphorylated forms of HA-Chk2 (25 ng) that were immunofinity-purified from untreated (T68-P negative inactive HA-Chk2) or NCS-treated (T68-P negative active HA-Chk2) HTC116 cells (fraction 13 in Fig. 3A and C) were incubated with 1200 ng of either fraction 2 or fraction 6 of either Chk2-FLAG(WT) or Chk2-FLAG (D347A) from A and C, respectively. The mixtures were immunoprecipitated (IP) with anti-FLAG antibody cross-linked to agarose beads, resolved by SDS-PAGE, and then immunoblotted (IB) with anti-HA antibody. F, fractions 3 (20 ng) and 5 (25 ng) from either 2 or 6 shown in B were incubated with 0.5, 1, and 2 μg of GST-Cdc25C (200–256) in protein kinase buffer containing [γ-32P]ATP. Mixtures were analyzed as described in Fig. 2.

position of the dimers. Thus, a dynamic equilibrium exists between unphosphorylated Chk2 monomers and dimers, whereas Thr-68-phosphorylated Chk2 dimers are stable.

To further investigate Thr-68 phosphorylation-independent dimerization we subjected a baculovirally derived kinase-inactive mutant form of Chk2 (D347A)-FLAG (5) to gel filtration. Chk2 (D347A)-FLAG protein could also be separated into dimer and monomer forms, and again only the former contained Thr-68 phosphorylated protein (Fig. 6C). When the extent of Thr-68 phosphorylation of wild-type and mutant dimers was compared, 25 ng of wild-type dimers and 50 ng of mutant dimers were phosphorylated to a similar level. This shows that at least in the mutant form Chk2 dimers can consist of populations that contain and lack phosphorylation at Thr-68 (Fig. 6D).

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from the dimer fraction from the first round of gel filtration were 2-fold more active than those from the initial monomer fraction. Here, too, given that unphosphorylated dimers and monomers can interconvert, we were not able to assess the extent to which each form can function as a kinase.

**DISCUSSION**

Although aspects of Chk2 structure and function have been described, there are still many gaps in our understanding of its biochemical properties. In this study we examined some of the characteristics of human Chk2 purified from insect and human cells. The experiments yielded several findings. First, unstressed cells produce only inactive Thr-68-unphosphorylated monomers. Second, upon DNA damage, Chk2 comprises stable Thr-68-phosphorylated dimers as well as Thr-68-unphosphorylated Chk2 dimers that can exist in a state of dynamic equilibrium with monomers. Third, Thr-68 phosphorylation is not required for maintenance of Chk2 kinase activity. Our data taken together suggest a model by which Chk2 phosphorylation at Thr-68 is a priming event for subsequent events leading to activation of the protein but that maintenance of phosphorylation at that site is not required for dimerization or activity (Fig. 7). It is acknowledged that our data to support the latter hypothesis rest partly on experiments using Chk2 isolated from insect cells and that further confirmation awaits the ability to isolate non-Thr-68-phosphorylated Chk2 dimers from DNA-damaged human cells. Nevertheless all of our data indicate that insect cell-derived Chk2 is virtually identical to that from DNA-damaged human cells.

Wu et al. (20) report that wild-type Chk2 mainly exists as a protein complex of 200 kDa in human cells. They speculated that this complex might contain other cellular proteins. Based upon their results, our initial goal was to identify the components of the protein complex. However, when we characterized and compared the biochemical and biophysical properties of recombinant Chk2 purified from both baculovirus-infected insect cells and mammalian cells, it became evident that highly purified Chk2 exists as monomers and homodimers. Gel filtration and sedimentation analyses of the protein suggest that it has a rather unusual shape and that the 200-kDa protein complex is the dimeric form of Chk2. Chk2 is not terribly unique in possessing a non-globular shape; a sample of asymmetric proteins includes p53 (35), calreticulin (36), and the HSC70 co-chaperone, HIP (37), among others.

