

# Threonine 68 Phosphorylation by Ataxia Telangiectasia Mutated Is Required for Efficient Activation of Chk2 in Response to Ionizing Radiation<sup>1</sup>

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## Abstract

Eukaryotic cells activate an evolutionarily conserved set of proteins that rapidly induce cell cycle arrest to prevent replication or segregation of damaged DNA before repair is completed. In response to ionizing radiation (IR), the cell cycle checkpoint kinase, Chk2 (hCds1), is phosphorylated and activated in an ataxia telangiectasia mutated (ATM)-dependent manner. Here we show that the ATM protein kinase directly phosphorylates T68 within the SQ/TQ-rich domain of Chk2 *in vitro* and that T68 is phosphorylated *in vivo* in response to IR in an ATM-dependent manner. Furthermore, phosphorylation of T68 was required for full activation of Chk2 after IR. Together, these data are consistent with the model that ATM directly phosphorylates Chk2 *in vivo* and that this event contributes to the activation of Chk2 in irradiated cells.

## Introduction

Mutations in the *ATM*<sup>3</sup> gene are responsible for the pleiotropic recessive disorder AT, which is characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, predisposition to cancer, and extreme sensitivity to IR (1). Cell lines derived from AT patients are also radiosensitive and exhibit defects in various cell cycle checkpoint responses to IR including p53-dependent G<sub>1</sub> cell cycle arrest and rapid p53-independent S and G<sub>2</sub> phase arrests (2, 3). *ATM* encodes a M<sub>r</sub> 370,000 protein kinase, the activity of which is enhanced in response to DNA strand breaks, and targets several effectors of checkpoint control including Brca1, p95/nbs1, p53, and Mdm2 and, therefore, may be a key regulator of checkpoint responses when cells are exposed to IR (4–12).

Chk2 (hCds1) is a mammalian homologue of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 checkpoint protein kinases (13–16). In response to IR, Chk2 is phosphorylated and activated in an ATM-dependent manner, and this event has been linked to the regulation of p53 stability and maintenance of G<sub>2</sub> cell cycle arrest (13–19). Here we present evidence that Chk2 is a direct target of the ATM protein. Through site-directed mutagenesis of various Chk2 residues, we identified T68 as the major site of phosphorylation by the ATM kinase *in vitro*. In response to IR, Chk2 is specifically phosphorylated on T68 in an ATM-dependent manner consistent with a recent report from Zhou *et al.* (20). In this report, we provide additional evidence that phosphorylation of T68 is necessary for efficient activation of the Chk2 kinase in irradiated cells, thus

further establishing a direct link between ATM and Chk2 in checkpoint control.

## Materials and Methods

**Cell Lines, DNA Transfections, and Retroviral Transductions.** Human neuroblastoma SY5Y cells (kindly provided by G. Brodeur, The Children's Hospital of Philadelphia, Philadelphia, PA) and 293T cells were transiently transfected with the indicated expression vectors using FuGene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's suggestions. Under these conditions, ~90% transfection efficiency is obtained. A29 (p53<sup>-/-</sup>) and A38 (p53<sup>-/-</sup> ATM<sup>-/-</sup>) mouse embryo fibroblasts (21) were infected with retrovirus generated in 293T cells after transient transfection with either pBabePuro3' mycChk2 wt or T68A mutant constructs plus an ecotropic packaging construct. After 2 days, infected cells were selected in culture with 4 μg/ml Puromycin (Sigma) and used for further experimentation.

**Plasmids.** To generate NH<sub>2</sub>-terminal FLAG-tagged Chk2 expression constructs, wt and kd Chk2 cDNA fragments were excised from GST-Chk2 and GST-Chk2 (D347A; kindly provided by S. Elledge, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX) and ligated into a derivative of the mammalian expression vector, pSG5 (Stratagene) such that an NH<sub>2</sub>-terminal FLAG-epitope would be linked to Chk2. A T68A derivative of FLAG-Chk2 was then generated using the QuickChange Site Directed Mutagenesis kit (Stratagene). COOH-terminal myc-tagged Chk2 wt, kd, and T68A mutant expression constructs were generated by PCR amplifying each respective cDNA using Pfu polymerase (Stratagene) and ligating PCR products into a pSG5 vector derivative such that a COOH-terminal myc epitope would be linked to Chk2. 3' myc Chk2 cDNAs were then transferred to the retroviral pBabePuro vector. A prokaryotic GST-Chk2<sub>1–80</sub> fusion protein expression vector was made by linking the first 80 amino acids of Chk2 to GST of pGEX-3X (Pharmacia). Various mutants of pGEX-Chk2<sub>1–80</sub> (S19A, S26A, T28A, S33A, S35A, S50A, and T68A) were made using the QuickChange Site-Directed Mutagenesis protocol. The T68A derivative of pGEX2TN-hCds1 kd (D368N) was generated as discussed above using site-directed mutagenesis.

