Intrinsic Kinase Activity and SQ/TQ Domain of Chk2 Kinase as Well as N-terminal Domain of Wip1 Phosphatase Are Required for Regulation of Chk2 by Wip1*§

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Akinori Yoda†1, Xiao Zhou Xu‡1, Nobuyuki Onishi§2, Kyoko Toyoshima‡, Hiroko Fujimoto‡, Naoko Kato‡, Isao Oishi*, Takeshi Kondo‡, and Yasuhiro Minami‡3

From the †Department of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017 and the §Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

The anti-oncogenic Chk2 kinase plays a crucial role in DNA damage-induced cell cycle checkpoint regulation. Recently, we have shown that Chk2 associates with the oncogenic Wip1 (PPM1D) phosphatase and that Wip1 acts as a negative regulator of Chk2 during DNA damage response by dephosphorylating phosphorylated Thr-68 in activated Chk2 (Fujimoto, H., Onishi, N., Kato, N., Takekawa, M., Xu, X. Z., Kosugi, A., Kondo, T., Imamura, M., Oishi, I., Yoda, A., and Minami, Y. (2006) Cell Death Differ. 13, 1170–1180). Here, we performed structure-function analyses of Chk2 and Wip1 by using a series of deletion or amino acid-substituted mutant proteins of Chk2 and Wip1. We show that nuclear localization of both Chk2 and Wip1 is required for their association in cultured cells and that the serine-glutamine (SQ)/threonine-glutamine (TQ) domain of Chk2, containing Thr-68, and the N-terminal domain of Wip1, comprising about 100 amino acids, are necessary and sufficient for the association of both molecules. However, it was found that an intrinsic kinase activity of Chk2, but not phosphatase activity of Wip1, is required for the association of full-length Chk2 and Wip1. Interestingly, we also show that the mutant Wip1 proteins, bearing the N-terminal domain of Wip1 alone or lacking an intrinsic phosphatase activity, exhibit dominant negative effects on the functions of the wild-type Wip1. i.e. ectopic expression of either of these Wip1 mutants inhibits dephosphorylation of Thr-68 in Chk2 by Wip1 and anti-apoptotic function of Wip1. These results provide a molecular basis for developing novel anti-cancer drugs, targeting oncogenic Wip1 phosphatase.

The Chk2 tumor suppressor is an evolutionarily conserved nuclear protein serine/threonine kinase that plays a crucial role during DNA damage response and helps guard the integrity of the genome by regulating cell cycle checkpoints, DNA repair, and apoptosis (1–10). Upon DNA damage, Chk2 is phosphorylated and activated by ataxia telangiectasia-mutated (ATM)4 kinase (11–15). Activated Chk2 then phosphorylates its downstream effectors, including the tumor suppressors p53, BRCA1, PML, E2F-1, and Cdc25 phosphatases (16–21), thereby regulating cellular responses following DNA damage. Chk2 kinase consists of several evolutionarily conserved functional domains. They include the N-terminal serine-glutamine (SQ)/threonine-glutamine (TQ) domain, which contains multiple SQ/TQ phosphorylation sites preferred for ATM, FHA (fork head-associated) domain, which binds to phosphopeptides, protein kinase domain, and the C-terminal nuclear localization signal (NLS) (2, 22). The regulation of Chk2 kinase activation following γ-irradiation has been well studied. In response to γ-irradiation, ATM phosphorylates Chk2 at Thr-68 (13, 14), and the phosphorylated Thr-68 in Chk2 associates with the FHA domain of another Chk2 molecule, leading to the formation of Chk2 dimers (23–25). Chk2 dimerization results in autophosphorylation of Chk2 at Thr-383/Thr-387 within the activation loop of the Chk2 kinase domain and at Ser-516 in the C-terminal region of Chk2 (26–28), and these phosphorylation events are known to be required for activation of Chk2. Chk2 dimerization may further regulate the function of Chk2 kinase. In fact, it has been reported that Chk2 can also autophosphorylate serine/threonine residues within its FHA domain in vitro and that these phosphorylation events at the FHA domain result in the dissociation of Chk2 dimers in vitro (24, 25). Mutations within the Chk2 gene have been identified in a variant form of Li-Fraumeni syndrome, a highly penetrant familial cancer phenotype normally associated with p53 mutations, and in sporadic human cancers (3, 29). Aberrant epigenetic regulation of Chk2 gene expression was also found in several cell lines from lymphoid malignancies (30). Recently, it has been shown that accumulation of phosphorylated Chk2 and

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‡ Both authors contributed equally to this work.
§ Research fellow of the Japan Society for the Promotion of Science.
□ To whom correspondence should be addressed: Dept. of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5560; Fax: 81-78-382-5579; E-mail: minami@kobe-u.ac.jp.

4 The abbreviations used are: ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; ER, estrogen receptor; FHA, fork head-associated; GST, glutathione S-transferase; HA, hemagglutinin; MBP, maltose-binding protein; NLS, nuclear localization signal; PP2C, protein phosphatase 2C; WCL, whole-cell lysate; WT, wild-type; aa, amino acid; Gy, gray; 4OHT, 4-hydroxytamoxifen; FCS, fetal calf serum; PBS, phosphate-buffered saline; DK, dead kinase.
enhanced apoptosis are found in early precursor lesions of tumors, indicating that Chk2 functions as an anti-cancer barrier at early stages of tumorigenesis (31, 32).

In mammals, the PP2C family of protein phosphatases consists of at least seven distinct isofoms and has been implicated in stress response signaling (33–35). Among the members of the PP2C family protein phosphatases, Wip1 (PPM1D or PP2Cδ) possesses unique biological characteristics. Wip1 is induced by DNA damage in a p53-dependent manner and inhibits ultraviolet (UV) irradiation-induced activation of p38 mitogen-activated protein kinase by dephosphorylating Thr-180 in p38, thereby inhibiting the function of p53 (35, 36). It has also been reported that Wip1 dephosphorylates the nuclear form of uracil DNA glycosylase (UNG2), thereby suppressing base excision repair (37). In the case of UNG2 and p38, Wip1 dephosphorylates the phosphorylated threonine within a TXY motif of these proteins. In vitro kinetic analysis has revealed that Wip1 recognizes a doubly phosphorylated TXY motif (pTXpY) more selectively than a mono-phosphorylated TXY motif (pTXY) (38). More recently, it has been reported that Wip1 dephosphorylates the two ATM/ATR (ATM and Rad-3-related) targets, Chk1 and p53, thereby suppressing intra-S and G2/M checkpoint regulations (39). It has been shown that the Wip1 (PPM1D) gene is amplified or overexpressed in various human cancers, including breast cancers (40–43), and that overexpression of Wip1 (PPM1D) cooperates with the oncogenic protein. It has also been reported that mice deficient for Wip1 show defects in spermatogenesis, lymphoid function, and cell cycle regulation (44). In addition, a recent study using Wip1-deficient mice has revealed that blocking its function results in enhanced apoptosis in Ras and Erbb2-induced breast tumors and impairs tumor formation (45, 46).

In yeast, Pct2 and Pct3, the two members of the PP2C family protein phosphatases, bind to Rad53, a yeast homolog of Chk2, and inactivate the Rad53-dependent pathways (47). We have also shown that Wip1 associates with Chk2 both physically and functionally in the nuclei of human cells (48). Wip1 dephosphorylates Thr-68 in activated Chk2 and inactivates Chk2 kinase. Interestingly, Wip1 appears to dephosphorylate preferably a phosphorylated serine or threonine followed by a glutamine in Chk2 as well as Chk1 and p53 (39, 48). Furthermore, inhibition of Wip1 expression by RNA interference results in abnormally sustained Thr-68 phosphorylation and kinase activation of Chk2 and enhanced apoptosis in response to DNA damage. These results indicate that Wip1 acts as a negative regulator of Chk2 during DNA damage responses. However, it remains largely unknown about the structure-function relationship between Chk2 and Wip1 during DNA damage responses.

