Chk2 Oligomerization Studied by Phosphopeptide Ligation

IMPLICATIONS FOR REGULATION AND PHOSPHODEPENDENT INTERACTIONS

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Chk2/CHEK2/hCds1 is a modular serine-threonine kinase involved in transducing DNA damage signals. Phosphorylation by ataxia telangiectasia-mutated kinase (ATM) promotes Chk2 self-association, autophosphorylation, and activation. Here we use expressed protein ligation to generate a Chk2 N-terminal regulatory region encompassing a forhead-associated (FHA) domain, a stoichiometrically phosphorylated Thr-68 motif and intervening linker. Hydrodynamic analysis reveals that Thr-68 phosphorylation stabilizes weak FHA-FHA interactions that occur in the unphosphorylated species to form a high affinity dimer. Although clearly a prerequisite for Chk2 activation in vivo, we show that dimerization modulates potential phosphodependent interactions with effector proteins and substrates through either the pThr-68 site, or the canonical FHA phosphobinding surface with which it is tightly associated. We further show that the dimer-occluded pThr-68 motif is released by intra-dimer autophosphorylation of the FHA domain at the highly conserved Ser-140 position, a major pThr contact in all FHA-phosphopeptide complex structures, revealing a mechanism of Chk2 dimer dissociation following kinase domain activation.

Chk2 (also known as CHEK2 or hCds1) was originally discovered as a Saccharomyces cerevisiae Rad53-related kinase that is primarily activated by the ataxia telangiectasia-mutated kinase (ATM)3 after DNA damage (1–5). Many substrates and interacting partners have since been identified (6) and through this increasingly diverse array of interactions, Chk2 appears to act not only as a regulator of DNA damage/stress-response signaling and cell cycle checkpoint activation, but also apoptosis, senescence, viral infectivity, and other pathways. In particular, its apparent role in regulating p53 (7–9), and the discovery of mutant Chk2 alleles in patients with wild-type p53 but who suffer from Li-Fraumeni syndrome, suggested that Chk2 may itself be a tumor suppressor (10). Although this remains controversial, recent studies have shown that Chk2 mutations may cause increased susceptibility to a wide variety of cancers, and it has emerged as a potentially important anti-cancer drug target (11, 12).

The significance of Chk2 in the DNA damage response and checkpoint regulation has inspired a number of studies of its regulation and its interactions with target molecules. Chk2 is a member of a family of kinases that includes budding yeast Rad53p and Dun1p, fission yeast Cds1p and Drosophila DMNK. Overall, these molecules share a related architecture, and contain two main regions of sequence homology that comprise a C-terminal Ser/Thr kinase domain and an N-terminal (FHA) domain. Rad53 is an exception and is the only family member with a second, C-terminal FHA. It is well established that FHA domains mediate protein-protein interactions primarily through binding to short phosphothreonine-containing motifs in target proteins. The importance of the FHA domain in Chk2 function is exemplified by its absolute requirement for Chk2 activation and the identification of several point mutations in this region in various cancer cells types. In addition, the InsZ Chk2 transcript variant encoding only the FHA domain and N-terminal regulatory region may act as a dominant negative allele in late stage breast cancer (13).

Chk2 is a phosphoprotein in vivo, and its full activation requires phosphorylation on Thr-68. This is located within a partially conserved N-terminal region (14–18) containing a series of Ser-Gln or Thr-Gln pairs (SCD, Ref. 19). While these represent preferred sites for PI3-kinase-related kinases such as ATM, ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), they may also be targeted by other kinases, such as Mps1, MRK, and Polo-like kinases (Plk) (20–23). A single mutation of Thr-68 to alanine in the Chk2 SCD is sufficient to severely reduce ATM-dependent Chk2 activation after DNA damage (14–16), and abolish interactions of IR-activated Chk2 with recombinant Chk2 FHA domain (24). In contrast, mutations at the six other potential PIKK sites have little or no effect alone or in combination (14–16). A model for activation of Chk2 family
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kinases has emerged that involves self-association through intermolecular binding of the FHA domain to phosphorylated Thr-68 and possibly other sites in the SCD. This generates oligomers that are proficient for autophosphorylation of the kinase domain activation loop in trans (24–28). Although structures of both the Chk2 FHA-phosphopeptide complex, and the Chk2 kinase domain have been determined individually (27, 29), a structure of any full-length Chk2 orthologue remains elusive. Furthermore, efforts to tease out details of the activation mechanism have been complicated by the fact that Chk2 is highly phosphorylated at many sites (30–32), of which only a subset have been characterized in terms of their contribution to Chk2 function and regulation in vivo. The experimental challenge is in generating Chk2 molecules with precisely defined phosphorylation states. A powerful alternative to enzymatic modification is expressed protein ligation (EPL) of recombinant proteins with synthetic peptides containing specific and stoichiometric post-translational modifications of many kinds. In the case of phosphorylation, chemical, and intein-mediated ligation has been previously used to great effect to generate semi-synthetic analogues of active TGF-β kinase (33) and phosphorylated substrate proteins, particularly R-smad MH2 domains (34). To this end, we have used EPL technology to generate a semi-synthetic molecule in which the Chk2 FHA domain is appended to its N-terminal regulatory region containing a unique pThr-68 phosphorylation site, as a model of ATM-phosphorylated full-length Chk2 protein. Using this chemically defined species in combination with hydrodynamics, mass spectrometry, and in vitro binding assays, we investigate protein-protein interactions involved in Chk2 activation in vitro, their regulation by autophosphorylation and implications for Chk2 interactions with regulators and target molecules.

