Phosphorylation of Threonine 68 Promotes Oligomerization of the Chk2 Protein Kinase via the Forkhead-associated Domain*

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Phosphorylation of Thr-68 by the ataxia telangiectasia-mutated is necessary for efficient activation of Chk2 when cells are exposed to ionizing radiation. By an unknown mechanism, this initial event promotes additional autophosphorylation events including modifications of Thr-383 and Thr-387, two amino acid residues located within the activation loop segment within the Chk2 catalytic domain. Chk2 and related kinases possess one or more Forkhead-associated (FHA) domains that are phosphopeptide-binding modules believed to be crucial for their checkpoint control activities. We show that the Chk2 FHA domain is dispensable for Thr-68 phosphorylation but necessary for efficient autophosphorylation in response to ionizing radiation. Phosphorylation of Thr-68 promotes oligomerization of Chk2 by serving as a specific ligand for the FHA domain of another Chk2 molecule. In addition, Chk2 phosphorylates its own FHA domain, and this modification reduces its affinity for Thr-68-phosphorylated Chk2. Thus, activation of Chk2 in irradiated cells may occur through oligomerization of Chk2 via binding of the Thr-68-phosphorylated region of one Chk2 to the FHA domain of another. Oligomerization of Chk2 may therefore increase the efficiency of trans-autophosphorylation resulting in the release of active Chk2 monomers that proceed to enforce checkpoint control in irradiated cells.

The maintenance of genomic integrity following DNA damage requires the coordinated actions of DNA repair and cell cycle checkpoint control. The Chk2/Cds1 protein kinase is activated by DNA damage and phosphorylates several known modulators of cell cycle control including the tumor suppressor proteins, p53 and BRCA1, and Cdc25A phosphatase (1–9). Mutations in the CHK2 gene have been identified in human hereditary and sporadic cancers suggesting that post-translational modifications within the FHA domain of Chk2 and thereby facilitate RAD53 trans-autophosphorylation and activation (21). Therefore, based on the structural and functional similarities between Chk2 and RAD53, the FHA domain may play an important role in the regulation of Chk2 activity in irradiated cells.

Received for publication, January 25, 2002, and in revised form, March 4, 2002
Published, JBC Papers in Press, March 18, 2002, DOI 10.1074/jbc.M200822200

* This work was supported by National Institutes of Health Grant CA88861 and by the American Lebanese Syrian Associated Charities of the St. Jude Children’s Research Hospital. 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.
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1 The abbreviations used are: ATM, ataxia telangiectasia-mutated; IR, ionizing radiation; FHA, forkhead-associated; LFS, Li-Fraumeni syndrome; GST, glutathione S-transferase; wt, wild type; kd, kinase-dead; Gy, gray.
Thr-68 Phosphorylation Promotes Chk2 Oligomerization

![Diagram showing mutations within the FHA domain of Chk2](Image)

**Fig. 1.** Mutations within the proposed phosphopeptide-binding region of the Chk2 FHA domain prevent efficient activation of Chk2. A, sequence alignment of the FHA domains found in *S. pombe* Cds1 (SpCds1), *S. cerevisiae* RAD53 (ScRad53) (FA1), and *Homo sapiens* Chk2 (HsChk2). NCBI Protein Database accession numbers for protein sequences used in the alignment are as follows: gi:12644396, gi:134835, and gi:6005850, respectively. Residues believed to participate in Chk2 (HsChk2). Arrows indicate the positions of sites mutated within the Chk2 FHA domain. B, HCT-15 cells expressing wild type (wt) or mutant Chk2 proteins were exposed to 0 or 6 Gy IR and incubated for 30 min. Whole cell extracts were prepared and separated by SDS-PAGE. Immunoblots were probed with either anti-FLAG monoclonal antibody (top panel) or anti-phosphothreonine 68 (P-T68) rabbit polyclonal antibodies (bottom panel).