As an attempt to understand the structure-function relationship between Chk2 kinase and Wip1 phosphatase, we generated a series of Chk2 and Wip1 mutant proteins. Here we show that Chk2 and Wip1 associate directly in vitro and that the SQ/TQ domain of Chk2 and the N-terminal domain of Wip1, comprising about 100 amino acids (aa), are necessary and sufficient for the association between Chk2 and Wip1 in vivo. It was also found that an intrinsic kinase activity and the NLS sequence of Chk2, but not a phosphatase activity of Wip1, are required for the association of full-length Chk2 and Wip1. Interestingly, expression of the mutant Wip1 protein bearing the N-terminal domain of Wip1 alone or of the phosphatase-deficient mutant Wip1 protein inhibits both suppression of Thr-68 phosphorylation in Chk2 and anti-apoptotic function mediated by the wild-type Wip1, indicating a dominant negative function of these Wip1 mutants in the regulation of Chk2.

We discuss the possible application of our findings to develop novel anti-cancer drugs targeting Wip1.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Mammalian expression plasmids pEBG-Chk2(WT) (encoding glutathione S-transferase (GST)-HA-Chk2), pcDNA-FLAG-Wip1(WT), and pcDNA-FLAG-Wip1(D/A) were constructed as described previously (48). Base-substituted and/or internal deletion mutants were generated by PCR-based site-directed mutagenesis using pEBG-Chk2 and pcDNA-FLAG-Wip1 as templates (48). pEBG-Chk2(ΔFH/A) was constructed by ligating Chk2 cDNAs, corresponding to Chk2 (1–86 aa) and to Chk2 (190–543 aa), respectively, in-frame. Prokaryotic expression vectors were constructed in pGEX (GE Healthcare) or pMAL-C2 (New England Biolabs). A plasmid expressing a tamoxifen-regulated form of Wip1, pECE-HA-Wip1(1–516)-ER, was constructed by ligation of the cDNA encoding for amino acids 1–516 of Wip1 at the 3’ end to a cDNA encoding a mutated ligand-binding domain of estrogen receptor (ER) in pECE-HA vector (49, 50). Plasmid constructs were verified by sequence analysis. Primer sequences and detailed cloning strategies are available upon request.

Antibodies, Cells, and DNA Transfection—The mouse monoclonal antibody M2 (Sigma) recognizes the Flag peptide sequence (DYKDDDDK). The mouse monoclonal antibody 16B12 (Babco) and rabbit polyclonal anti-HA antibody Y-11 (Santa Cruz Biotechnology) recognize the peptide sequence (YPYDVPDYA) derived from the human influenza hemagglutinin (HA) protein. Rabbit polyclonal anti-Chk2 antibody and rabbit polyclonal anti-Wip1 antibody were prepared as described (48). Rabbit polyclonal anti-phospho-Chk2 (Thr-68 and Ser-516) antibodies were purchased from Cell Signaling; mouse monoclonal anti-Chk2 antibody (DCS-270) was from Sigma; mouse monoclonal anti-Wip1 antibody (WC10) was from Trevigen, Inc.; goat polyclonal anti-GST antibody was from GE Healthcare; and Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rabbit IgG, green) were from Invitrogen. HEK293T (293T), A549, and MCF7 cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% (v/v) fetal calf serum (FCS). Transient cDNA transfection was performed using the calcium phosphate method (10) or Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions.

In Vitro Binding Analyses—The GST or maltose-binding protein (MBP) fusion proteins were expressed in Escherichia coli BL21 and purified by the use of glutathione-Sepharose beads (GE Healthcare) or amylose resin beads (New England Biolabs), respectively. Purified GST or GST fusion proteins (1 μg each) were immobilized on glutathione-Sepharose beads.
Then MBP-Chk2(WT) or -Chk2(SQ/TQ) (1 μg each) was applied to the GST fusion protein-immobilized beads equilibrated with PBS containing 0.1% Triton X-100 and 1% bovine serum albumin and incubated for 1 h at 4°C. Pelletized beads were washed three times with PBS containing 0.1% Triton X-100, eluted with Laemmli sample buffer, and subjected to SDS-PAGE followed by immunoblot analyses.

Preparation of Cell Lysates and Co-(immuno)precipitation/Immunoblot Analyses—The cells were solubilized with lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and cell lysates were prepared by centrifugation at 12,000 × g for 15 min at 4°C. The cell lysates were pre-cleared with protein A-Sepharose (GE Healthcare) for 1 h at 4°C. The pre-cleared supernatants were then co-(immuno) precipitated with glutathione-Sepharose beads or with antibodies conjugated to protein A-Sepharose beads for 2 h at 4°C. The (immuno) precipitates were washed five times with lysis buffer and eluted with Laemmli sample buffer. Proteins either from the co-(immuno) precipitation analysis or whole-cell lysates (WCLs) were separated by SDS-PAGE (10% PAGE), and transferred onto polyvinylidene difluoride membrane filters (Immobilon, Millipore). The membranes were immunoblotted with the respective antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against mouse or rabbit IgGs (Bio-Rad) using chemiluminescence reagents (Western Lightning, PerkinElmer Life Sciences).

Immunofluorescence Analysis—293T cells grown on coverslips coated with rat tail collagen were fixed with 4% paraformaldehyde/PBS for 15 min at room temperature and then permeabilized with PBS containing 0.1% (v/v) Triton X-100 for 15 min at room temperature. After blocking with PBS containing 10% (v/v) FCS for 30 min, cells were incubated with primary antibodies, anti-FLAG monoclonal antibody (M2, 1:500), and/or anti-HA polyclonal antibody (Y-11, 1:200), in PBS containing 10% (v/v) FCS for 30 min at room temperature. Cells were washed twice with PBS and then incubated with secondary antibodies, Alexa Fluor 546 (goat anti-mouse IgG, 1:500) and/or Alexa Fluor 488 (goat anti-rabbit IgG, 1:200) in PBS, 10% (v/v) FCS for 30 min at room temperature. After two washes with PBS, cells were mounted with Pristine Mount (Research Genetics) and analyzed with an inverted confocal laser microscope (Zeiss). The nuclei were stained with 4′,6-diamidino-2-phenylindole. Nuclear foci of phosphorylated Chk2 were visualized by using anti-phospho-Chk2(Thr-68) antibody according to the manufacturer’s instructions.

In Vitro Phosphatase Assay—To prepare phosphorylated GST-HA-Chk2(WT), 293T cells were transfected with the expression vector encoding GST-HA-Chk2(WT). 24 h after transfection, cells were exposed to 10 Gy γ-irradiation and cultured for 1 h prior to harvest. Phosphorylated GST-HA-Chk2(WT) was immunoprecipitated with anti-HA antibody, washed five times with lysis buffer, washed once with phosphatase buffer (50 mM Tris-HCl (pH 7.5), 30 mM MgCl2, 1 mg/ml bovine serum albumin, 0.05% 2-mercaptoethanol), and resuspended in phosphatase buffer. The in vitro phosphatase reaction was initiated by addition of purified GST-Wip1(WT) to phosphorylated GST-HA-Chk2(WT) in the presence or absence of MBP-Chk2(SQ/TQ) or MBP and allowed to incubate for 3 h at 30°C. Pelletized beads were washed once with lysis buffer, eluted with Laemmli sample buffer, and subjected to SDS-PAGE followed by immunoblot analyses.

Apoptosis Assay—A549 or MCF7 cells grown on coverslips in 6-well plates were transfected with pEBG-Chk2(WT) (1 μg/well), pEBG-Chk2(SQ/TQ) (1 μg/well), pEBG-Chk2(DK) (1 μg/well), pEBG-Chk2(Δkinase) (1 μg/well), pcDNA-FLAG-Wip1(N) (1 μg/well), pcDNA-FLAG-Wip1(D/A) (1 μg/well), or pcDNA-FLAG-Wip1(WT) (0.1 μg/well), pEGFP (Clontech) (1 μg/well) alone or in combination. The total amount of plasmid DNAs in each transfection was adjusted to 2.1 μg/well with pcDNA3 empty vector. 24 h after transfection cells were exposed to γ-irradiation (10 Gy), 10 h after γ-irradiation, cells were stained as described above. The fractions of apoptotic cells were determined by dividing the number of FLAG- and HA-positive cells that exhibit apoptotic morphology (round cell shape with condensed nucleus) by the number of FLAG- and HA-positive cells. At least 200 FLAG- and HA-positive cells were counted. The assays were performed in triplicate.