EXPERIMENTAL PROCEDURES

Strains and Reagents—Escherichia coli strain BL21 (DE3) RIL (Stratagene) was used for GST fusion protein expression and NovaBlue SinglesTM competent cells (Novagen) were used for plasmid construction and maintenance. The pGEX-6P1 expression vector was obtained from Amersham Biosciences. All reagents used in the SPR measurements, EDC (N-ethyl-N’-(3-dimethyl-amino propyl)-carbodiimide hydrochloride), NHS (N-hydrosuccinimide), ethanolamine.HCl, HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% (3-dimethyl-amino propyl)-carbodiimide hydrochloride, NHS hydrodynamics, mass spectrometry, and

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FHA domain fragment appropriate for chemical ligation, residues

73–219 of Chk2 were PCR-amplified and cloned into pGEX-

6P1. The 5’ primer was designed so as to generate a mutation of Ser-73 to cysteine (S73C) and to place a cleavage site for Factor Xa immediately upstream. Following purification of this fusion protein, cleavage with Factor Xa yields the FHA domain fragment with a free N-terminal cysteine. The synthetic C-terminal thiouester peptide at a concentration of 1 mM was incubated with 100 μM purified Factor Xa, digested FHA proteins overnight at room temperature in 200 mM phosphate buffer, pH 8.0, 150 mM NaCl, 10 mM TCEP, 122 mM 2-mercaptoethanesulfonic acid (MESNA) to form the ligated pThr-68 FHA product (pT68 FHAlig).

Analytical Ultracentrifugation (AUC)—Sedimentation velocity experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics. Chk2 FHA-(63–219) and pT68 FHAlig were and were prepared with dilution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 0.5 mM TCEP). The aluminum double sector centerpieces were filled with 400 μl of the protein sample and 420 μl the dilution buffer, respectively. Samples were centrifuged at a speed of 50,000 rpm and a temperature of 20 °C using an An50-Ti rotor. Scans were acquired at a wavelength of 280 nm in time intervals of 600 s. The partial specific volume of 0.72 ml/g for FHA proteins was calculated based on the amino acid composition (36). Sedimentation velocity data were analyzed using SEDFIT (37).

Size Exclusion Chromatography Multiangle Laser Scattering (SEC-MALLS)—Molecular weights and molecular weight distributions were determined using on line multi-angle laser light scattering coupled with size exclusion chromatography (SEC-MALLS). Samples were applied to a Superdex 75 10/300 GL column equilibrated in 20 mM Tris–HCl, 150 mM NaCl 0.5 mM virus 3C protease. The Polo box-binding domain (PBD) of human Plk1 was expressed and purified as described (35). Site-directed mutagenesis was carried out using the QuikChange system (Stratagene). Chk2 GST fusion proteins were affinity-purified on glutathione-4B resin (Amersham Biosciences) and cleaved from the affinity resin with rhinovirus 3C protease. Where appropriate, the eluted protein fragments were further purified using Q-Sepharose FF anion exchange resin (Amersham Biosciences). Fractions containing overexpressed protein fragments were pooled and purified with gel-filtration chromatography on Superdex 75 in 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM TCEP (Tris(2-carboxyethyl)phosphine).

Isothermal Titration Calorimetry—ITC experiments were performed in 20 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM β-mercaptoethanol at 20 °C using a VP-ITC microcalorimeter (MicroCal Inc.). Chk2 FHA-(63–219) at a concentration of 60 μM was titrated against 50 × 5 μl injections of Chk2 pThr-68 peptide (TVSTpQELYLS) at a syringe concentration of 880 μM. Following subtraction of the heat of dilution, data were analyzed assuming a single-site binding model using ORIGIN software.