**In Vitro Kinase Assays and Immunoblot Analysis.** FLAG-tagged wt and kd ATM were immunoprecipitated from transiently transfected 293T cells and subjected to an *in vitro* kinase assay as described (10, 22) using 1 μg of each respective GST-Chk2 (hCds1) substrate purified from bacteria (22). For Chk2 kinase assays, SY5Y cells transiently expressing FLAG-tagged Chk2 were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, 0.5% Tween 20, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM DTT. FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 monoclonal antibody-agarose affinity gel (Sigma). Kinase reactions contained immunoprecipitated FLAG-tagged Chk2, 1 μg of GST-Cdc25C motif, 20 mM HEPES (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 10 μM ATP, and 10 μCi [<sup>32</sup>P]ATP for 15 min at 30°C. Kinase reactions were then subjected to SDS-PAGE and transferred to nitrocellulose membrane. Radiolabeled proteins were visualized by PhosphorImager analysis and quantitated using ImageQuant software (Molecular Dynamics). Two dimensional phosphoamino acid analysis of hydrolyzed, radiolabeled GST-Chk2<sub>1–80</sub> was carried out using the Hunter System (C.B.S. Scientific) as described (23). Antibodies used for immunoblot analysis were anti-FLAG M5 (Sigma) and anti-GST (Roche Molecular Biochemicals). Phosphothreonine-containing proteins were detected with rabbit anti-phosphothreonine antibodies (New England Biolabs).

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<sup>3</sup> The abbreviations used are: ATM, ataxia telangiectasia mutated; IR, ionizing radiation; wt, wild type; kd, kinase dead; MEF, mouse embryo fibroblast; GST, glutathione S-transferase; Chk, checkpoint kinase.

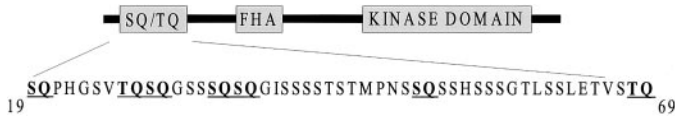


Fig. 1. Domain structure of human Chk2 showing the placement of the SQ/TQ-rich domain with respect to the fork head-associated (FHA) and kinase domain. Potential ATM phosphorylation sites within the SQ/TQ region are *underlined* and in *bold*.

The c-myc antibody (9E10) used for both immunoprecipitation and Western blotting was obtained from Roche Molecular Biochemicals.

## Results and Discussion

Structurally, Chk2 protein is composed of three domains: one rich in SQ/TQ motifs, one fork head-associated domain, and a kinase domain (Fig. 1; Refs. 13–16). ATM has been shown to preferentially phosphorylate SQ/TQ motifs, and the SQ/TQ-rich domain was, therefore, used as a substrate for ATM *in vitro* (22). Immunoprecipitated FLAG-tagged, wt ATM phosphorylated GST-Chk2<sub>1–80</sub> protein, whereas catalytic-inactive (kd) FLAG-tagged ATM did not confirming that the activity observed here is intrinsic to ATM (Fig. 2A). To map the site of ATM phosphorylation, each of the seven potential serines or threonines adjacent to a glutamine within GST-Chk2<sub>1–80</sub> were individually substituted with alanine. Analysis of these mutated GST-Chk2<sub>1–80</sub> substrates revealed that T68 is the major target of ATM *in vitro* (Fig. 2A). Further phosphoamino acid analysis demonstrated that GST-Chk2<sub>1–80</sub> was predominantly phosphorylated on threonine residues, although some serine phosphorylation was also seen, suggesting that ATM may target an additional SQ motif on GST-Chk2<sub>1–80</sub> (Fig. 2B). Full-length Chk2 (hCds1) protein fused to GST was also predominantly phosphorylated on T68 by ATM *in vitro* (Fig. 2C).

To determine whether T68 is phosphorylated *in vivo* in an ATM-dependent manner, A29 (p53<sup>-/-</sup> ATM<sup>+/+</sup>) and A38 (p53<sup>-/-</sup> ATM<sup>-/-</sup>) mouse embryo fibroblasts were infected with retrovirus encoding wt or T68A mutated myc-tagged Chk2. Cells were exposed to 0–6 Gy IR, and the exogenous Chk2 was immunoprecipitated from cells 1 h later. Immunoblot analysis of myc-Chk2 immunoprecipitates revealed a significant increase in phosphothreonine content of wt but not T68A mutated Chk2 in A29 cells, demonstrating that T68 is phosphorylated *in vivo* in response to IR (Fig. 3A). Although less myc-tagged Chk2 protein was expressed in A38 cells, IR-induced phosphorylation of T68 was not detectable. Together, these results

suggest that ATM may directly phosphorylate T68 of Chk2 in response to IR.