RESULTS

Chk2 Associates with Wip1 in Vivo—We have previously reported that Chk2 associates with Wip1 when both molecules are expressed ectopically in 293T cells, and that endogenous Chk2 and Wip1 associate in MCF7 cells that express Wip1 at a higher level (48). To confirm further their association in vivo, we examined the association of endogenous Chk2 and Wip1 in A549 cells that express Wip1 at a relatively a lower level than MCF7 cells using anti-Chk2 and anti-Wip1 antibodies (see “Experimental Procedures”). As shown in Fig. 1A, endogenous Chk2 and Wip1 also associated in A549 cells. To examine whether Chk2 directly binds to Wip1, we performed in vitro binding analyses using maltose-binding protein (MBP)-Chk2(WT) and GST-Wip1(WT) purified from E. coli, respectively. As shown in Fig. 1B, MBP-Chk2(WT) associated specifically with GST-Wip1(WT), but not with GST, indicating that the Chk2 and Wip1 can associate directly.

It has been shown that the Thr-68-phosphorylated Chk2 forms distinct nuclear foci in response to γ-irradiation (51). To examine the functional significance of the association between Chk2 and Wip1 in this γ-irradiation-induced formation of nuclear foci, wild-type (WT) or a phosphatase-deficient (D/A) Wip1 protein was expressed in A549 cells, and nuclear foci of phospho-Chk2 were monitored by immunofluorescence analysis using anti-phospho-Chk2(Thr-68) antibody following γ-irradiation. As shown in Fig. 1C, expression levels of Chk2 protein were unaffected in the presence or absence of transfected Wip1. However, ectopic expression of Wip1(WT), but not Wip1(D/A), resulted in decreased numbers of foci detected by anti-phospho-Chk2(Thr-68) antibody (Fig. 1C), suggesting that Wip1 phosphatase activity can counteract Chk2 phosphorylation. A similar result was obtained when MCF7 cells, transfected with Wip1(WT) or Wip1(D/A), were subjected to a similar immunofluorescence analysis following γ-irradiation (data not shown).
Structure-Function Analysis of Chk2 and Wip1

A

Blot: anti-Chk2
Blot: anti-Wip1

B

| GST pull down | 5% Input |
|--------------|----------|
| MBP-Chk2(WT) | GST      |
| MBP-Chk2(SQ/TQ) | GST-Wip1(WT) |
|              | GST-Wip1(A) |

Blot: anti-Chk2

C

anti-Flag  anti-phospho-Chk2(Thr68)  DAPI  anti-Flag  anti-Chk2  DAPI

Flag-Wip1(WT)
Flag-Wip1(D/A)
Mock
The SQ/TQ Domain, Kinase Activity, and Nuclear Localization of Chk2 Are Required for the Association of Chk2 with Wip1 in Vivo—To determine a region(s) within Chk2 that is required for its association with Wip1 in vivo, we generated a series of truncated, internal deletion or amino acid-substituted mutant Chk2 constructs (Fig. 2A). GST-HA-tagged wild-type or the respective mutant Chk2 proteins were expressed transiently in 293T cells along with FLAG-Wip1(WT), and the transfected cells were solubilized, and protein association was examined by co-precipitation with glutathione-Sepharose, followed by anti-FLAG immunoblotting. As shown in Fig. 2B, GST-HA-Chk2(WT) and GST-HA-Chk2(ΔFHA) could associate with Wip1, indicating that the FHA domain of Chk2 is dispensable for the Chk2-Wip1 association. Consistent with the result, GST-HA-Chk2(WT) and GST-HA-Chk2(ΔFHA) were co-localized with FLAG-Wip1(WT) primarily in the nuclei (Fig. 2C). On the other hand, GST-HA-Chk2(ΔSQ/TQ) failed to associate with Wip1 (Fig. 2B), although GST-HA-Chk2(ΔSQ/TQ) exhibited nuclear localization, similar to Wip1(WT) (Fig. 2C). In addition, GST-HA-Chk2(Δkinase) also failed to associate with Wip1, and GST-HA-Chk2(Δkinase) localized primarily in the cytoplasm (Fig. 2C). This result is consistent with the previous finding that the nuclear localization signal (NLS) is localized in the C-terminal region of Chk2 (22). Collectively, these results indicate that the SQ/TQ and C-terminal region (195–543 aa) of Chk2 are required for the association of Chk2 with Wip1 in vivo.

It has been shown that γ-irradiation or ectopic overexpression of Chk2 by itself in cultured cells results in Thr-68 phosphorylation and modification (activation) of Chk2 (11–15, 25). We have shown previously that Thr-68 phosphorylation of Chk2, induced by ectopic expression of Chk2 or γ-irradiation, is inhibited by co-expression of Wip1(WT), but not Wip1(D/A), in cultured cells (48). Hence, we examined the Thr-68 phosphorylation status of wild-type or the respective mutant Chk2 proteins in 293T cells in the presence or absence of FLAG-Wip1(WT) by immunoblotting with anti-phospho-Chk2(Thr-68). As shown in Fig. 2B (lower panel), ectopic expression of GST-HA-Chk2(WT) or GST-HA-Chk2(ΔFHA) resulted in Thr-68 phosphorylation of Chk2, and their Thr-68 phosphorylation was inhibited by co-expression of FLAG-Wip1(WT) in 293T cells. These results suggest that the FHA domain of Chk2 is dispensable for the association of Chk2 with Wip1 and for the Wip1-mediated inhibition of Thr-68 phosphorylation of Chk2. Although GST-HA-Chk2(Δkinase) possesses the SQ/TQ domain, its Thr-68 phosphorylation was not detected, indicating that the C-terminal region (195–543 aa) of Chk2, containing the kinase domain and the NLS, is required for Thr-68 phosphorylation of Chk2 overexpressed ectopically in cultured cells.

The C-terminal region of Chk2 contains both the kinase domain and the NLS (515–522 aa) (22). Thus, we examined whether or not the association of Chk2 with Wip1 requires its intrinsic kinase activity and/or nuclear localization of Chk2. To this end, NLS mutant (KR/AA: K520A and R521A) or the dead-kinase mutant (DK) of GST-HA-Chk2 proteins was expressed in 293T cells along with FLAG-Wip1, and their association was examined. As shown in Fig. 2D, FLAG-Wip1 was not co-precipitated efficiently with Chk2(KR/AA) or Chk2(DK). We also examined the Thr-68 phosphorylation status of these mutant GST-HA-Chk2 proteins in 293T cells in the presence or absence of FLAG-Wip1(WT). As shown in Fig. 2D (lower panel), ectopic expression of GST-HA-Chk2(KR/AA) or GST-HA-Chk2(DK) resulted in Thr-68 phosphorylation of Chk2. GST-HA-Chk2(KR/AA) and GST-HA-Chk2(Δkinase) lack the NLS and exhibit cytoplasmic localization (Fig. 2C); however, unlike Chk2(KR/AA), Chk2(Δkinase) failed to be phosphorylated on Thr-68, presumably because of the lack of its intrinsic kinase activity. Unlike Chk2(Δkinase), Chk2(DK) was detected exclusively in the nuclei and was phosphorylated on Thr-68 as reported previously (24, 28). Importantly, the levels of Thr-68 phosphorylation of Chk2 in cells expressing Chk2(KR/AA) or Chk2(DK) were not affected by the presence of FLAG-Wip1. Collectively, the results indicate that both the intrinsic kinase activity and nuclear localization of Chk2 are required for the association of Chk2 with Wip1.