Peptide Synthesis and Phosphopeptide Ligation—The peptide thioester with sequence H2N-LETVSpQELY-SBn (pThr-68-SBn) was prepared using the sulfonamide safety-catch linker strategy (supplemental information). To produce a Chk2 FHA fragment appropriate for chemical ligation, residues 73–219 of Chk2 were PCR-amplified and cloned into pGEX-6P1. The 5’ primer was designed so as to generate a mutation of Ser-73 to cysteine (S73C) and to place a cleavage site for Factor Xa immediately upstream. Following purification of this fusion protein, cleavage with Factor Xa yields the FHA domain fragment with a free N-terminal cysteine. The synthetic C-terminal thiouester peptide at a concentration of 1 mM was incubated with 100 μM purified Factor Xa, digested FHA proteins overnight at room temperature in 200 mM phosphate buffer, pH 8.0, 150 mM NaCl, 10 mM TCEP, 122 mM 2-mercaptoethanesulfonic acid (MESNA) to form the ligated pThr-68 FHA product (pT68 FHAlig).
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TCEP, pH 8.0 at a flow rate of 0.5 ml/min. The column was mounted on a Jasco HPLC controlled by the Chrompack software package. The scattered light intensity of the column eluent was recorded at sixteen angles (over the range 32°–147°) using a DAWN-HELOS laser photometer (Wyatt Technology Corp., Santa Barbara, CA). The protein concentration of the eluent was determined from the refractive index change (dn/dc = 0.186) using an OPTILAB-rEX differential refractometer equipped with a Peltier temperature-regulated flow cell, maintained at 25 °C (Wyatt Technology Corp.). The wavelength of the laser in the DAWN-HELOS, and the light source in the OPTILAB-rEX was 658 nm. The weight-averaged molecular mass of material contained in chromatographic peaks was determined using the ASTRA software version 5.1 (Wyatt Technology Corp.). Briefly, at 1-s intervals throughout the elution of peaks the scattered light intensities together with the corresponding protein concentration were used to construct Debye plots (KC/R versus sin²(θ/2)). The weight-averaged molecular mass was then calculated at each point in the chromatogram from the intercept of an individual plot. An overall average molecular weight and polydispersity term for each species was calculated by combining and averaging the data from the individual measurements.

Surface Plasmon Resonance Measurements—For phosphopeptide binding studies by surface plasmon resonance (SPR), a streptavidin sensor chip was used for immobilization of a biotinylated peptide, Biotin-T68pL (Biotin-T_{68}SSSLLET-VSTpQELYSIPEr_{70}), derived from the N-terminal region of human Chk2. Steady state measurements were performed using a Biacore 2000 instrument. Samples were injected at a flow rate of 5 μl/min for 12 min to ensure that steady state was established. The sensor chip surface was regenerated with a 12-min wash step. The Plk1 PBD protein was diluted in HBS-EP buffer as a series of analytes (78 nM, 156 nM, 312 nM, 625 nM, 1.25 μM, 2.5 μM, 5.0 μM, 10.0 μM). Responses (RU) from the ligand-immobilized flow cell with the corresponding blank flow cell. These were plotted against analyte concentration and the data fitted with non-linear single-site binding model in Equation 1,

\[ Y = \frac{BX}{K_D + X} \]  

(Eq. 1)

where \( Y \) is the specific binding at steady state, \( B \) is the maximum binding capacity of the surface, \( X \) is the analyte concentration, and \( K_D \) is the equilibrium dissociation constant.

Competition experiments using the Plk1 PBD protein were measured using steady state methods. A series of analytes were prepared that contained a constant PBD concentration (50 nM) and increasing concentrations of competitor proteins/peptides (0 nM, 9.75 nM, 19.5 nM, 39 nM, 78 nM, 156 nM, 312.5 nM, 625 nM, 1.25 μM, 2.5 μM, 5 μM, 10 μM). The percentages of steady state signal remaining were calculated using the response from binding of 50 nM Plk1 PBD alone as 100%. These were plotted against logarithm of concentration of the competitor. Where appropriate, data were fitted with non-linear one-site competition model in Equation 2,

\[ Y = A_2 + \frac{A_1 - A_2}{1 + 10^{X - \log X_0}} \]  

(Eq. 2)

where \( Y \) is percentage of specific binding, \( A_2 \) is the fit minimum, \( A_1 \) is the fit maximum, \( X \) is the logarithm of competitor concentration, and \( X_0 \) is the EC_{50}.

Autophosphorylation Assays—Autophosphorylation/rephosphorylation assays (32) were carried out at a Chk2 concentration of 20 μM in a buffer containing 50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM CaCl₂, and 3 mM ATP at room temperature for 2 h. For FHA phosphorylation assays, ligated or unligated wild-type FHA fragments were included at equimolar concentration and incubated at room temperature for 4 h followed by overnight incubation at 4 °C.