**MATERIALS AND METHODS**

**Antibodies**—Rabbit polyclonal antibodies (Zymed Laboratories Inc.) specific to phospho-Thr-68 of Chk2 were raised against a peptide containing ETVST(-PO4)QELY5 and purified using antigen peptide conjugated to Sepharose (Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital). Antibodies were further purified by passage through Thr-68 peptide (ETVSTQELYS)-Sepharose to deplete contaminating antibodies that are reactive with Chk2 not phosphorylated at Thr-68. Commercial antibodies used in this study included monoclonal anti-FLAG M5 (Sigma), monoclonal anti-GST and c-Myc (Roche Molecular Biochemicals), and polyclonal goat anti-Chk2 N-17 (Santa Cruz Biotechnology).

**Cell Culture**—293T/17 and HCT-15 (which contains a heterozygous R145W mutation within the FHA domain of Chk2 (10)) cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s media containing 10% fetal calf serum. For retrovirus production, 293T/17 cells were co-transfected with pBabe/puro FLAG Chk2 and the amphotropic pEQPAM3 packaging construct using calcium phosphate. After 48 h, media were collected, and HCT-15 cells were exposed three times to retrovirus in the presence of 4 μg/ml Polybrene. After 2 days, retrovirus-infected cells were selected in 10 μg/ml puromycin and used for the further experimentation. Where indicated, cells were exposed to ionizing radiation from a 137Cs source delivered at a dose rate of ~1.2 Gy/min.

**Plasmid Constructs**—The pET-15b containing full-length wild type (wt) and catalytic inactive (D368N) Chk2 (hsCds1) cDNAs were kindly provided by H. Piwnica-Worms (28). pGEX-Chk2(1–220) has been described previously (18) and consists of the first 80 amino acids of Chk2 comprising the SQ/TQ-rich domain fused to GST. cDNA fragments containing the FHA domain (amino acids 60–225) or catalytic inactive kinase domain (amino acids 222–543, D368N) of Chk2 were amplified by PCR and cloned into the pGEX-4T-1 (Amersham Biosciences) and/or pET-28a (Novagen). Site-directed mutagenesis of pSG5-FLAG Chk2 (18) was performed by a two-step overlap PCR approach using oligonucleotide primers containing the desired mutation and primers corresponding to the 5’ and 3’ end of Chk2. FLAG-tagged wild type and mutant Chk2 cDNAs were excised from pSG5 and subcloned into the pBabe/puro retrovirus vector. All oligonucleotide synthesis and plasmid sequence analyses were performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital.

**Recombinant Protein Production**—Escherichia coli BL21/DDE cells were induced to express recombinant His6 or GST-tagged proteins with 0.4 mM isopropyl-1-thio-β-galactopyranoside overnight at 16°C. Cells expressing GST-tagged proteins were lysed in STE (150 mM NaCl, 50 mM TRIS, 10 mM β-mercaptoethanol, 0.5% NP-40) and separated by SDS-PAGE. Immunoblots were probed with either anti-FLAG monoclonal antibody (top panel) or anti-phosphothreonine 68 (P-T68) rabbit polyclonal antibodies (bottom panel).

It has been reported previously that the Chk2 FHA domain is required for DNA damage-dependent Thr-68 phosphorylation and activation of Chk2 (19). Thus far, only the LFS-associated mutations (I157T or R145W) within the FHA domain have been characterized as to their ability to be phosphorylated on Thr-68 and activated by IR. Others have shown (27) that the I157T mutant is efficiently phosphorylated on Thr-68 and activated by IR suggesting that this mutation does not affect upstream signaling to Chk2. Unlike the I157T mutant Chk2, the R145W mutant is poorly Thr-68-phosphorylated and activated by IR consistent with this mutation abrogating Chk2 function (19, 27). However, this mutant is also very unstable and may exist in a large, inactive complex that renders it inaccessible to upstream signaling cascades initiated by DNA damage (26, 27). Therefore, it is difficult to interpret data obtained with the R145W mutant because it is unclear whether this mutation solely affects FHA domain function or induces a more drastic conformational change that alters the entire structure of Chk2.

