Characterization of tumor-associated Chk2 mutations

Xianglin Wu¹, Shelley R. Webster¹, Junjie Chen¹²

¹Guggenheim 1342
Division of Oncology Research
Mayo Clinic
200 First Street, S.W.
Rochester, Minnesota 55905

²Correspondence author: Junjie Chen
E-MAIL Chen.junjie@mayo.edu; FAX (507)-284-3906; Phone (507)-538-1545

Running title: Multiple mechanisms for Chk2 inactivation

The abbreviations used are: Chk2: checkpoint kinase 2; ATM, ataxia telangiectasia mutated protein; LFS: Li-Fraumeni syndrome; FHA: forkhead homology-associated.
Summary:

The integrity of DNA damage response pathway is essential for the prevention of neoplastic transformation. Several proteins involved in this pathway, including p53, BRCA1 and ATM, are frequently mutated in human cancer. Chk2 is a DNA damage activated protein kinase that lies downstream of ATM in this pathway. Recently, heterozygous germ-line mutations in \textit{Chk2} have been identified in a subset of patients with Li-Fraumeni syndrome, a highly penetrant familial cancer phenotype, suggesting that \textit{Chk2} is a tumor suppressor gene. Here, we reported the biochemical characterization of all four tumor-associated Chk2 mutants. Two of the reported Chk2 mutations identified in Li-Fraumeni syndrome result in loss of Chk2 kinase activity. While one mutation within Chk2 forkhead homology-associated (FHA) domain, R145W, retains some basal kinase activity, this mutant cannot be phosphorylated at Thr-68, an ATM-dependent phosphorylation site, and cannot be activated following gamma radiation. Wild type Chk2 exists mainly in a protein complex of Mr ~200,000 Da, whereas the R145W mutant forms a larger, presumably inactive, complex in the cell. The other FHA domain mutant, I157T, behaves as wild type Chk2 in all the assays used here. Since the FHA domain is involved in protein-protein interactions, this mutation may affect associations of Chk2 with other proteins. Additionally, we have shown that Chk2 can also be inactivated by down-regulation of its expression in cancer cells. Thus, Chk2 may be inactivated by multiple mechanisms in the cell.
Introduction:

The maintenance of genomic integrity following DNA damage depends on the coordination of DNA repair and the controls of cell cycle progression. Chk2/hcds1, a mammalian homologue of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 genes, plays a critical role in DNA damage signaling pathways (1-5). Downstream of ATM in response to gamma radiation (1,4,6), Chk2 directly phosphorylates and regulates the functions of p53 and BRCA1 (7-11). Moreover, heterozygous germ-line mutations in *Chk2* have been identified in a subset of patients with Li-Fraumeni syndrome, a highly penetrant familial cancer phenotype (12). These studies strongly suggest that, like *p53*, *Chk2* is a tumor suppressor gene.

Several mutations of Chk2 were identified in patients with Li-Fraumeni syndrome and in sporadic colon cancer. Although it has been speculated that these Chk2 mutants are defective in their tumor suppressor functions (12), this possibility has not been addressed directly. Here, we report the biochemical characterization of all four reported Chk2 mutations.
Materials and methods

Constructs:

Dr. Jann Sarkaria kindly provided plasmid for the expression of HA-tagged Chk2 in mammalian cells (13). Site-directed mutagenesis (Promega) was performed to introduce mutations in Chk2 coding sequence. For expressing wild type or mutant Chk2 as glutathione-S-transferase (GST)-fusion proteins in insect cells, wild type or mutant Chk2 coding sequence was cloned into pDONR201 vector (Gibco BRL). Gateway cloning technology (Gibco BRL) was used to sub-clone these coding sequences into pDEST20 vector, a vector for baculovirus expression of GST-fusion proteins. Recombinant baculoviruses encoding GST-fused wild type and mutants of Chk2 were generated using Bac-to-Bac baculovirus system (Gibco BRL).