Phosphorylation Site Mapping—Identification of autophosphorylation sites was performed essentially as described (38). Briefly, excised SDS-PAGE protein bands were reduced with dithiothreitol and alkylated using iodoacetamide. The bands were dried and reswollen in a sufficient volume to cover 2 ng/μl chymotrypsin (sequencing grade Roche Diagnostic, Germany) in 5 mM ammonium bicarbonate. After overnight digestion at 32 °C, the supernatant was acidified by the addition of a 1/10th volume of 4% trifluoroacetic acid. A phosphoprotein purification kit (Qiagen) was used to isolate the phosphorylated peptides, which were then fractionated with a Model 130A syringe pump HPLC system from Applied Biosystems using a 250-mm 5-mm Reliasil C18 BDX column (Column Engineering, Ontario). Peptide mass fingerprinting was performed using a Reflex III MALDI time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with a nitrogen laser and a Scout-384 probe, to obtain positive ion mass spectra of digested protein with pulsed-ion extraction in reflectron mode. An accelerating voltage of 26 kV was used with detector bias gating set to 2 kV and a mass cutoff of m/z 650. Matrix surfaces were prepared using recrystallized α-cyano-4-hydroxycinnamic acid and nitrocellulose. 0.4 μl of digestion supernatant was deposited on the matrix surface and allowed to dry prior to desalting with water. Peptide mass fingerprints were searched against the non-redundant protein data base placed in the public domain by the National Centre for Biotechnology Information (NCBI) using the program MASCOT (39). Nanospray mass spectra were acquired on an LCQ “classic” quadrupole ion trap mass spectrometer (ThermoQuest, Austin, TX) equipped with a nanospray source (Protaba, Odense, Denmark) operated at a spray voltage of 800 V and a capillary temperature of 150 °C. Dried HPLC fractions were dissolved in 60% v/v methanol, 0.1% v/v formic acid, and 2 μl transferred to an Econo12 nanospray needle (New Objective Inc, Cambridge, MA). Daughter ion spectra were typically acquired at a collision energy of 30% and an isolation width of 4 Da.

RESULTS

Chk2 FHA Binds to pThr-68 Peptides with Low Affinity—Of the two threonines present within TQ dipeptide motifs in the
human Chk2 SCD (Thr-26 and Thr-68), Thr-68 appears to be the preferred site for DNA damage-dependent phosphorylation by ATM. Thr-68 is unique in the Chk2 SCD in that it resides within a sequence Thr68-Gln69-Glu70-Leu71 that is conserved in all mammalian Chk2 orthologues (Fig. 1B). This sequence resembles the optimal Chk2 FHA-interacting motif identified by oriented peptide library screening, which showed a marked preference for medium hydrophobic residues (Ile/Leu/Val) at the position three residues C-terminal to the phosphorylated threonine (pT) (29). A peptide encompassing the pThr-68 motif has been reported to bind to the Chk2 FHA domain (29, 40). We now show by ITC that it does so with an apparent $K_d$ of around 30 $\mu$M (Fig. 1A) consistent with a potential role for pThr-68-FHA interactions in Chk2 self-association and activation. Many studies of FHA domain phosphodependent binding activity and specificity, including that of Chk2 (41) have demonstrated a profound binding preference for pThr-containing motifs. Therefore it seems unlikely that Chk2 activation can be efficiently achieved through serine phosphorylation at SQ or other sites in the SCD, and this appears to be the case (14–16). The remaining TQ pair in the human Chk2 SCD at Thr-26 is efficiently phosphorylated by ATM/Rad3-related (ATR) but not by ATM (16). More significantly, it is not conserved in mammalian Chk2 orthologues (Fig. 1C) and has a glutamine in the pT+3 position rendering it an unlikely FHA binding site. Similarly, the *Schizosaccharomyces pombe* Chk2 orthologue Cds1 contains two phosphorylated threonines in its SCD region but only one of these, Thr-11, is necessary and sufficient for activation (42). For these reasons we chose to focus on the role of Thr-68 phosphorylation in Chk2-Chk2 interactions.

**A Semisynthetic Model of pThr-68/FHA-mediated Chk2-Chk2 Interactions**—To circumvent problems of heterogeneous autophosphorylation of recombinant Chk2, and our currently incomplete understanding of the effects of secondary autophosphorylation on kinase activity and oligomerization, we used EPL technology to generate an uniquely Thr-68-phosphorylated fragment of Chk2 encompassing the FHA domain and intervening linker sequences (Fig. 2A). Time course studies at two different peptide/protein ratios were used to monitor the efficiencies of the ligation reactions (Fig. 2B). A 10:1 peptide/protein molar ratio produced greater than 80% ligation efficiency over a 20-h period, and these conditions were used in all subsequent experiments. For further purification and analysis, we employed gel-filtration chromatography of ligation products on Superdex 75 (Fig. 2C). Elution profiles showed the presence of well separated fast and slow migrating peaks consistent with self-association of the ligated product. The difference in retention time of the two species appeared to be directly attributable to Thr-68 phosphorylation as treatment with phosphatase converted the ligated product to a form with the expected molecular mass of the dephosphorylated product that migrated identically to unphosphorylated Chk2 FHA (63–219). The faster eluting protein was confirmed as the ligated product by mass spectrometry with no detectable contaminating unphosphorylated species.