We created a series of defined mutations within the FHA domain of Chk2 to further address whether it is necessary for the activation of Chk2 by ATM. Individual point mutations of theoretical phosphopeptide-binding residues within the Chk2 FHA domain did not alter the stability of the protein nor the ability of ATM to phosphorylate Thr-68. Instead, these mutations negatively affected autophosphorylation. We also demonstrate that the FHA domain of Chk2 binds specifically to the SQ/TQ-rich region of Chk2 in a Thr(P)-68-dependent manner. This association in turn may promote autophosphorylation at multiple sites including the FHA domain resulting in dissociation of this complex. Together, these data suggest that Thr-68 phosphorylation by ATM promotes association of two or more Chk2 molecules by enhancing the affinity of the SQ/TQ-rich domain of one Chk2 molecule for the Chk2 FHA domain of another. Oligomer formation may then facilitate trans-autophosphorylation and activation of Chk2 in irradiated cells.
mm Tris-HCl, pH 8.1, 1 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride and 2% Triton X-100. GST-tagged proteins were then bound to glutathione-Sepharose beads (Sigma). Cells expressing His$_5$-tagged FHA or kinase domain were lysed in 50 mM Na$_2$PO$_4$, pH 8.0, 10 mM imidazole, 300 mM NaCl, 0.3% Sarkosyl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml pepstatin, and 5 μg/ml leupeptin). Nonidet P-40 (0.6%) was added, and His-tagged proteins were bound to nickel-nitrilotriacetic acid-agarose (Qiagen), washed, and eluted with 50 mM Na$_2$PO$_4$, pH 8.0, 300 mM NaCl, and 250 mM Imidazole. Eluted proteins were then dialyzed overnight in 50 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM dithiothreitol. His$_5$-tagged full-length Chk2 was purified as above except that Sarkosyl was omitted from the lysis buffer.

**GST-FHA Binding Assay**—Whole cell extracts were prepared from 293T/17 cells or HCT-15 cells expressing wild type or T68A mutant FLAG-tagged Chk2 in NTN buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na$_2$VO$_4$, and protease inhibitors). One mg of whole cell extract was incubated with 5 μg of GST-FHA pre-bound to glutathione-agarose for 2 h at 4°C. Bound fractions were washed three times with NTN buffer and then analyzed by SDS-PAGE.

**Peptide Binding Assay**—ETVST(F)-PO$_4$QELYLS (Thr(P)-68) and ETVSTQELYLS (Thr-68) peptides were synthesized and conjugated to Sepharose at a concentration of 15 μmol of peptide/d (Hartwell Center forBioinformatics and Biotechnology at St. Jude Children’s Research Hospital). E. coli expressing GST, GST-FHA, or GST-FHA containing the N166A mutation (GST-N166A) was lysed in STE buffer containing 2% Triton X-100, protease inhibitors, and 1 mM dithiothreitol. Four mg of each lysate was incubated with 25 μl of Thr-68 or Thr(P)-68-Sepharose at 4°C for 2 h. Mammalian whole cell extracts were prepared in NTN buffer as above. One mg of each extract was incubated with peptide beads for 2 h at 4°C. The bound fraction of each extract was washed three times with STE (E. coli) or NTN (mammalian) buffer and then analyzed by SDS-PAGE.

**In Vitro Kinase Assay**—Reactions were performed in kinase buffer (10 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 0.5 mM EGTA, 1 mM dithiothreitol, and 10 μCi of [γ-32P]ATP) for 30 min at 30°C and contained 1 μg of purified His-tagged wild type (wt) or catalytic inactive (kd) Chk2 plus 2 μg of purified protein substrates. Kinase reactions were then subjected to 10% SDS-PAGE and transferred to nitrocellulose. Radiolabeled proteins were detected by PhosphorImager analysis (Molecular Dynamics). For binding assays using Chk2-phosphorylated GST-FHA, 10 μg of GST-FHA bound to glutathione-agarose was incubated with wt or kd Chk2 in the same buffer without [γ-32P]ATP for 30 min at 30°C. Bound GST-FHA was washed three times with NTN buffer and once with NTN buffer containing 500 mM LiCl. One-half of each sample was directly used for the GST-FHA binding assay, whereas the other half was eluted with 10 mM glutathione and used for the peptide binding assay. Both assays were performed as described above.