Highly purified HA-Chk2 proteins from human cells after treatment with NCS also consist of homodimers and monomers. There did not appear to be detectable quantities of larger forms of Chk2. This suggests that there is no highly stable interaction between Chk2 and other cellular factors as a result of DNA damage. We cannot rule out transient or weak interaction between such proteins, however, which would not be sustained in our immunoaffinity purification. The recombinant Chk2 FHA domain was found to interact with BRCA1 in both phosphorylation-dependent and -independent manners (15, 38). BRCA1 is a part of BASC, a supercomplex of a BRCA1-associated genome surveillance complex comprised of tumor suppressors, DNA damage repair proteins, and DNA replication factors (39). Therefore, Chk2 may well participate in huge macromolecular complexes. In fact Ward et al. (40) report that Thr-68-phosphorylated Chk2 is localized to sites of DNA strand breaks after ionizing radiation. The interacting partner(s) of Chk2 in such conditions have yet to be identified.

There are some differences between what we have observed and others have reported regarding Chk2 dimerization. Ahn et al. (28) report that Chk2 was activated through transient dimerization after phosphorylation at Thr-68 after DNA damage to cells. They were not able to observe a stable interaction between two wild-type Chk2 proteins differentially epitope-tagged at the N terminus by immunoprecipitation from transiently transfected mammalian cells after ionizing radiation. However, they obtained stable complexes of catalytic inactive mutant and wild-type Chk2 independently of ionizing radiation to the cells, leading them to propose that Chk2 exists as an active monomer that is phosphorylated at Thr-68 as a response to DNA damage in cells. On the other hand, Xu et al. (29) report that two wild-type Chk2 constructs differentially tagged at their N termini could form a stable complex using similar conditions to those of Ahn et al. (28). Xu et al. (29) also observed dimerization of Chk2 in the absence of ionizing radiation with a concomitant increase in dimerization and phosphorylation of the SCD domain after DNA damage. Both groups proposed that homooligomerization of the proteins arises from the intermolecular interaction between the phosphorylated SCD domain of one Chk2 molecule and the FHA domain of the other molecule. By contrast, neither Wu et al. (20) nor ourselves was able to detect interactions between endogenous Chk2 and HA-Chk2 from HCT116 cells by immunoprecipitation with anti-HA antibody. Our results strongly suggest that the anti-HA antibody, when bound to the N-terminal HA epitope, interferes with the intermolecular interaction between the SCD domain and the FHA domain probably by steric hindrance. A possible explanation for the differences in our results and those of Xu et al. (29) was that their Chk2 construct had multiple tandem epitopes that could result in antibody binding at the extreme N terminus without perturbing the SCD-FHA domain interaction. Additionally, a possible explanation as to why other groups observed oligomerization even without having irradiated cells is because they used transient transfection to overexpress Chk2 proteins, and this process has been shown to cause stress to cells that is akin to DNA damage and activate a DNA damage response pathway (41, 42). We have not tested whether the process of transfection of HCT116 cells would yield dimeric Thr-68-phosphorylated Chk2.

In the SCD domain of Chk2 are seven SQ/TQ sites. Thr-68 phosphorylation is the presumed priming event that activates the protein upon DNA damage to cells (16–19). We observed the most stable forms of dimers are phosphorylated at Thr-68. Interestingly, our results showed that active forms of Chk2 protein lacking Thr-68 phosphorylation still formed dimers, although the interaction is not stable as judged by their elution profile from gel filtration chromatography and resistance to chemical cross-linking. Xu et al. (29) also show that a mutant form of Chk2 (T68A) forms dimers but to a lesser extent than wild-type proteins. Because the mutant protein used in our study is defective in autophosphorylation, Thr-68 phosphorylation-independent dimerization cannot be due to autophosphorylation during activation of kinase domain. Secondary dimerization determinants may be other phosphorylation sites in the SCD domain. The existence of Chk2 protein from DNA-damaged cells that is not phosphorylated at Thr-68 implies that such proteins arise from dimers from which phosphorylation at Thr-68 has been removed but which retain phosphates necessary and sufficient for activation of the kinase activity.

Our results pose a number of questions. 1) If secondary dimerization is due to phosphorylation at other sites, is it mediated by ATM or other unknown kinases? 2) What is the activity that selectively removes phosphate from Thr-68 after DNA damage? 3) If both monomer and dimer forms of Chk2 are active in DNA-damaged cells, what are their respective purposes? Answers to these questions will provide the impetus for future work on this interesting checkpoint kinase.

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