Cell Lines and Culture Conditions

All cell lines were obtained from American Tissue Culture Collection (ATCC) and cultivated in RPMI 1640 (Biofluids) supplemented with 10% fetal bovine serum. To establish cell lines stably expressing HA-tagged wild type or mutant Chk2, HCT116 cells were transfected with plasmids encoding the indicated HA-tagged sequences. G418 resistant clones were isolated and analyzed by Western blotting using either anti-HA antibody or anti-Chk2 antibody. Clones that express HA-tagged Chk2 at levels similar to that of endogenous Chk2 were used in this study. Where indicated, cells were exposed to gamma radiation from a $^{137}$Cs source at a dose of 6.4 Gy/min. Following irradiation, cells were returned to the incubator and harvested one hour later.

Sf9 insect cells were cultivated in Grace’s insect media supplemented with 10% fetal bovine serum. For protein expression, Sf9 cells were infected with baculoviruses encoding GST-
fused wild type or mutant Chk2. Cells were collected and lysed 48 hours after viral infection. Wild type or mutant Chk2 was purified using glutathione affinity chromatography.

**Immunoprecipitation, immunoblotting and kinase assays**

Preparation of cell lysates, immunoprecipitation and immunoblotting were performed as described previously (14). Anti-HA antibody HA11 was purchased from Babco. Antibodies against Chk2 were raised against Glutathione-S-transferase (GST)-fusion proteins containing full length Chk2 (mAB#7) or raised against a GST-fusion protein containing C-terminus of Chk2 (residues 193-543; anti-Chk2B). Anti-Chk2 Thr-68 phospho-specific antibodies were provided by Dr. Bin-Bing Zhou. Chk2 kinase assays were performed as described previously (13).

**Size fractionation of native Chk2 complexes**

HCT116 and derivative cells were harvested and lysed in NETN buffer [150 mM NaCl, 20 mM TrisHCl (pH8.0), 1 mM EDTA and 0.05% NP40]. Whole cell extracts were loaded onto a Superdex 200 HR 10/30 (Pharmacia) column equilibrated with NETN and run in the same buffer with a flow-rate of 0.5 ml/minute. For each run, a sample of 500 µl was injected and 500 µl fractions were collected. For column equilibration, low- and high- molecular-weight gel filtration calibration kits (Pharmacia) were used and the column was run under identical conditions.
Results:

Frameshift mutations at the C-terminus of Chk2 lead to the loss of Chk2 kinase activity.

Since Chk2 is a DNA damage-activated protein kinase that participates in the phosphorylation of several substrates, including Cdc25C, p53 and BRCA1, we first examined the kinase activity of Chk2 mutants. Using site-directed mutagenesis, we generated four Chk2 mutants (Figure 1) that have been previously reported (12). Wild type and mutant GST-Chk2 proteins were expressed in insect cells, purified using glutathione-Sepharose beads, and kinase activities were assessed using GST-Cdc25C (residues 200-256) as a substrate. As shown in Figure 2, one FHA domain mutant (I157T) exhibits wild type activity. In contrast, the other FHA domain mutant (R145W) has reduced catalytic activity, and the two frame-shift mutants lack kinase activity.

R145W mutant of Chk2 is not phosphorylated or activated following gamma radiation.

Chk2 is activated following DNA damage (1). Although Chk2 FHA domain mutants (R145W and I157T) retain some kinase activity (Figure 2), they may not be activated by DNA damage. To explore this possibility, we established HCT116 derivative cell lines that stably express comparable levels of HA epitope-tagged wild type or mutant Chk2 (Figure 3a). The expression levels of HA-tagged Chk2 in these cells are similar to that of endogenous Chk2 (Figure 3a). Like wild type Chk2, the I157T Chk2 mutant was activated following gamma radiation, as demonstrated by its ability to autophosphorylate
and to phosphorylate Cdc25C (Figure 3b). However, R145W Chk2 mutant was not activated following DNA damage (Figure 3b).