**Immunoprecipitation Assays**—293T/17 cells were transiently co-transfected with GST, FLAG- and Myc-tagged Chk2 constructs (18) using LipofectAMINE reagent (Invitrogen). Thirty six hours after transfection, cells were exposed to 0 or 6 Gy IR, harvested 30 min later, and then lysed in NTN buffer. Extracts were clarified by centrifugation at 14,000 rpm for 20 min at 4°C, and soluble fractions were collected and diluted with an equal volume of 20 mM Tris-Ci, pH 8.0, and 1 mM EDTA. One mg of diluted whole cell extract was incubated with anti-FLAG (M2)-agarose (Sigma) or 10 μg of anti-Myc antibody (9E10, Genetics) plus 25 μl of protein A/G-agarose (Oncogene) for 2 h at 4°C. Immunoprecipitated proteins were washed with 0.5× NTN buffer and then analyzed by SDS-PAGE.

**RESULTS AND DISCUSSION**

The FHA Domain of Chk2 Is Necessary for Efficient Autophosphorylation in Response to IR but Not Necessary for Thr-68 Phosphorylation—A series of individual mutations within the FHA domain were generated to determine the role of this domain in IR-induced autophosphorylation of Chk2. The crystal structure of the first FHA domain of RAD53 bound to a phosphothreonine-containing peptide reveals that this complex consists of a multiple strand β-sandwich containing short α-helical loops that extend out and make contact with a phosphothreonine-containing peptide (24). These structural data were used to produce a modified sequence alignment of several FHA domains including Chk2 (24). Based on this information, we created mutations at conserved residues that are located within the β-sandwich (G116E, H143A, and R145W) or proposed α-helical loops that are expected to make contact with a phosphopeptide ligand (R117A, S140A, K141A, N166A, and I157T) (Fig. 1A). Mutations of residues believed to form the β-sandwich structure of the Chk2 FHA domain (G116E and H143A) appeared to destabilize the protein (data not shown). These results are similar to what has already been documented for the LFS-associated R145W (10, 26, 27) and suggest that mutations located within the β-sandwich are likely to destabi-
lize Chk2 protein. Mutations within the α-helical loops did not affect protein stability, and these Chk2 mutants were therefore used for further investigation.

To determine whether mutations expected to disrupt phosphopeptide binding by the Chk2 FHA domain alter Chk2 activation by IR, we infected HCT-15 cells with retrovirus encoding various FLAG-tagged mutant cDNAs of Chk2. This cell line was chosen because endogenous Chk2 levels and activity are undetectable, and HCT-15 cells do not display normal Chk2 function (9, 27). FLAG-tagged wild type Chk2 protein exhibited the characteristic phosphorylation-dependent mobility shift upon SDS-PAGE that correlates with kinase activation following DNA damage (1, 2) (Fig. 1B). Consistent with previous reports (16–18, 27), the T68A mutant was defective in this response, and the LFS-associated I157T mutant was hyper-phosphorylated just as efficiently as wild type Chk2. Mutation of the highly conserved Arg-117, Ser-140, or Asn-166 residue to alanine was the most disruptive to DNA damage-induced phosphorylation of Chk2, whereas mutation of the less conserved Lys-141 residue to alanine had the least impact. All mutants were phosphorylated on Thr-68 suggesting that the integrity of the FHA domain is not necessary for ATM-dependent phosphorylation of Chk2. These observations contrast those observed for the unstable R145W mutant (19, 27). Interestingly, the catalytic inactive (kd) Chk2 protein containing the D368N mutation did not exhibit a mobility shift even though it showed strong Thr-68 phosphorylation after DNA damage. Therefore, changes in mobility of Chk2 protein during SDS-PAGE are caused by additional phosphorylation events that depend upon intrinsic kinase activity and an intact FHA domain but is independent of Thr-68 phosphorylation.