**Thr-68-phosphorylated Chk2 FHA Forms a Tight Dimer**—The solution molecular weight and hydrodynamic properties

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**FIGURE 1.** Regulatory interactions in the Chk2 N-terminal region. A, an affinity of $30 \mu$M was determined for Chk2 FHA-(63–219) binding to a phosphopeptide encompassing Thr-68 by ITC as described under “Experimental Procedures.” Raw data are shown in the upper panel and integrated heat changes shown in the lower panel as open circles, along with the curve fit to a 1:1 binding model represented by the solid line. B, schematic overview of Chk2 architecture (top), and alignment of mammalian Chk2 sequences (bottom) showing the SCD (gray), FHA domain (white), and kinase domains (black). The *Thr*-Gln-Glu-Leu* Thr binding motif (solid bar) is the only threonine-containing PIKK phosphorylation site within the Chk2 SCD that is conserved in known mammalian Chk2 sequences.
of non-phospho (Chk2 FHA-(63–219)) and the Thr-68-phosphorylated (pT68 FHAlig) protein generated by EPL were examined by a combination of AUC and light scattering methods. Initially, the solution molecular weight of unmodified Chk2 FHA-(63–219) was estimated using SEC-MALLS (Fig. 3A). These data report a weight-averaged molecular mass of 22.2 kDa, significantly greater than that expected for the protein monomer (18.4 kDa). This anomalously high solution molecular weight was further investigated using sedimentation velocity AUC. Typical sedimentation coefficient C(S) and molar mass C(M) distributions produced from sedimentation velocity experiments employing 18 μM non-phosphorylated Chk2 FHA-(63–219) and 24 μM pT68 FHAlig are shown in Fig. 3, C and D, respectively. Inspection of the C(S) function derived from sedimentation of Chk2 FHA-(63–219) reveals that around 70% of the material is contained within a peak with S20,w of 1.9, Mw = 28.6 kDa, where the best fit weight-averaged frictional ratio (f/fo)w for the whole distribution function has a value of 1.85. The remainder of the signal is distributed among several smaller peaks that have sedimentation coefficients ranging from 3 S to 10 S and are presumably the result of some aggregation in the sample. Because of the slight heterogeneity, the (f/f0)w for the 1.9 S peak alone was determined by inspection of the contour plot of the two-dimensional distribution function of sedimentation coefficients and frictional ratios C(S,f/f0)w (data not shown). The most populated frictional ratio within the 1.9 S peak determined by this method has a value of 1.7. Applying this frictional ratio, an apparent weight-averaged molecular weight of 24.8 kDa is then obtained from the C(S) distribution function (Fig. 3C) and a similar value of 25.0 kDa from the integration of the C(M) function (Fig. 3D). Thus, both the SEC-MALLS and sedimentation velocity measurements are consistent with weak, but significant FHA-FHA interactions in the absence of SCD phosphorylation.

In contrast to the non-phosphorylated fragment, AUC showed that at a loading concentration of 24 μM the ligated FHA sediments as single species comprising greater than 95% of the signal in the C(S) distribution (Fig. 3C). The molecule has an S20,w of 2.9 and an apparent mass of 35.9 kDa, within 2.5% of the expected dimeric mass of 36.8 kDa, with no observable monomeric species present. Furthermore, analysis of the self-association of the phosphorylated species by SEC-MALLS shows no observable dissociation at an on-column peak concentration of ~4.8 μM (Fig. 3B). We, therefore, conclude that Thr-68-phosphorylated Chk2 forms a dimeric complex with high apparent affinity. This notion is supported by experiments designed to examine the accessibility of the pThr-68 motif in Chk2 dimers described below.

**Chk2 Dimerization Impedes pThr-68- and FHA-dependent Interactions with Effectors**—Several studies have suggested that pThr-68 can serve as a docking/recognition motif for