The increase of Chk2 kinase activity coincidences with its phosphorylation following gamma radiation (1). Both activation and phosphorylation of Chk2 depend on intact ataxia telangiectasia mutated (ATM) kinase, strongly suggesting that ATM may phosphorylate Chk2 and activate its kinase activity following DNA damage (1). In vivo, Thr-68 of Chk2 is phosphorylated in an ATM-dependent manner following gamma radiation (6). Thus, we examined whether the Chk2 mutants were phosphorylated at Thr-68 following gamma radiation. Cells expressing either wild type or mutant HA-tagged Chk2 were irradiated. Wild type and mutant Chk2 were immunoprecipitated using either anti-Chk2 or anti-HA antibody. Phosphorylation of Chk2 at Thr-68 was detected by Western blotting using anti-Thr-68 phospho-specific antibody. In agreement with its activation following gamma radiation, ectopically expressed HA-Chk2 was phosphorylated at Thr-68 (Figure 3c). Also consistent with the above findings that HA-Chk2 harboring I157T mutation, but not R145W mutation, can be activated by DNA damage (Figure 3b), the I157T mutant was phosphorylated at Thr-68 whereas the R145W mutant was not (Figure 3c). In addition, expression of the R145W mutant did not affect the phosphorylation of endogenous Chk2 in these cells (Figure 3c), suggesting that the mutant protein may not exhibit dominant negative activity.

**FHA domain mutants of Chk2 localize normally in nuclei.**

Chk2 normally localizes to nuclei. We examined the sub-cellular localization of wild type and mutant HA-tagged Chk2 stably expressed in HCT116 derivative cell lines.
Immunostaining using anti-HA antibodies revealed that wild type, R145W and I157T mutants of Chk2 all localized normally to nuclei (Figure 4). Furthermore, the localization of wild type or mutant Chk2 does not change following gamma radiation (data not shown).

**Chk2 exists as a protein complex of apparent Mr ~200 kDa in the cell.**

Since Chk2 R145W and I157T mutants are missense mutations within the forkhead homology-associated (FHA) domain, a domain involved in protein-protein interaction (15,16), we speculated that these Chk2 mutations might affect the association of Chk2 with other proteins. To investigate this possibility, we first used size exclusion chromatography to determine the native size of Chk2 in HeLa and HCT116 cells. As shown in Figure 5, endogenous Chk2 eluted from a Superdex 200 column mainly as a protein complex with an apparent Mr ~200,000 Da, although a smaller portion of Chk2 eluted as a protein complex of Mr ~600,000 Da. Because only a very small amount of Chk2 eluted where monomeric Chk2 is predicted to elute, we conclude that the majority of Chk2 exists in complex(es) with other proteins. Alternatively, Chk2 may multimerize.

We then examined the elution profiles of the stably expressed Chk2 mutants in these HCT116 derivative cell lines. As a control, HA-tagged wild type Chk2 eluted with an apparent molecular weight identical to endogenous Chk2 (Figure 5). The Chk2 I157T mutant eluted in fractions similar to that of wild type Chk2. In contrast, the Chk2 R145W mutant eluted as a much larger protein complex (Figure 5), suggesting that this mutant may affect the association of Chk2 with other proteins.
Chk2 expression is down-regulated in HCT15 cells.

The R145W mutation of Chk2 was identified in a colon cancer cell line HCT15 (12). Based on sequence analysis, HCT15 carries one mutant allele (R145W) and one wild-type allele of Chk2 (12). It is speculated that the Chk2 mutation in HCT15 may contribute to tumorigenesis either as a result of reduced gene dosage or through a dominant negative effect (12).