In budding yeast, the FHA2 domain of RAD53 interacts with RAD9 in both a DNA damage- and phosphorylation-dependent manner, and this interaction facilitates trans-autophosphorylation and activation of RAD53 (21, 25). Like RAD9, mammalian BRCA1 and 53BP1 contain carboxyl-terminal tandem BRCT (BRCA1 carboxyl terminus) motifs, and it has been speculated that either protein could be a functional homologue of RAD9 (29). Of interest, both BRCA1 and 53BP1 can associate with and/or co-localize with Chk2 and therefore may facilitate Chk2 activation by serving as a scaffold for Chk2 trans-autophosphorylation (8, 30). Cells expressing mutant BRCA1 (HCC1937 (31)) efficiently activated Chk2 in response to IR (data not shown) establishing that BRCA1 is not required for IR-induced activation of Chk2. Furthermore, our attempts to determine whether Chk2 and 53BP1 directly interact through co-immunoprecipitation studies were unsuccessful (53BP1 reagents were kindly provided by J. Chen). Therefore, other BRCA1- and 53BP1-independent mechanisms may exist that facilitate Chk2 autophosphorylation in irradiated cells.

Chk2 Specifically Interacts with Its Own FHA Domain following Modification by DNA Damage—We considered the possibility that autophosphorylation is facilitated through direct oligomerization of Chk2 which is dependent upon both Thr-68 phosphorylation and a functional FHA domain. Binding assays were performed using recombinant GST protein fused to the Chk2 FHA domain (GST-FHA) as bait and whole cell extracts for a source of Chk2 protein to determine whether Chk2 directly interacts with its own FHA domain. Chk2 protein present in whole cell extract prepared from irradiated cells specifically bound to GST-FHA immobilized on glutathione-agarose (Fig. 2A). The interaction between Chk2 and GST-FHA was decreased if extract made from irradiated cells was pretreated with λ protein phosphatase consistent with the findings that protein interactions with FHA domains are phosphorylation-dependent (23) (Fig. 2A). Therefore, binding of Chk2 from irradiated cells to GST-FHA required DNA damage-induced phosphorylation in addition to a functional FHA domain because mutating a highly conserved residue within the FHA domain of Chk2 (GST-N166A) abolished specific binding of Chk2 obtained from irradiated cells (Fig. 2B).

A Peptide Representing the Phosphothreonine 68 Region of Chk2 Is a Ligand for the Chk2 FHA Domain—Thr-68 phosphorylation by ATM is believed to be the first event in the pathway leading to full activation of Chk2 in irradiated cells. To determine whether Thr-68 phosphorylation was required for Chk2 binding to GST-FHA, we prepared whole cell extracts from HCT15 cells expressing FLAG-tagged wild type or T68A Chk2 protein and subjected them to the in vitro GST-FHA binding assay. T68A mutant Chk2 protein present in irradiated cells failed to interact with GST-FHA suggesting that phosphorylation of Thr-68 is necessary for this DNA damage-dependent interaction (Fig. 2C). To confirm that the FHA domain of Chk2 can specifically bind to Thr(P)-68 Chk2, additional binding assays were performed using extracts made from E. coli ex-
pressing GST, GST-FHA, and GST-N166A and a 10-amino acid peptide representing the Thr-68 region of Chk2 conjugated to Sepharose. Only peptide containing Thr(P)-68 bound GST-FHA, and as expected, the N166A FHA domain mutant did not bind to Thr(P)-68 peptide-Sepharose (Fig. 3A). Therefore, a peptide consisting of the Thr(P)-68 region of Chk2 is a phosphospecific ligand for the Chk2 FHA domain, and the N166A mutation disrupts Chk2 FHA domain function without negatively affecting the stability of the protein.

Mutations within the FHA domain that alter efficient activation of Chk2 in irradiated cells (R117A, S140A, K141A, and N166A) significantly reduced the ability of Chk2 to bind to Thr(P)-68 peptide in vitro (Fig. 3B). Consistent with its ability to be efficiently autophosphorylated and activated following IR, the I157T mutant bound to Thr(P)-68 peptide as well as wild type (Fig. 3B). Therefore, the efficiency by which Chk2 is autophosphorylated in response to IR grossly correlates with whether Chk2 can bind to P-Thr-68 peptide via the FHA domain. To determine whether the interaction between Thr(P)-68 and Chk2 depends upon a modification induced by IR, 293T/17 cells were irradiated or mock-irradiated, and whole cell extracts were incubated with either Thr-68 or Thr(P)-68 peptide-Sepharose. Chk2 from control rather than irradiated cell extracts bound to Thr(P)-68 peptide-Sepharose suggesting that the affinity of the FHA domain is reduced after cells are exposed to IR (Fig. 3C). The interaction between Thr(P)-68 peptide and full-length Chk2 depends upon an intact FHA domain but is diminished when Chk2 is modified in DNA-damaged cells.