The N-terminal Domain of Wip1, but Not Phosphatase Activity of Wip1, Is Required for the Association of Wip1 with Chk2 in Vivo—To determine a region(s) within Wip1 that is required for its association with Chk2 in vivo, a series of truncated or internal deletion mutant constructs of Wip1 was generated (Fig. 3A) and expressed transiently in 293T cells along with wild-type GST-HA-Chk2. The transfected cells were then solubilized, and protein association was examined by immunoprecipitation with anti-FLAG antibody, followed by anti-HA immunoblotting. As shown in Fig. 3B, Wip1(WT), Wip1(AC), and Wip1(AB), containing the domain A (1–98 aa), could associate with Chk2. Consistent with this result, Wip1(AC) and Wip1(AB) could be detected in the nuclei, although Wip1(AB) was rather distributed throughout inside the cells (Fig. 3C). On the other hand, Wip1(BC), lacking the domain A, failed to associate with Chk2(WT) or Chk2(SQ/TQ) (Fig. 3, B and E), although Wip1(BC) exhibited nuclear localization, similar to Wip1(WT) (Fig. 3C). It is worth noting that subcellular distribution of the respective mutant Wip1 proteins was unaffected by the presence or absence of GST-HA-Chk2 (data not shown). These results indicate that the N-terminal domain A of Wip1 is required for its association with Chk2 in vivo.

FIGURE 1. Chk2 kinase associates with Wip1 phosphatase in vivo. A, the association between Chk2 and Wip1 in vivo. WCLs or immunoprecipitates (IPs) with rabbit polyclonal anti-Wip1 or control (preimmune) antibodies prepared from A549 cells were subjected to rabbit polyclonal anti-Chk2 (upper panel) or mouse monoclonal anti-Wip1 (lower panel) immunoblotting. B, the association between Chk2 and Wip1 in vitro. Purified MBP-Chk2(WT) and MBP-Chk2(SQ/TQ) were incubated with either GST, GST-Wip1(WT), or GST-Wip1(A) immobilized on glutathione-Sepharose beads. After the beads were washed extensively, the precipitates were subjected to immunoblotting with anti-Chk2 antibody (DCS-270) or stained with Coomassie Brilliant Blue (CBB). C, formation of nuclear foci of Thr-68-phosphorylated Chk2 can be inhibited by Wip1. A549 cells grown on coverslips were transfected with wild-type (WT) or a phosphatase-deficient mutant (D/A) of FLAG-Wip1 protein. 24 h after transfection, cells were exposed to γ-irradiation (10 Gy), and 1 h after γ-irradiation, cells were fixed and permeabilized, and protein association was examined by co-precipitation with glutathione-Sepharose, followed by anti-FLAG immunoblotting. As shown in Fig. 2D, FLAG-Wip1 was not co-precipitated with Chk2(KR/AA) or Chk2(DK). We also examined the Thr-68 phosphorylation status of these mutant GST-HA-Chk2 proteins in 293T cells in the presence or absence of FLAG-Wip1(WT). As shown in Fig. 2D (lower panel), ectopic expression of GST-HA-Chk2(KR/AA) or GST-HA-Chk2(DK) resulted in Thr-68 phosphorylation of Chk2. GST-HA-Chk2(KR/AA) and GST-HA-Chk2(Δkinase) lack the NLS and exhibit cytoplasmic localization (Fig. 2C); however, unlike Chk2(KR/AA), Chk2(Δkinase) failed to be phosphorylated on Thr-68, presumably because of the lack of its intrinsic kinase activity. Unlike Chk2(Δkinase), Chk2(DK) was detected exclusively in the nuclei and was phosphorylated on Thr-68 as reported previously (24, 28). Importantly, the levels of Thr-68 phosphorylation of Chk2 in cells expressing Chk2(KR/AA) or Chk2(DK) were not affected by the presence of FLAG-Wip1. Collectively, the results indicate that both the intrinsic kinase activity and nuclear localization of Chk2 are required for the association of Chk2 with Wip1.

The C-terminal region of Chk2 contains both the kinase domain and the NLS (515–522 aa) (22). Thus, we examined whether or not the association of Chk2 with Wip1 requires its intrinsic kinase activity and/or nuclear localization of Chk2. To this end, NLS mutant (KR/AA: K520A and R521A) or the dead-kinase mutant (DK) of GST-HA-Chk2 proteins was expressed in 293T cells along with FLAG-Wip1, and their association was examined. As shown in Fig. 2D, FLAG-Wip1 was not co-precipitated efficiently with Chk2(KR/AA) or Chk2(DK). We also examined the Thr-68 phosphorylation status of these mutant GST-HA-Chk2 proteins in 293T cells in the presence or absence of FLAG-Wip1(WT). As shown in Fig. 2D (lower panel), ectopic expression of GST-HA-Chk2(KR/AA) or GST-HA-Chk2(DK) resulted in Thr-68 phosphorylation of Chk2. GST-HA-Chk2(KR/AA) and GST-HA-Chk2(Δkinase) lack the NLS and exhibit cytoplasmic localization (Fig. 2C); however, unlike Chk2(KR/AA), Chk2(Δkinase) failed to be phosphorylated on Thr-68, presumably because of the lack of its intrinsic kinase activity. Unlike Chk2(Δkinase), Chk2(DK) was detected exclusively in the nuclei and was phosphorylated on Thr-68 as reported previously (24, 28). Importantly, the levels of Thr-68 phosphorylation of Chk2 in cells expressing Chk2(KR/AA) or Chk2(DK) were not affected by the presence of FLAG-Wip1. Collectively, the results indicate that both the intrinsic kinase activity and nuclear localization of Chk2 are required for the association of Chk2 with Wip1.
**FIGURE 2.** The SQ/TQ domain, kinase activity, and nuclear localization of Chk2 are required for its association with Wip1 in vivo. A, schematic representation of the wild-type and a series of mutant Chk2 proteins. The numbers point out the positions of the amino acid residues, and the putative functional domains within Chk2 are indicated. The Chk2(DK) mutant lacks an intrinsic kinase activity because of a substitution of the conserved residue within the kinase domain. Lys-520 and Arg-521 within the NLS of Chk2 were replaced with alanines in the Chk2(KR/AA) mutant. B, the SQ/TQ and kinase domains of Chk2 are required for the association of Chk2 with Wip1 in vivo. Wild-type or the respective mutant Chk2 proteins were expressed transiently in 293T cells along with FLAG-Wip1(WT) protein. WCLs were prepared from the respective transfectants and were subjected to affinity precipitation with glutathione-Sepharose. WCLs and co-precipitates (co-ppts) were immunoblotted with the indicated antibodies.

C, subcellular localization of wild-type or the respective mutant Chk2 proteins. 293T cells expressing wild-type or the respective mutant Chk2 proteins along with FLAG-Wip1(WT) were fixed and permeabilized as described under “Experimental Procedures.” After blocking, samples were incubated with anti-FLAG monoclonal antibody (M2, red) and anti-HA polyclonal antibody (Y-11, green), followed by staining with Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rabbit IgG, green), respectively. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). D, an intrinsic kinase activity and the NLS of Chk2 are required for the association of Chk2 with Wip1 and for the Wip1-mediated inhibition of Thr-68 phosphorylation of Chk2. Wild-type (WT), C-terminal-region deleted mutant (Δ kinase), NLS mutant (KR/AA), or dead-kinase mutant (DK) of GST-HA-Chk2 proteins were expressed transiently in 293T cells along with FLAG-Wip1(WT). WCLs were prepared from the respective transfectants and were subjected to affinity precipitation with glutathione-Sepharose beads. WCLs and co-precipitates (co-ppts) were immunoblotted with the indicated antibodies.
FIGURE 2—continued

Structure-Function Analysis of Chk2 and Wip1

C

|        | Chk2 | Wip1(WT) | DAPI | Merge |
|--------|------|----------|------|-------|
| Chk2(WT) | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| Chk2(ΔSQ/TQ) | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| Chk2(ΔFHA) | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| Chk2(Δkinase) | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| Chk2(DK) | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| Chk2(KR/AA) | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) |
| Chk2(SQ/TQ) | ![Image](image25) | ![Image](image26) | ![Image](image27) | ![Image](image28) |

D

| GST-HA-Chk2(WT) | GST-HA-Chk2(Δkinase) | GST-HA-Chk2(KR/AA) | GST-HA-Chk2(DK) | Flag-Wip1(WT) |
|-----------------|----------------------|--------------------|-----------------|---------------|
| +               | +                    | +                  | -               | -             |