Expression of this R145W mutant in HCT116 cells does not affect the phosphorylation (see Figure 3c) or the activation (data not shown) of endogenous Chk2, arguing that this mutant may not behave as dominant negative mutant. We also observed that, when the same amounts of DNA encoding either wild type or R145W mutant of Chk2 were used in transient transfection experiments, the expression level of R145W mutant was only one-fifth to one-tenth that of wild type Chk2 (data not shown). These results suggest that R145W mutation of Chk2 may affect the stability of this mutant Chk2 protein. Thus, it is possible that this mutant contribute to tumorigenesis due to haploid-insufficient.

To examine whether mutation in HCT15 results in reduced level of Chk2 protein, we compared Chk2 protein level in HCT15 cells with that in K562, HCT116 or HeLa cells. If the presumed wild-type allele of Chk2 in HCT15 cells were expressed normally, we would expect to observe, at most, two fold reduction in Chk2 protein level. However, as shown in Figure 6a, Chk2 protein was barely detectable in extract of HCT15 cells, while Chk2 protein was readily detected in extracts of K562, HeLa and HCT116 cells. We estimate that the steady level of Chk2 in HCT15 cells is only 5-10% of that in other cell lines. Additionally, Chk2 kinase activity was undetectable in HCT15 cells (Figure 6b...
and data not shown). These data strongly suggest that the Chk2 expression from the second Chk2 allele is greatly reduced, if not absent, in HCT15 cells. The mechanism for the down-regulation of Chk2 expression is unknown. Since HCT15 carries inactivating mutations in both hMSH6 alleles, it is also possible that genomic instability in these cells may lead to the mutation in the second Chk2 allele. Such mutation, either at promoter or at coding sequence of Chk2, could result in the reduced level of Chk2 protein.
Discussion:

In this study, we have characterized all four reported Chk2 mutations. Two mutations identified in Li-Fraumeni patients that lead to frame-shifts at the C-terminal kinase domain, result in loss of kinase activity. In contrast, the R145W Chk2 mutant still retains some kinase activity \textit{in vitro}, but is incapable of being activated following gamma radiation \textit{in vivo}, most likely because it is not phosphorylated at Thr-68 by ATM kinase. This mutant also behaves different from wild type Chk2 in size fractionation experiments, suggesting that this mutation may also affect associations of Chk2 with other cellular proteins.

The I157T Chk2 mutant behaves as wild type Chk2 in all the assays used in this study. The I157T mutation may be a rare polymorphism that does not affect Chk2 functions. Alternatively, this mutation may affect associations of Chk2 with certain cellular proteins in a way that does not result in apparent changes in the sizes of Chk2-containing protein complexes revealed by size exclusion chromatography. Identification of Chk2-associated proteins will provide us some insights in this regard.

It is interesting that the two mutations with the Chk2 FHA domain behave differently in our assays. The FHA domain of Chk2 may have multiple functions. FHA domain is involved in protein-phosphoprotein interaction (15,16). These interactions may be essential for transmitting DNA damage signals to Chk2. Any alternation of association of Chk2 with upstream signaling proteins could lead to the failure of Chk2 activation following DNA damage, as in the case of R145W mutant. In addition, FHA domain may also mediate transmitting signals from Chk2 to downstream effectors, such as p53, BRCA1 and Cdc25C. It is reasonable to speculate that I157T mutant may be defective in
this aspect of Chk2 function. One could examine whether any Chk2-dependent events, such as stabilization of p53 following gamma radiation (8), are defective in cells that carry only the I157T mutant of Chk2. Such experiments will provide insights into the mechanism of this Chk2 mutant.
Acknowledgments:

We thank Drs. Scott Kaufmann, Larry Karnitz and Jann Sarkaria for stimulating conversations and Dr. Bin-Bing Zhou for antibodies against phosphorylated Thr-68 of Chk2. This work was supported by the Mayo Foundation, Mayo Cancer Center and Division of Oncology Research.
Figure legends:

Figure 1: Schematic diagram of Chk2 and its mutants. S/TQ-rich region, FHA domain and kinase domain are indicated, Corresponding Chk2 residues are marked. Black boxes indicate unrelated protein sequences encoded due to frameshift mutations.