Chk2 Phosphorylates Its Own FHA Domain in Vitro Resulting in Reduced Affinity for Thr(P)-68 Peptide—DNA damage-dependent phosphorylation of the Chk2 FHA domain may be one mechanism by which Chk2 binding to Thr(P)-68 peptide is reduced. To explore the possibility that Chk2 autophosphorylates within the FHA domain or other potential sites, we subjected each fragment to in vitro kinase assays using purified wt or kd Chk2 kinase. Consistent with Chk2 autophosphorylating the activation segment, recombinant wt Chk2 phosphorylated a catalytic inactive kinase domain fragment (Fig. 4A). The FHA domain was highly phosphorylated by wt Chk2, whereas the SQ/TQ-rich region of Chk2 does not appear to be a substrate for Chk2 phosphorylation in vitro (Fig. 4A). Phosphorylation of GST-FHA by wt Chk2 also reduced its ability to bind to Thr(P)-

![Fig. 4. Chk2 autophosphorylates its own FHA domain thereby reducing its interaction with Thr(P)-68. A, GST or His-tagged recombinant proteins consisting of the SQ/TQ-rich region (F1), the FHA domain (F2), or a catalytic inactive (kd) kinase domain (F3) of Chk2 were expressed in E. coli and purified. Recombinant full-length wild type (wt) and kd Chk2 proteins prepared from E. coli were subjected to an in vitro kinase assay using the various domains (F1–3) of Chk2 as substrates. Samples were separated by SDS-PAGE and transferred to nitrocellulose, and the amounts of phosphorylation of Chk2 itself (top left panel) and substrates (bottom left panel) were visualized by PhosphorImaging (32P incorporation). Total amounts of recombinant kd and wt Chk2 proteins (top right panel) or substrate Chk2 domain proteins (bottom right panel) contained in each assay were determined by fast green staining of nitrocellulose membrane. a.a., amino acids. B, purified GST-FHA protein was preincubated with either purified wt or kd Chk2 in the presence of Mg2+ATP. Chk2-phosphorylated GST-FHA proteins were then mixed with Thr(P)-68 peptide-Sepharose, and bound fractions were probed with anti-GST antibody after SDS-PAGE and transferred to nitrocellulose. C, whole cell extracts from 293T/17 cells were prepared after 6 Gy of ionizing radiation. GST-FHA protein was bound to glutathione-agarose, preincubated with Mg2+ATP and either wt or kd recombinant Chk2, and then mixed with cell extract. Bound fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Chk2.

![Fig. 5. Stable oligomer formation by catalytic inactive Chk2 requires both Thr-68 phosphorylation and an intact FHA domain. 293T/17 cells were transiently co-transfected with constructs encoding FLAG and Myc-tagged catalytic inactive (kd), T68A/kd, or N166A/kd Chk2. Cells were exposed to 0 or 10 Gy IR and harvested 30 min later. Whole cell extracts (WCE) were divided in half and then subjected to immunoprecipitation (IP) with either anti-FLAG or anti-Myc as indicated (bottom four panels). Anti-FLAG, anti-Myc, and anti-Thr(P)-68 immunoblots of whole cell extracts used for the immunoprecipitation assay are shown in the top three panels.](http://www.jbc.org/)

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Fig. 6. A model of Chk2 activation in irradiated cells. In response to IR, ATM targets Thr-68 within the SQ/TQ-rich domain of Chk2. Thr-68-phosphorylated Chk2 proteins form an oligomer through interactions between the phosphothreonine 68 region of one Chk2 molecule and the FHA domain of another. Chk2 proteins then trans-autophosphorylate at multiple sites including the FHA domain and activation segment within the kinase domain. Phosphorylation of an unknown site(s) within the FHA domain causes oligomer dissociation resulting in the release of fully active, hyperphosphorylated Chk2 monomers.