We then investigated whether T68 phosphorylation is necessary for the activation of Chk2 in response to IR. We transiently expressed FLAG-tagged wt, kd, and T68A mutant Chk2 in SY5Y human neuroblastoma cells. Cells were irradiated or mock-irradiated, and immunoprecipitated FLAG-tagged Chk2 activity was assessed using GST-Cdc25C<sub>200–256</sub> as a substrate (13–15). Consistent with reports analyzing endogenous Chk2, ectopically expressed wt FLAG-tagged Chk2 immunoprecipitated from irradiated cells showed a mobility shift after SDS-PAGE resolution and increased phosphorylation of GST-Cdc25C<sub>200–256</sub> *in vitro* (~3-fold; Fig. 3, B and C). This mobility shift and increase in activity were largely dependent upon T68 because the T68A mutant Chk2 showed reduced mobility shift and kinase activation in response to IR (Fig. 3, B and C). Immunoblot analysis of immunoprecipitated Chk2 with anti-phosphothreonine antibody confirmed that both wt and kd Chk2 are phosphorylated on T68 in response to IR in SY5Y cells (data not shown). The fact that the T68 mutant is partially defective for IR-induced mobility shift and activation suggests that phosphorylation of T68 alone is not sufficient for complete activation of Chk2. A minor serine residue within the SQ/TQ-rich domain of Chk2 may be targeted by ATM as well and contribute to the activation of Chk2 (Fig. 2B). Interestingly, catalytic inactive (kd) Chk2 failed to show a mobility shift in response to IR. Therefore, Chk2 mobility shifts on SDS-PAGE gels may represent intrinsic Chk2 kinase activity rather than phosphorylation of T68 alone. This result suggests that additional autophosphorylation events are required for complete activation of Chk2 after IR. How T68 phosphorylation influences Chk2 autophosphorylation activity is currently unknown.

In conclusion, these results suggest that ATM may directly phosphorylate Chk2 at T68 *in vivo*, and that this event appears to be necessary for complete activation of Chk2 in irradiated cells. Chk2 has been implicated as an ATM effector in several DNA damage response pathways. Both ATM and Chk2 are required for efficient stabilization of p53 in response to IR (19, 24). Chk2 and p53 have recently been genetically linked with the discovery of *Chk2* germ-line mutations among several Li-Fraumeni syndrome patients that failed to show the typical mutations in the *p53* gene (25). Chk2 may regulate the stability of p53 by phosphorylating serine 20 of p53, which in turn interferes with Mdm2 binding and associated p53 protein degradation (17, 19, 26, 27). Chk2 also associates with and phosphorylates the