WCL Blot: anti-Flag

co-ppt Blot: anti-GST

co-ppt Blot: anti-phospho-Chk2(Thr68)

FIGURE 2—continued
FIGURE 3. The SQ/TQ domain of Chk2 and the N-terminal domain of Wip1 are sufficient for the association of both molecules in vivo. A, schematic representation of the wild-type and a series of mutant Wip1 proteins. The numbers point out the positions of the amino acid residues, and the putative PP2C domain (catalytic domain: 18–372 aa) within Wip1 is indicated. Conserved residues required for the binding to metal ions and phosphate ions are indicated by black and white arrowheads, respectively. The Wip1(D/A) mutant lacks an intrinsic phosphatase activity because of a substitution of the conserved residue within the PP2C domain. B, the N-terminal domain of Wip1 is required for its association with Chk2 in vivo. Wild-type or the respective mutant Wip1 proteins were expressed transiently in 293T cells along with GST-HA-Chk2 protein. WCLs were prepared from the respective transfectants and were subjected to immunoprecipitation with anti-FLAG antibody. WCLs and immunoprecipitates (IPs) were immunoblotted with the indicated antibodies. C, subcellular localization of wild-type or the respective mutant Wip1 proteins. 293T cells expressing the respective mutant Wip1 proteins along with GST-HA-Chk2(WT) or GST-HA-Chk2(SQ/TQ) were fixed and permeabilized as described under “Experimental Procedures.” After blocking, samples were incubated with anti-FLAG monoclonal antibody (M2, red) and anti-HA polyclonal antibody (Y-11, green), followed by staining with Alexa Fluor 546 (goat anti-mouse IgG, red) and Alexa Fluor 488 (goat anti-rabbit IgG, green), respectively. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). D, an intrinsic phosphatase activity of Wip1 is dispensable for the association of Wip1 with Chk2 in vivo. WCLs were prepared from 293T cells expressing GST-HA-Chk2(WT), along with either FLAG-Wip1(WT) or FLAG-Wip1(D/A), and were subjected to affinity precipitation with glutathione-Sepharose beads. WCLs and precipitate (co-precip) were immunoblotted with the indicated antibodies. E, SQ/TQ domain of Chk2 and the N-terminal domain of Wip1 are sufficient for the Chk2-Wip1 association in vivo. WCLs were prepared from 293T cells expressing GST-HA-Chk2(WT) along with either GST-HA-Chk2(WT) or GST-HA-Chk2(SQ/TQ), and pEBG empty vector. F, nuclear localization of Wip1 is required for its function. A549 cells expressing the Wip1–estrogen receptor chimeric protein (Wip1(1–516)-ER) were incubated in the presence (+) or absence (−) of 4OHT (1 μM) for 5 h and exposed to γ-irradiation (10 Gy). One hour after γ-irradiation, cells were fixed and permeabilized as described under “Experimental Procedures.” The subcellular localization of Wip1(1–516)-ER and the nuclear foci of Thr-68-phosphorylated Chk2 were monitored by immunofluorescence using anti-HA and anti-phospho-Chk2(Thr-68) antibodies, respectively. The cells expressing Wip1(1–516)-ER were indicated by arrowheads.
We next examined whether or not the association of Wip1 with Chk2 requires an intrinsic phosphatase activity of Wip1. To this end, GST-HA-Chk2 along with FLAG-Wip1(WT) or FLAG-Wip1(D/A) was expressed in 293T cells, and their association was examined by glutathione-Sepharose precipitation followed by anti-FLAG immunoblotting. As shown in Fig. 3D, both FLAG-Wip1(WT) and FLAG-Wip1(D/A) could be co-precipitated with GST-HA-Chk2(WT), indicating that phosphatase activity of Wip1 is dispensable for its association with Chk2 in vivo.

We also examined the abilities of the respective mutant Wip1 proteins to inhibit Thr-68 phosphorylation of Chk2 (presumably reflecting their phosphatase activities) in vivo. WCLs from 293T cells expressing wild-type or the respective mutant Wip1 along with GST-HA-Chk2(WT) were immunoblotted with anti-phospho-Chk2(Thr-68) antibody. As shown in Fig. 3B, this phospho-specific antibody failed to detect GST-HA-Chk2 co-expressed with Wip1(AB) or Wip1(WT), indicating that Wip1(AB) as well as Wip1(WT) can inhibit Thr-68 phosphorylation of Chk2. The results suggest that the C-terminal
domain (domain C, 370–605 aa) of Wip1 is dispensable for both the association of Wip1 with Chk2 and phosphatase activity of Wip1 in vivo. In addition, Thr-68 phosphorylation of Chk2 was not inhibited in cells co-expressing Wip1(BC) or Wip1(AC) (Fig. 3B), suggesting that both mutant Wip1 proteins lack their catalytic activities. In fact, Wip1(BC) and Wip1(AC) lack some, if not all, of the metal ion-binding residues that are required for phosphatase activity of Wip1 (52, 53). To examine the phosphatase activity of Wip1(BC), we purified GST-Wip1(BC) from E. coli and performed in vitro phosphatase assay. GST-Wip1(BC) exhibited no phosphatase activity toward p-nitrophenyl phosphate (data not shown), confirming that the N-terminal domain of Wip1 is required for its phosphatase activity.

To further define a Chk2-binding site within the N-terminal domain A of Wip1 without affecting its metal ion-binding capability, we generated additional Wip1 deletion mutants (FLAG-Wip1(Δ71–99) and -Wip1(Δ37–99)) (supplemental Fig. S1A). As shown, both Wip1(Δ71–99) and Wip1(Δ37–99) could associate with Chk2 and inhibit Thr-68 phosphorylation of Chk2 (supplemental Fig. S1B), indicating that the N-terminal 36 amino acids of Wip1 (Wip1, aa 1–36) is required for its association with Chk2 and phosphatase activity. Thus, at present we cannot separate a Chk2-binding site and metal ion-binding sites within Wip1. It can be assumed that a Chk2-binding site within Wip1 may also contain metal ion-binding residues.

To address the question of whether nuclear localization of Wip1 is required for its function, we first examined putative NLSs in Wip1. We found that the domain C of Wip1 possesses putative NLSs, i.e. KRRTLEESNSGPLMKKHRR (535–552 aa) and RRRLRGGKKK (581–589 aa), and that the mutant Wip1 proteins, containing these putative NLSs (Wip1-(1–515–605)), exhibited nuclear localization (supplemental Fig. S2A). However, the other mutant Wip1 proteins, bearing amino acid-substituted mutations within these putative NLSs (Wip1(K535A/R536A/K548A/K549A/K588A/K589A), or Wip1-(1–516), lacking these putative NLSs, was also detected in the nuclei (supplemental Fig. S2A), albeit Wip1-(1–516) was also detected weakly in the cytoplasm.

To regulate nuclear and cytoplasmic localization of Wip1, we took advantage of the Wip1/estrogen receptor (ER) fusion protein that translocates from the cytoplasm to the nucleus in response to ligand. The ER portion of this fusion protein was localized exclusively in the nucleus and Wip1-(1–516)-ER resulted in decreased numbers of γ-irradiation-induced phospho-Chk2 foci in a ligand-dependent manner, suggesting that nuclear localization of Wip1 is required for its function.

The SQ/TQ Domain of Chk2 and the N-terminal Domain of Wip1 Are Sufficient for the Chk2-Wip1 Association—We examined whether or not the SQ/TQ domain of Chk2 by itself is sufficient for the association of Chk2 with Wip1 in vivo. For this purpose, FLAG-Wip1(WT) and/or GST-HA-Chk2(SQ/TQ), in combination, was expressed transiently in 293T cells. As shown in Fig. 2C, GST-HA-Chk2(SQ/TQ) was detected throughout inside the cells, presumably due to the absence of the NLS, and due to its molecular size (about 35 kDa) smaller than a cut-off size of proteins (about 50 kDa) for active nuclear transport (54). Furthermore, FLAG-Wip1 protein could be co-precipitated with GST-HA-Chk2(SQ/TQ), but not with GST, indicating specific association between the SQ/TQ domain of Chk2 and Wip1 (supplemental Fig. S3A). It is conceivable that GST-HA-Chk2(SQ/TQ) dimerizes with and is (trans)-phosphorylated by endogenous Chk2 efficiently. Alternatively, Thr-68 of GST-HA-Chk2(SQ/TQ) may be phosphorylated efficiently by the endogenous nuclear kinases, including ATM.