68-Sepharose and to interact with endogenous Chk2 present in extract obtained from DNA-damaged cells (Fig 4, B and C). Therefore, autophosphorylation within the FHA domain of Chk2 may induce rapid dissociation of Chk2 oligomers. It is unclear whether the FHA domain of Chk2 is required for interactions with the proteins it targets for phosphorylation. If this proves to be the case, autophosphorylation within the FHA domain may selectively affect Thr(P)-68 Chk2 binding and not all phosphoprotein interactions.

Catalytic Inactive Chk2 Forms an Oligomer in Vivo—FLAG-tagged and Myc-tagged catalytic inactive (kd) Chk2 proteins were co-expressed in 293T/17 cells to determine whether two or more Chk2 proteins associate with one another in vivo and whether Thr-68 phosphorylation and FHA domain integrity are required for this interaction. By using this approach, we have found that kd Chk2 forms a stable oligomer in 293T/17 cells based on the observation that both the FLAG and Myc antibodies can co-immunoprecipitate epitope-tagged Chk2 (Fig. 5). Despite DNA damage-induced Thr-68 phosphorylation of the N166A/kd double mutant, we were unable to detect oligomer formation by this mutant in irradiated cells suggesting that the integrity of the FHA domain is important for oligomer formation. Oligomer formation by the T68A/kd mutant is also deficient as compared with that exhibited by the kd Chk2 proteins (Fig. 5). Therefore, stable oligomer formation by catalytic inactive Chk2 requires both Thr-68 phosphorylation and an intact FHA domain.

Repeated attempts to co-immunoprecipitate two differentially tagged wild type Chk2 molecules using this approach have been unsuccessful (data not shown). The observation that only kd Chk2 formed oligomers suggests that autophosphorylation by Chk2 reduces the affinity of one Chk2 molecule for another. We have frequently found that transient overexpression of wild type Chk2 at high levels in 293T/17 cells is associated with Chk2 autophosphorylation and activation in the absence of DNA damage (data not shown). This phenomenon is analogous to what occurs when RAD53 is expressed at high concentrations in E. coli where it becomes hyperphosphorylated in the absence of RAD9 (25). Therefore, the inability to co-immunoprecipitate differentially tagged, wild type Chk2 proteins may be due to the fact that Chk2 is already activated and can no longer form stable oligomers once autophosphorylation occurs. This is consistent with our findings that recombinant GST-FHA phosphorylated by Chk2 in vitro exhibits reduced affinity for the Thr(P)-68 region of Chk2 and modified Chk2 expressed in irradiated cells (Fig. 4).

In addition to wild type Chk2 activated in a DNA damage-independent manner upon overexpression in 293T/17 cells, we have also observed high levels of constitutive Thr-68 phosphorylation of kd Chk2 under similar conditions (Fig. 5). High levels of Thr-68 phosphorylation may reflect an inability of a specific phosphatase to gain access to Thr-68-phosphorylated kd Chk2 because it is presumably shielded by the FHA domain. The fact that we have only been able to co-immunoprecipitate kd Chk2 suggests that once phosphorylated on Thr-68, oligomerization of kd Chk2 becomes very stable. This is most likely due to low autophosphorylation activity expressed by kd Chk2 that would in turn result in a lower dissociation rate of the oligomer complex.

Thus far, this is the first biologically relevant phosphorylation-dependent ligand described for the FHA domain of Chk2. Together, these data support the model that phosphorylation of Thr-68 by ATM promotes binding of the SQ/TQ region of one Chk2 molecule to the FHA domain of another forming oligomers. Oligomerization may effectively increase the local concentration of Chk2 such that trans-autophosphorylation becomes more favorable (Fig. 6). Trans-autophosphorylation within the FHA domain may then cause dissociation of the Chk2 complex. Active Chk2 monomers are released and proceed to phosphorylate effector molecules important for mammalian checkpoint control.

Acknowledgments—We thank Jing Wu for excellent technical assistance with retrovirus production, Elio Vanin for the pEQPAM3 retrovirus packaging construct, and Risa Kitagawa for critical reading of this manuscript.

Note Added in Proof—Similar work is described in Xu, X., Tsvetko, L. M., and Stern, D. F. (2002) Mol. Cell. Biol., in press.

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J. Biol. Chem. 2002, 277:19389-19395.
doi: 10.1074/jbc.M200822200 originally published online March 18, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200822200

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