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**FIGURE 2. Generation of a semisynthetic Thr-68-phosphorylated Chk2 FHA domain.** (A) Chk2 FHA-(73–219) S73C was expressed as a GST fusion protein containing a Factor Xa cleavage site immediately N-terminal to residue 73, which was mutated from Ser to Cys. After proteolytic cleavage, the Chk2 FHA was ligated to pThr-68-SH synthetic thioester peptide through the N-terminal Cys-73 to produce a Chk2 fragment containing residues 63–219, a phosphorylated Thr-68 and a Ser-73 to Cys mutation. B, time course of the ligation reaction at a peptide/protein ratio of 2:1 (left). The lower band is Chk2 FHA-(73–219) S73C, and the upper band is the ligated product. After 21 h at room temperature, around 30% ligation is observed. Increasing the peptide/protein molar ratio to 10:1 produces >80% ligation efficiency (right). C, gel-filtration profiles of the purified pT68 FHAlig treated for 5 min with 0 U (solid line), 7 U (dashed line), and 70 U (dotted line) of λ phosphatase at room temperature. The major peak for untreated pT68 FHAig is at 23.5 min. In contrast, the major peak for 70 U phosphatase-treated protein is at 23.7 min while the 70 U phosphatase-treated sample contains two major peaks at 25.9 and 27.0 min. The elution position of unphosphorylated Chk2-(63–219) is shown by the vertical arrow.
phosphodependent binding modules present within Chk2 binding partners (43, 44). We therefore wished to investigate the implications of the tight, pThr-68-dependent dimerization that we have described for downstream effector/substrate interactions. To do this we chose to use the PBD of human Plk1, which binds to the pThr-68 phosphopeptide with an affinity of 400 nM (Fig. 4A), as a probe of pThr-68 motif accessibility in monomeric and dimeric Chk2. We first attempted to compare PBD/Chk2 binding directly by SPR titration but this proved impractical due to either artifactual dissociation of the pThr-68-dimerized FHA domain or disruption of the PBD binding surface during cross-linking to activated carboxymethyl dextran BIAcore chips. Instead, we developed a solution competition assay in which a series of mixtures containing a constant concentration of the PBD and increasing concentrations of competitor proteins, were injected over a Biacore streptavidin chip coated with the biotinylated pThr-68 peptide. Binding of competitor to the Plk1 PBD reduces its interaction with the surface immobilized phosphopeptide. Thus for the control titration (Fig. 4B), PBD-pThr-68 interactions are effectively competed by increasing concentrations of a peptide (MAGPMQSpTPLNGAKK-Plktide) that we have previously shown to bind to the Plk1 PBD with a $K_d$ of 300 nM (35). For Chk2-derived competitors (Fig. 4C), only the pThr-68 peptide and a ligated Chk2 FHA domain with an alanine substitution at Ser-140 (pT68 FHAlig S140A) showed a continuous concentration-dependent competition for PBD binding to the chip. This is significant because Ser-140 makes a crucial and highly conserved interaction with the pThr moiety (29). Its mutation to alanine abrogates phosphopeptide binding (see Fig. 6B below) and therefore pThr-68-dependent dimerization. Similar observations were made for competition experiments using the FHA domain of Mdc1, which has also been proposed to bind Chk2 in a pThr-68-dependent manner, in place of the Plk1 PBD (44) (supplemental Fig. S1). In contrast, the other competitors, including Chk2 Kin-(205–538) that contains the kinase domain and only a single phosphorylated Ser-516 (data not shown), show a background reduction in signal that remains at around 80%. Most importantly, dimerized pT68 FHAlig only reduces binding to control levels, despite the high affinity of the PBD for the isolated pThr-68 motif. This is consistent with our

**FIGURE 3. Hydrodynamic analyses of phosphodependent self-association.** SEC-MALLS analysis of non-phospho (A) and pThr-68 phosphorylated (B) proteins. Normalized dRI signal (red) and UV signal (black) were plotted against elution time. The molar mass distribution (green) was calculated as described under “Experimental Procedures.” C, C(S) distribution profiles and D, C(M) distribution profiles from sedimentation velocity AUC. Samples of pT68 FHAlig (black line) and Chk2-(63–219) (dotted line) were centrifuged at 50,000 rpm at 20 °C. The moving boundary data were analyzed in terms of either discrete components or the size distribution functions C(S), C(S,f/f_o) (not shown) and C(M), implemented in the program Sedfit (37).
contention that tight dimerization of Chk2 effectively renders it unable to interact with downstream effectors/binding partners through either the pThr-68 motif or the FHA phosphobinding site.

**FHA Domain Phosphorylation at a Highly Conserved pThr Contact Site**—Modification of the Chk2 FHA domain by autophosphorylation may play a role in promoting the formation of Thr-68-phosphorylated Chk2 monomers after DNA damage (24). However, autophosphorylation site(s) that may promote dissociation of active Chk2 dimers have not been identified. With these observations in mind, we performed phosphosite mapping by MALDI-TOF mass spectrometry on recombinant Chk2 purified from *E. coli*. Similar studies of recombinant Chk2 autophosphorylation sites have revealed a number of modified residues spread throughout the molecule (30, 31), several of which have been confirmed in vivo. Our efforts have thus far identified nine distinct proteolytic peptides that contain at least a single phosphorylation site.4 Two such peptides were identified within the FHA domain, one containing Ser-120 that has been observed previously (30–32) and another spanning residues 138–144. In this case, the site of phosphorylation was confirmed by tandem mass spectrometry to be Ser-140 (Fig. 5A) that occurs within a sequence RTYS140 resembling preferred Chk2 phosphorylation motifs (45).