We next examined whether or not the N-terminal domain of Wip1 (1–98 aa), Wip1(A), is sufficient for the association of Wip1 with Chk2 in vivo. To this end, FLAG-Wip1(A) or FLAG-Wip1(WT) was expressed transiently in 293T cells along with GST-HA-Chk2(WT), and protein association was examined by immunoprecipitation with anti-FLAG antibody, followed by anti-HA immunoblotting. As shown in Fig. 3C, FLAG-Wip1(A) was detected throughout inside the cells. In addition, Chk2 was co-immunoprecipitated efficiently with Wip1(A) and Wip1(WT), indicating that the N-terminal domain A of Wip1 is sufficient for the association with Chk2 in vivo (supplemental Fig. S3B). We examined further whether or not the domain A of Wip1 by itself can associate with Chk2(SQ/TQ). Wip1(A) protein, co-localized with Chk2(SQ/TQ) (Fig. 3C), was co-precipitated with both Chk2(WT) and Chk2(SQ/TQ) but not with GST (Fig. 3E), indicating that the N-terminal domain A of Wip1 associates with the SQ/TQ domain of Chk2. The result suggests that the SQ/TQ domain of Chk2 and the N-terminal domain of Wip1 are sufficient for the association between Chk2 and Wip1. In vitro binding analyses revealed that MBP-Chk2(SQ/TQ) associated specifically with GST-Wip1(WT) or -Wip1(A) but not with GST, further indicating the direct binding of Chk2(SQ/TQ) with Wip1(A) (Fig. 1B).

Wip1(A) and Wip1(D/A) Mutants Exhibit a Dominant Negative Effect on Wip1(WT)-mediated Inhibition of Thr-68 Phosphorylation of Chk2 Following γ-Irradiation—We have shown previously that Thr-68 phosphorylation of Chk2(WT), induced by ectopic overexpression by itself or by γ-irradiation, is inhibited by Wip1(WT) (48). Considering our present findings that Wip1(A) and Wip1(D/A), both lacking an intrinsic phospho-
tase activity, can associate with Chk2, it was envisaged that both mutant Wip1 proteins might exhibit a dominant negative effect(s) on the function of Wip1(WT). To test this possibility, we examined the effects of expression of FLAG-Wip1(A) or FLAG-Wip1(D/A) on FLAG-Wip1(WT)-mediated inhibition of Thr-68 phosphorylation of GST-HA-Chk2(WT) in transfected 293T cells. As shown in Fig. 4A, GST-HA-Chk2(WT), expressed in 293T cells, was heavily phosphorylated on Thr-68, and this Thr-68 phosphorylation of Chk2 was inhibited drastically by co-expression of FLAG-Wip1(WT). Interestingly, the antagonistic function of FLAG-Wip1(WT) on Thr-68 phosphorylation of GST-HA-Chk2(WT) was inhibited by further co-expression of FLAG-Wip1(A) or FLAG-Wip1(D/A), but not of FLAG-Wip1(BC), in a dose-dependent manner (Fig. 4A). The result indicates that Wip1(A) and Wip1(D/A) act as dominant negative mutants for Wip1 and that the N-terminal domain of Wip1 (1–98 aa) is sufficient for this dominant negative function.

It has been reported that Thr-68 phosphorylation of endogenous Chk2 increases following γ-irradiation and then decreases rapidly concomitant with the accumulation of endogenous Wip1 protein in p53-proficient cells (A549 and MCF7 cells) (48). Therefore, we also examined whether or not Wip1(A) or Wip1(D/A) can inhibit antagonistic function of endogenous Wip1 on Thr-68 phosphorylation of endogenous Chk2. FLAG-Wip1(A) or FLAG-Wip1(D/A) was expressed transiently in A549 cells, and the levels of Thr-68 phosphorylation of endogenous Chk2 before or after γ-irradiation were monitored by immunoblotting with anti-phospho-Chk2(Thr-68). In cells transfected with the control vector, Thr-68 phosphorylation of Chk2 increased following γ-irradiation and then decreased rapidly thereafter, although increased Thr-68 phosphorylation of Chk2 was abnormally sustained in cells transfected with either Wip1(A) or Wip1(D/A) following γ-irradiation (Fig. 4B). These results support the notion that Wip1(A) or Wip1(D/A) expressed ectopically can exhibit a dominant negative effect(s) on endogenous Wip1, resulting in sustained Thr-68 phosphorylation of Chk2 at relatively higher levels in cells transfected with Wip1(A) or Wip1(D/A).

Because Chk2(SQ/TQ) associated with Wip1, we examined whether or not Chk2(SQ/TQ) can inhibit the function of Wip1(WT) in vitro. To this end, purified GST-Wip1(WT) proteins were subjected to in vitro phosphatase assay using Thr-68-phosphorylated GST-HA-Chk2 as a substrate in the presence of MBP-Chk2(SQ/TQ) or MBP. As shown in Fig. 4C, dephosphorylation of phospho-Chk2 by Wip1(WT) was suppressed in the presence of MBP-Chk2(SQ/TQ), but not MBP, indicating that Chk2(SQ/TQ) inhibits the function of Wip1 at least in vitro.

Wip1(A) and Wip1(D/A) Mutants Inhibit an Anti-apoptotic Function of Wip1(WT)—It was well established that Chk2 functions as a positive regulator in γ-irradiation-induced apoptosis (20, 21, 27, 55–59). Recently, we have found that ectopic expression of Wip1(WT), but not Wip1(D/A), suppresses Chk2-mediated apoptosis in MCF7 cells (48). To confirm an anti-apoptotic function of Wip1 in other cells, GST-HA-Chk2(WT) was expressed transiently in A549 cells along with either FLAG-Wip1(WT), FLAG-Wip1(D/A), or FLAG-Wip1(A), and apoptotic cells were scored after γ-irradiation (see “Experimental Procedures”). Consistent with our previous findings (48), enhanced apoptosis of A549 cells was observed when GST-HA-Chk2(WT) was expressed in the cells, and this Chk2-mediated apoptosis was remarkably inhibited by co-expression of FLAG-Wip1(WT), but not FLAG-Wip1(A) or FLAG-Wip1(D/A) (Fig. 5A). The result suggests that Wip1 regulates negatively Chk2-mediated apoptosis following γ-irradiation and that this inhibition by Wip1 requires its phosphatase activity.

We next examined whether or not Wip1(A) or Wip1(D/A) can exhibit a dominant negative effect(s) on anti-apoptotic function of Wip1(WT) in vivo. As shown in Fig. 5B, GST-HA-Chk2-mediated apoptosis of A549 cells was again inhibited by co-expression of FLAG-Wip1(WT). Interestingly, this antagonistic function of Wip1(WT) on Chk2-mediated apoptosis was inhibited by additional co-expression of Wip1(A) or Wip1(D/A) (Fig. 5B), indicating that Wip1(A) and Wip1(D/A) indeed act as dominant negative mutants for Wip1.

Furthermore, we examined whether or not Wip1(A) or Wip1(D/A) can suppress anti-apoptotic function of endogenous Wip1, induced upon γ-irradiation of p53-proficient cells, A549 and MCF7 cells. To this end, FLAG-Wip1(A) or FLAG-Wip1(D/A) was expressed transiently in A549 or MCF7 cells, and apoptotic cells were scored following γ-irradiation. As shown in Fig. 5C, enhanced apoptosis of A549 cells was observed when the cells were transfected with FLAG-Wip1(A) or FLAG-Wip1(D/A) but not FLAG-Wip1(WT). Essentially, similar results were obtained when MCF7 cells were analyzed under the same experimental conditions (data not shown). These results further support the notion that the mutant Wip1 proteins, bearing the N-terminal domain of Wip1 alone or lacking a phosphatase activity, exhibit a dominant negative effect(s) on anti-apoptotic function of Wip1.