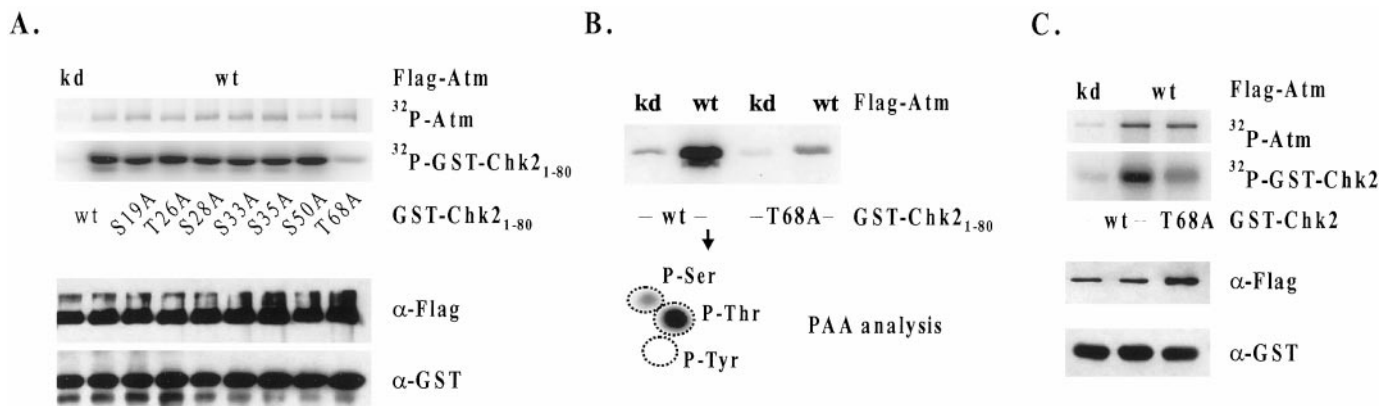


Fig. 2. The ATM kinase directly phosphorylates the SQ/TQ rich domain on T68 *in vitro*. A, immunoprecipitated wt or catalytic-inactive (kd) ATM were subjected to *in vitro* kinase assays with GST-Chk2<sub>1–80</sub> wt and SQ/TQ mutant proteins. Equal amounts of ATM and GST-fusion proteins contained in each reaction were confirmed by Western analysis with anti-FLAG or anti-GST antibodies. B, GST-Chk2<sub>1–80</sub> wt protein phosphorylated by wt ATM was subjected to two-dimensional phosphoamino acid analysis. The positions of unlabeled serine, threonine and tyrosine standards are circled. C, full-length GST-hCds1 and T68A mutant proteins were tested as substrates for ATM. Because Chk2/hCds1 exhibits a relatively high level of autophosphorylation, catalytic-inactive D368N GST-hCds1 or D368N/T68A GST-hCds1 proteins were used.

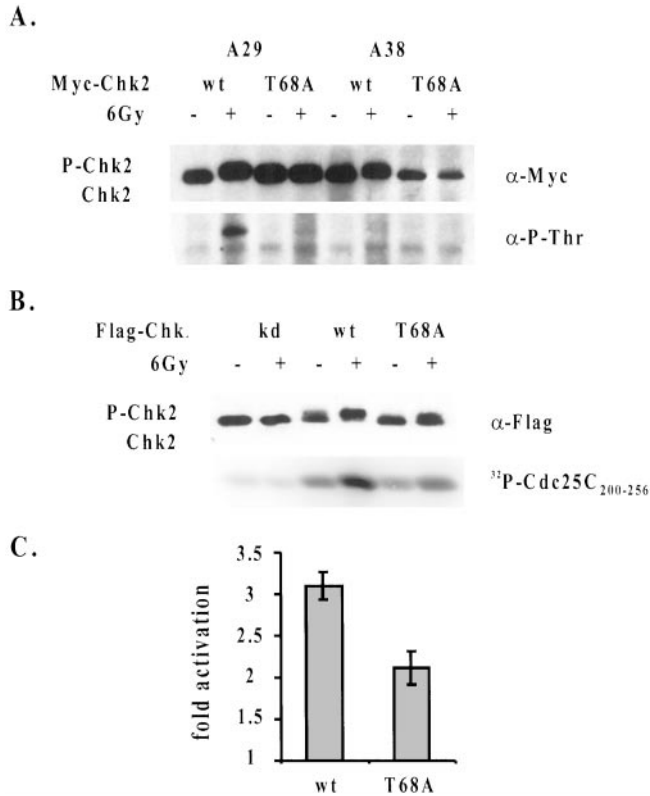


Fig. 3. IR-induced T68 phosphorylation of Chk2 by ATM is required for optimal activation. *A*, A29 (p53<sup>-/-</sup> ATM<sup>+/+</sup>) and A38 (p53<sup>-/-</sup> ATM<sup>-/-</sup>) mouse embryo fibroblasts were infected with retrovirus encoding either myc-tagged Chk2 wt or T68A. Cells were exposed to 0–6 Gy IR and harvested 1 h later. Chk2 proteins were immunoprecipitated with anti-myc antibody and Western blotted with anti-phosphothreonine antibody (*bottom panel*) or anti-myc (*top panel*). *B*, FLAG-tagged wt, kd, or T68A mutant Chk2 proteins were transiently expressed in SY5Y cells. Cells were exposed to 0–6 Gy IR. Chk2 was then immunoprecipitated 1 h later with anti-FLAG and subjected to *in vitro* kinase assays using GST-Cdc25C<sub>200–256</sub> as a substrate (*bottom panel*). Total levels of Chk2 contained in FLAG immunoprecipitates were determined with anti-FLAG (*top panel*). *C*, the ratio of <sup>32</sup>P incorporation into GST-Cdc25C<sub>200–256</sub> by FLAG-Chk2 immunoprecipitated from irradiated *versus* unirradiated cells is shown graphically. Background <sup>32</sup>P incorporation into GST-Cdc25C was subtracted prior to ratio calculations. Data are expressed as the mean fold activation from three independent experiments; bars, SE.

Brc1 breast cancer gene product and may participate in the regulation of the G<sub>2</sub>-M checkpoint pathway by targeting the cdc25C phosphatase (13–16, 19, 20, 28). Future studies will be directed at understanding the involvement of Chk2 in mediating the various functions of ATM.

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### Note Added in Proof

Similar results showing that ATM-dependent T68 phosphorylation of Chk2 is required for activation in response to IR have been reported recently by Matsuoka *et al.* (Proc. Natl. Acad. Sci. USA, 97: 10389–10394, 2000) and Melchionna *et al.* (Nat. Cell Biol., 2: 762–765, 2000).

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