To facilitate further studies of Ser-140 phosphorylation in vitro, we raised polyclonal antibodies against a pSer-140 peptide. As expected, the affinity-purified antibody clearly detects recombinant Chk2-(63–543) and full-length human Chk2 produced in insect cells in Western blots but fails to detect a kinase-dead version harboring a D347A mutation (Fig. 5B). Fig. 5B also shows that both baculovirus Chk2 and *E. coli* produced

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4 J. Li and S. J. Smerdon, unpublished observations.
Chk2 (63–543) are phosphorylated on Thr-68, a known site of low-level autophosphorylation in recombinant Chk2 preparations, and on the T-loop (anti-pThr-387). Specificity of the anti-pS140 antiserum was confirmed using full-length Chk2 variants containing S120A or S140A mutations. To minimize background autophosphorylation of non-physiological sites, for example during folding of recombinant Chk2 in the bacterial host, we purified these proteins from strains that also express λ phosphatase from a compatible plasmid vector. Under this regime, all three proteins are devoid of any detectable phosphorylation (Fig. 5B). However, following incubation with ATP/Mg2++, phosphorylation at Thr-68 and Thr-387 is restored in all three Chk2 variants. Most significantly, a strong signal is seen for the anti-pSer-140 antibody in only the wild-type and S120A proteins and not the S140A mutant, effectively eliminating pSer-120 as the cognate epitope. Because all three proteins show substantial levels of overall rephosphorylation by mass spectrometry (supplemental Fig. S2) we conclude that the anti-pSer-140 antiserum are specific for this site.

FHA Phosphorylation as a Regulator of ATM-dependent Chk2 Dimerization—Of the two FHA domain autophosphorylation sites identified, the functional significance of Ser-120 phosphorylation is unclear. This residue is not conserved in mammalian Chk2 orthologues and the x-ray structure of the Chk2 FHA domain (29) shows that it is located in a solvent exposed region of the FHA sandwich, remote from the site of phosphopeptide interaction (Fig. 6A, left). Consistent with these observations, we observe no significant effect of a S120A mutation on binding to surface-immobilized pThr-68 peptide (Fig. 6B). In contrast, Ser-140 occupies a highly conserved position at the N-terminal end of β5 immediately juxtaposed to the ligand-binding surface. Here, its side chain makes a direct hydrogen-bonding contact to the pThr phos-

FIGURE 5. FHA domain autophosphorylation. A, tandem mass spectrometry unambiguously identifies Ser-140 as a site of autophosphorylation in recombinant Chk2 (lower). The upper panel shows that Ser-140 is highly conserved in FHA domains from yeast (Rad53 FHA1 and 2) and humans (Chk2, Ki67). In the FHA domains from the related Rad53 and Chk2 kinases, this serine occurs in a motif that contains basic residues in the −2 or −3 positions consistent with the expected kinase domain specificity of these enzymes. B, left, immunoblot of recombinant His-tagged baculovirus expressed Chk2, and E. coli-expressed Chk2-(63–543) and a kinase-dead version, with phosphospecific antibodies against previously characterized sites (pThr-68 and pThr-387) and the novel pSer-140 site. Right, full-length, unphosphorylated Chk2 wild-type (WT), S120A, and S140A mutants were incubated in the presence or absence of ATP and analyzed for rephosphorylation of Thr-68, Ser-140, and Thr-387 by immunoblotting with appropriate antibodies.

* F. J. Ivins and S. J. Smerdon, unpublished data.
phoryl oxygen, and additional stabilizing interactions with main-chain atoms from the β6-β7 turn. Electrostatic and steric effects of phosphorylation of this residue would therefore be incompatible with phosphopeptide binding (Fig. 6A, right) consistent with the significance of this residue for pThr recognition and the loss of interaction observed for the S140A mutant (Figs. 4C and 6B). MALLS analysis also confirms that Ser-140 substitution abrogates tight homodimerization (Fig. 6C) but also suggests that activated pThr-68/pSer-140 monomers are able to interact with Thr-68 unphosphorylated Chk2 to form partially stabilized dimers (Fig. 6C).

**DISCUSSION**

The importance of Chk2 in a variety of DNA damage response and other cell cycle-dependent pathways has engendered considerable interest in the mechanisms of its activation and regulation. ATM phosphorylation of Thr-68 is prominent in most extant models of Chk2 regulation. This is largely founded on several independent observa-
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**FIGURE 7.** Phosphothreonine 68-dependent phosphorylation of Ser-140. A, unphosphorylated (lane 1) full-length Chk2 (20 μM) was incubated with 3 mM ATP alone (lane 2) and in the presence of wild-type or S140A mutant unligated (lanes 3 and 4) or ligated (lanes 5 and 6) Chk2 FHA (all at 20 μM) and analyzed for Thr-68, Ser-140, and Thr-387 rephosphorylation by immunoblotting. Coomassie treatment of the filter showed that transfer and staining efficiency is similarly low for all four FHA domains and the scanned image was therefore enhanced using the contrast filter in Adobe Photoshop CS2 for clarity. Comparison of lanes 3 and 5 clearly shows that efficient Ser-140 phosphorylation requires prior phosphorylation of Thr-68. B, model for Chk2 FHA autophosphorylation. In these two scenarios, the FHA domain is represented as an oval, the kinase domain as a large circle and Thr-68 phosphorylation as smaller circles. In trans phosphorylation of Ser-140 by assembled, active Chk2 dimers (top) should result in anti-pSer-140 staining of the unligated Chk2 FHA-(73–219) which is not the case (panel A, lane 3). Staining is only observed for pT68 FHAlig (panel A, lane 5), consistent with intradimer Ser-140 autophosphorylation (bottom).