We also examined whether or not Chk2(SQ/TQ) or Chk2(DK) can suppress anti-apoptotic function of Wip1 in vivo. To this end, GST-HA-Chk2(SQ/TQ) or GST-HA-Chk2(DK) was expressed transiently in A549 or MCF7 cells, and apoptotic cells were scored following γ-irradiation. Unexpectedly, inhibition of apoptosis of A549 cells was observed when the cells were transfected with GST-HA-Chk2(SQ/TQ) or GST-HA-Chk2(DK) (Fig. 5D). In contrast, expression of GST-HA-Chk2(ASQ/TQ) in the cells failed to inhibit γ-irradiation-induced apoptosis (Fig. 5D). Essentially, similar results were obtained when MCF7 cells were analyzed under the same experimental conditions (data not shown). To elucidate the molecular mechanism underlying this anti-apoptotic function of Chk2(SQ/TQ), we examined the status of Thr-68 and Ser-516 phosphorylation of Chk2(WT), both of which are required for Chk2-dependent apoptosis following γ-irradiation, in the presence or absence of Chk2(SQ/TQ) or Chk2(DK). Myc-Chk2(WT) exhibited enhanced phosphorylation (Thr-68 and Ser-516) and its electrophoretic mobility shift in a γ-irradiation-dependent manner. Interestingly, this enhanced phosphorylation and electrophoretic mobility shift were inhibited by co-expression of GST-HA-Chk2(SQ/TQ) or GST-HA-Chk2(DK), indicating that both Chk2(SQ/TQ)
Structure-Function Analysis of Chk2 and Wip1

A

GST-HA-Chk2(WT)
Myc-Wip1(WT)
Flag-Wip1(D/A)
Flag-Wip1(A)
Flag-Wip1(BC)

WCL Blot: anti-phospho-Chk2(Thr68)
WCL Blot: anti-HA
WCL Blot: anti-Wip1
WCL Blot: anti-Wip1
WCL Blot: anti-Wip1

B

Relative phosphorylation levels of Chk2(Thr68)

Flag-Wip1(WT)
Flag-Wip1(D/A)
Flag-Wip1(A)

WCL Blot: anti-phospho-Chk2(Thr68)
WCL Blot: anti-Chk2
WCL Blot: anti-Flag
WCL Blot: anti-Flag

C

GST-HA-Chk2(WT)
MBP-Chk2(SQ/TQ)
MBP
GST-Wip1(WT)
GST

IP: anti-HA
Blot: anti-phospho-Chk2(Thr68)
IP: anti-HA
Blot: anti-HA

CBB

100
75
50
37
25

GST

MBP-Chk2(SQ/TQ)
MBP
Wip1(WT)
**DISCUSSION**

Disruption of cellular machineries that regulate cell cycle checkpoints, DNA repair, and apoptosis results in genomic instability, leading to the development of cancer in multicellular organisms. The protein kinases ATM and ATR and their downstream substrates, Chk1 and Chk2, are central players in checkpoint activation in response to DNA damage (3, 4). Although Chk1 and Chk2 are structurally unrelated, it has been revealed that they are inactivated by a p53-inducible nuclear protein phosphatase, Wip1 (39, 48). Chk2 appears to be largely inactive in the absence of DNA damage, and it is activated mainly by ATM in response to DNA damage. We have shown previously that Wip1 inhibits Chk2 kinase activity by dephosphorylating serine/threonine residues, including phosphorylated Thr-68 within the SQ/TQ domain of Chk2, and thereby inhibits Chk2-mediated apoptosis (48). In this study we performed the structure and function analyses of Chk2 and Wip1, and we found that the SQ/TQ domain, kinase activity, and nuclear localization of Chk2 as well as the N-terminal domain of Wip1 are required for the association of Chk2 with Wip1 (Figs. 2 and 3). Furthermore, we show that Wip1(A) and Wip1(D/A) mutants that lack a phosphatase activity, yet can associate with Chk2, exhibit a dominant negative effect(s) on Wip1(WT)-mediated inhibition of Thr-68 phosphorylation of Chk2 and on an anti-apoptotic function of Wip1(WT) (Figs. 4 and 5). On the other hand, Chk1 is active even in ordinary cell cycles, and it is further activated in response to DNA damage or stalled replication. Recently, we and Lu et al. (39, 48) have found that Wip1 also inhibits Ser-317/Ser-345 phosphorylations within the SQ sites of Chk1 and suppresses Chk1 kinase activity, resulting in reduced cellular intra-S and G2/M checkpoint activities in response to DNA damage. In addition, it has been shown that the N-terminal domain of Wip1 (1–101 aa) is

**FIGURE 4.** Wip1(A) and Wip1(D/A) mutants exhibit a dominant negative effect on Wip1(WT)-mediated inhibition of Thr-68 phosphorylation of Chk2. A, dominant negative effect of Wip1(A) or Wip1(D/A), but not Wip1(BC), on Wip1(WT)-mediated inhibition of Thr-68 phosphorylation of Chk2. 293T cells were transfected with GST-HA-Chk2(WT) (2nd to 12th lanes, 0.05 μg/plate), FLAG-Wip1(WT) (1st and 3rd to 12th lanes, 0.02 μg/plate), FLAG-Wip1(D/A) (4th lane, 0.1 μg/plate; 5th lane, 0.2 μg/plate; 6th lane, 0.3 μg/plate), FLAG-Wip1(A) (7th lane, 0.2 μg/plate; 8th lane, 0.4 μg/plate; 9th lane, 0.6 μg/plate), or FLAG-Wip1(BC) (10th lane, 0.6 μg/plate; 11th lane, 1.2 μg/plate; 12th lane, 1.8 μg/plate) in combination. Total amount of plasmid DNA in each transfection experiment was adjusted to 1.87 μg/plate with pcDNA3 empty vector. WCLs were subjected to SDS-PAGE (10% PAGE) and were immunoblotted with the indicated antibodies. B, dominant negative effect of Wip1(A) or Wip1(D/A) on endogenous Wip1-mediated inhibition of Thr-68 phosphorylation of endogenous Chk2 following γ-irradiation. A549 cells were transfected with either FLAG-Wip1(WT), FLAG-Wip1(A), or pcDNA3 empty vector. One day after transfection, cells were exposed to 10 Gy γ-irradiation. At the indicated time points after γ-irradiation, WCLs from the respective transfectants were subjected to immunoblotting with anti-phospho-Chk2(Thr-68) or anti-Chk2 antibodies, and intensities of the respective bands were quantified. Relative levels of Thr-68 phosphorylation of Chk2 were determined by normalization with levels of Chk2 proteins in the respective samples. Data are expressed as the mean ± S.D. (relative to the levels in nonirradiated cells with control vector) in three independent experiments. C, Chk2(SQ/TQ) inhibits the function of Wip1 in vitro. Thr-68-phosphorylated GST-HA-Chk2 was immunoprecipitated and incubated with purified GST-Wip1(WT) (2nd to 4th lanes, 1 μg), GST (1st lane, 1 μg), MBP-Chk2(SQ/TQ) (3rd lane, 1 μg), or MBP (4th lane, 1 μg) in combination, and in vitro phosphatase assay was performed (see “Experimental Procedures”). Samples were subjected to SDS-PAGE, and then immunoblotted with anti-phospho-Chk2(Thr-68) or anti-HA antibodies or stained with Coomassie Brilliant Blue (CBB). It was also found that MBP-Chk2(SQ/TQ) suppresses dephosphorylation of phospho-Chk2 by Wip1(WT) in a dose-dependent manner (data not shown).
required for the association of Wip1 with Chk1 or UNG2 (37, 39), raising a possibility that the N-terminal domain of Wip1 may possess a more generalized role in the association of Wip1 with its substrates. Further study will be required to clarify this issue.