Intradimer Ser-140 autophosphorylation (top) should result in anti-pSer-140 staining of the unligated Chk2 FHA-(73–219) which is not the case (panel A, lane 3). Staining is only observed for pT68 FHAlig (panel A, lane 5), consistent with intradimer Ser-140 autophosphorylation (bottom).
derived from library selection studies (35, 47). Nonetheless, our SPR assays show that the pThr-68-dimerized Chk2 FHA is unable to compete with either Plk1 PBD or the Mdc1 FHA for binding to immobilized pThr-68 peptide. Thus, interactions involving either direct binding to pThr-68 or to the phosphobinding surface on the FHA domain following Chk2 activation must be subject to additional regulatory mechanisms.

It has been shown previously that Chk2 autophosphorylation of its FHA domain can disrupt dimerization, and that Chk2 kinase-dead dimers are more stable than wild-type kinase following DNA damage (24). A similar dependence on kinase activity for disassembly of Rad53 oligomers has been reported (28) indicating that FHA phosphorylation may be a conserved mechanism for regulation of Rad53/Chk2 family kinases. We have now been able to show autophosphorylation of Ser-140 that makes direct contact to the phosphothreonine residue in the Chk2 FHA complex structure (29). This residue is highly conserved across the FHA domain family and is present in all known Chk2/Rad53 family kinases supporting it as a strong candidate site for autophosphorylation-dependent control of dimerization. Antiser specific for the pSer-140 motif identify this modification in full-length human Chk2 produced in insect cells, which has been reported to be indistinguishable in terms of its phosphorylation status and oligomerization behavior from activated Chk2 immunoprecipitated from DNA-damaged HCT116 cells (46). Furthermore, repophosphorylation of phosphatase-treated recombinant Chk2 appears to robustly identify, physiologically significant in vivo phosphorylation sites (32), and our observation of Ser-140 repophosphorylation supports the contention that it is a functionally relevant site of Chk2 autoregulation.

In addition to FHA autophosphorylation, phosphodependent dimer stability also appears to be controlled by a p53-regulated Ser/Thr phosphatase, Wip1 through dephosphorylation of Thr-68 in Chk2 (48, 49). So, while autophosphorylation directly disrupts the phosphobinding site and releases pThr-68 monomers, Wip1 activity potentially generates monomeric, active Chk2 with intact FHA phosphobinding activity but with unmodified Thr-68. Cells expressing kinase-dead Chk2 accumulate abnormally high levels of pThr-68 dimeric forms (24). Therefore, some degree of weakening of the dimer by autophosphorylation at Ser-140 (this study) or other accessory sites may be required for full accessibility of phosphatases to pThr-68, as it now appears to be for pThr-68-interacting partners. This also may explain the apparent requirement of Chk2 kinase activity for interaction with Wip1 (50). Because Wip1 contains no recognizable phosphobinding domain, Chk2 dimerization may occlude a phosphoindependent Wip1 interaction surface on the FHA domain, as proposed previously for Chk2 interactions with Brc1, Cdc25, and p53 (29). Such a mechanism may provide a safety-catch whereby rapid and stable dimerization protects pThr-68 against dephosphorylation until Chk2 becomes fully activated. Similarly, the fact that Ser-140 autophosphorylation only occurs within the context of pThr-68-dependent dimers may explain how the kinase domain gains transient access to the protected Ser-140 site by virtue of a local concentration effect. It also potentially ensures that monomerization does not short-circuit the Chk2 activation pathway. Finally, the observation that pThr-68/pSer-140 di-phosphorylated monomers can bind and potentially activate unphosphorylated Chk2 molecules, provides a means of amplifying DNA damage signals, and/or prolonging Chk2 signaling in the absence of ATM activity.

The results reported here, together with previous studies, present a rather complex picture in which Chk2 exists in a variety of oligomerization states arising from the combined effects of autophosphorylation, and the activities of SCD-specific kinases and phosphatases. Our data further suggest that such complexity may be necessary to allow for downstream interactions mediated by the FHA domain and/or phosphorylated SCD motifs that would otherwise be inaccessible in stable dimeric forms. Many questions remain concerning the potential role of scaffolding molecules in Chk2 activation, and the overall structural organization of the pre-activated dimer along with monomeric and dimeric activated forms. However, the application of EPL methods described here has provided new biochemical and biophysical insights into Chk2 self-association, and establishes this approach as a powerful means to further dissect the molecular basis of activation of Chk2 and its interactions with substrates, effectors, and regulators.

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