In this study, it was found that GST-HA-Chk2(SQ/TQ), but not GST-HA-Chk2(ΔSQ/TQ), can associate with Wip1, showing that the SQ/TQ domain is necessary and sufficient for the association of Chk2 with Wip1 (Figs. 2B and 3E). In addition, we show that GST-HA-Chk2(ΔFHA) can associate with Wip1 and that the Thr-68 phosphorylation of GST-HA-Chk2(ΔFHA) is inhibited by Wip1, indicating that the FHA domain of Chk2 is dispensable for the Chk2-Wip1 association (Fig. 2B), although the FHA domain of Chk2 has been proposed to mediate Chk2 dimerization via direct association of the FHA domain with the phosphorylated SQ/TQ domain of Chk2 (24, 25). On the other hand, it has been shown that Rad53, the Saccharomyces cerevisiae homolog of Chk2, can associate with the PP2C-like protein phosphatases, Ptc2 and Ptc3, via its FHA domain and that phosphorylation of Ptc2 is required for the association of Rad53 with Ptc2 (47). These results suggest that the molecular mechanisms of the Chk2-Wip1 (or Rad53-Ptc2, 3) association are different between human and yeast.

In this study, we found that Chk2(KR/AA), bearing mutations within the NLS, was detected exclusively in the cytoplasm and failed to associate with Wip1 in vivo (Fig. 2, A, C, and D). Chk2(KR/AA) can be phosphorylated on Thr-68, presumably because of its intrinsic kinase activity, yet Thr-68 phosphorylation of Chk2(KR/AA) cannot be inhibited by Wip1 (Fig. 2D). Chk2(Δkinase), lacking the kinase domain and NLS, was also detected in the cytoplasm and failed to associate with Wip1 (Fig. 2, A, C, and D). Although, like Chk2(SQ/TQ), Chk2(Δkinase) retains the SQ/TQ domain, it failed to associate with Wip1 because of the lack of its nuclear localization (Figs. 2, C and D, 3E, and S3A). Unlike Chk2(KR/AA), Chk2(Δkinase) cannot be phosphorylated on Thr-68, presumably because of the lack of its intrinsic kinase activity (Fig. 2D). In contrast, Chk2(DK), lacking its kinase activity, but retaining the intact NLS, was detected exclusively in the nuclei and failed to associate with Wip1 (Fig. 2, A, C, and D). Interestingly, Chk2(DK) lacks its intrinsic kinase activity similar to Chk2(Δkinase), which is localized in the cytoplasm; Chk2(DK) can be phosphorylated on Thr-68 (Fig. 2D), presumably because of its localization in the nuclei where endogenous Chk2 and/or other kinases (e.g. ATM), which can phosphorylate Chk2(DK), exist. These results suggest that both nuclear localization and the intact NLS of Chk2 are necessary, but not sufficient, for its association with Wip1, and that the intrinsic kinase activity of Chk2 is also necessary for the association of full-length Chk2 with Wip1 (see below for more detail). Further study will be required to determine clearly which is important for Chk2-Wip1 association, nuclear localization of Chk2, the intact NLS of Chk2, or both.

Unlike Chk2, the mechanism for the nuclear localization of Wip1 remains largely unknown. Because Wip1(BC) and Wip1(AC), but not Wip1(AB), exhibit nuclear localization exclusively (Fig. 3C), the C-terminal domain (domain C, 376–605 aa) of Wip1 appears to be required for the nuclear localization of Wip1. Although domain C of Wip1 possesses putative NLSs (KRTLEENSPLMKKHRR (535–552 aa) and RRRL- RGQKK (581–589 aa)), Wip1 protein, bearing amino acid-substituted mutations within these NLSs, is still localized in the nuclei exclusively (supplemental Fig. S2A). Further study will be required to elucidate the mechanism for the nuclear localization of Wip1. Importantly, we show by using the Wip1(1–516)-ER chimeric protein that nuclear localization of Wip1(1–516)-ER in the presence of 4OHT, but not cytoplasmic localization of Wip1(1–516)-ER in the absence of 4OHT, results in the inhibition of phospho-Chk2 foci formation in the nuclei following γ-irradiation (Fig. 3F), indicating that nuclear localization of Wip1 is indeed required for the regulation of Chk2 by Wip1.

Although, like Chk2(WT), Chk2(DK) can be phosphorylated on Thr-68, Thr-68 phosphorylation of Chk2(DK) was unaffected by the presence or absence of Wip1 in vivo (Fig. 2D), presumably because of its inability to associate with Wip1. These results suggest that the kinase activity of Chk2 is required for the association of full-length Chk2 with Wip1. Ahn et al. (24) have shown that Thr-68-phosphorylated Chk2 forms a transient dimer, resulting in trans-phosphorylation of Chk2 at multiple sites, including the FHA domain and activation loop within the kinase domain. These phosphorylation events, in particular, at the FHA domain of Chk2 then induce the dissociation of a Chk2 dimer(s), eventually leading to the release of fully active Chk2 monomers (24). It has been postulated that Chk2(DK) can be phosphorylated by endogenous Chk2 or other kinases (e.g. ATM) and can form a stable dimer(s) in the nuclei (24). Therefore, it is possible that monomeric Chk2 (inactive or active), but not dimeric Chk2, would associate with Wip1. Alternatively, the intrinsic kinase activity of Chk2 may be required for a proper steric configuration of full-length Chk2 to associate with Wip1. Further biochemical and/or structural biological analyses will be required to distinguish these possibilities.

In our previous study, it has been shown that Wip1(WT), but not Wip1(D/A), dephosphorylates serine/threonine residues, including Thr-68, on phosphorylated Chk2 in vitro, resulting in the inhibition of Chk2 kinase activity (48). Here we show that the phosphate-deficient mutants, Wip1(A) and Wip1(D/A), can associate with Chk2 via its SQ/TQ domain and suppress Wip1(WT)-mediated inhibition of Thr-68 phosphorylation of Chk2 (Fig. 4, A and B). The results support an idea that Wip1(A) or Wip1(D/A) can compete with Wip1(WT) for the association with Chk2. Furthermore, antagonistic function of Wip1(WT) on Chk2-mediated apoptosis can be inhibited by co-expression of Wip1(A) or Wip1(D/A) (Fig. 5, A–C). These results indicate that Wip1(A) and Wip1(D/A) indeed act as dominant negative mutants for Wip1 at least in the regulation of Chk2. On the other hand, although Chk2(SQ/TQ) act as dominant negative mutants for Chk2 (Fig. 4C), Chk2-mediated apoptosis can be inhibited by co-expression of Chk2(SQ/TQ) or Chk2(DK) (Fig. 5D), and that Chk2(SQ/TQ) and Chk2(DK) can suppress Thr-68 and Ser-516 phosphorylation of Chk2 following γ-irradiation (supplemental Fig. S4). These results indicate that Chk2(SQ/TQ) and Chk2(DK) act as dominant negative mutants for Chk2(WT).
can be assumed that Chk2(SQ/TQ) and Chk2(DK) may inhibit Thr-68 phosphorylation of Chk2(WT) either by inhibiting ATM-mediated phosphorylation of Chk2(WT) or by inhibiting the dimer formation of Chk2(WT).

Importantly, Wip1 phosphatase has been considered to be an oncogenic protein on the basis of the findings that the gene encoding Wip1 (PPM1D) is amplified in human malignancies, including breast cancers (40–42), and that inactivation of Wip1 inhibits mammary tumorigenesis (45, 46). Thus, it can be assumed that the inhibition of Wip1 phosphatase activity or of Wip1 expression would suppress tumorigenesis mediated by Wip1. Considering our finding that Wip1(A) or Wip1(D/A) acts as dominant negative mutants for Wip1 in the regulation of Chk2, the functions of Wip1 can be suppressed by inhibiting the association of Wip1 with Wip1 substrates, including Chk2, in place of inhibiting phosphatase activity of Wip1. Therefore, it is anticipated that a small compound(s), which associates with the N-terminal domain of Wip1, thereby inhibiting the association of Wip1 with Chk2, can be a powerful and useful drug applicable to human malignancies related to Wip1. Future structural analyses for the N-terminal domain of Wip1 will contribute further to the development of a small compound(s) targeting Wip1.

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