Figure 2: In vitro kinase activities of wild type or mutant Chk2. Wild type or mutant GST-Chk2 proteins were expressed and purified from insect cells. Upper panel: Commassie blue stained gel indicating amounts of GST-Chk2 proteins used. Middle panel: autoradiograph indicating incorporation of $^{32}$P into the substrate GST-Cdc25C by kinases in upper panel. Lower panel: Commassie blue stained gel (the same gel showed in middle panel) indicating equal substrate in each kinase reaction.

Figure 3: R145W mutant of Chk2 is not activated following gamma radiation. a) HCT116 derivative cell lines that stably express HA-tagged wild type or mutant Chk2. Whole cell extracts were prepared from indicated cell lines and immunoblots were probed with anti-Chk2 mAb or with anti-HA mAb HA11. b) R145W mutant is not activated following gamma radiation. Extracts were prepared from indicated cell lines before and 1 hour after gamma radiation. HA-tagged wild type or mutants Chk2 were immunoprecipitated using anti-HA antibodies and kinase reactions were performed using GST-Cdc25C as substrate. c) Thr-68 is not phosphorylated in R145W mutant following gamma radiation. Extracts were prepared as described above. Immunoprecipitates with anti-Chk2 antibodies or anti-HA antibodies were immunoblotted with anti-phospho-Thr 68 antibodies and anti-Chk2 or anti-HA antibodies.
Figure 4: Nuclear localization of exogenously expressed wild type or mutant Chk2. HCT116 and its derivative cells were permeabilized, fixed and stained with DAPI (left panel) and anti-HA mAb HA11 to reveal the localization of HA-Chk2 proteins (right panel).

Figure 5: Native sizes of wild type or mutant Chk2 in the cell. Extracts prepared from HeLa, HCT116 and HCT116 derivative cells were analyzed by size exclusion chromatography. Immunoblots were probed with either anti-Chk2 antibody or anti-HA antibody.

Figure 6: Down-regulation of Chk2 in HCT15 cells. a) Chk2 expression is down-regulated in HCT15 cells. Extracts prepared from HeLa, HCT15, HCT116 and K562 cells were subjected to Western blotting using two independent, anti-Chk2 antibodies (anti-Chk2#7 and anti-Chk2B). b) HCT15 has no detectable Chk2 kinase activity before or after gamma radiation. Extracts were prepared from indicated cell lines before and 1 hour after gamma radiation. Kinase reactions were performed using anti-Chk2B immunoprecipitates.
References:


Figure 1:

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>Nucleotide change</th>
<th>Codon change</th>
<th>S/TQ-rich</th>
<th>FHA</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT15</td>
<td>C433T</td>
<td>R145W</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFS-variant MGH005</td>
<td>T470C</td>
<td>I157T</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFS MAB1</td>
<td>C1100del</td>
<td>T366FS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LFS-variant DF591</td>
<td>T1422del</td>
<td>R475FS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2:

GST-Chk2

**Coomassie blue staining (input kinases)**

**Autoradiography**

**Coomassie blue staining (input substrate)**

GST-Chk2

GST-Cdc25C

P-GST-Cdc25C
Figure 3:

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R145W</th>
<th>I157T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell Extract</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA Western</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**a.**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R145W</th>
<th>I157T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell Extract</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA Western</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**b.**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R145W</th>
<th>I157T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell Extract</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA Western</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**c.**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R145W</th>
<th>I157T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell Extract</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA Western</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4:

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>anti-HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Chk2  (Wild type)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HA-Chk2  (R145W)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HA-Chk2  (I157T)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5:

Size (kDa): 669 440 232 158 67
Fraction: 15 16 17 18 19 20 21 22 23 24 25 26 27 load

Western: anti-Chk2

HeLa

HCT116 (HA-Chk2 Wild type)

HCT116 (HA-Chk2 R145W)

HCT116 (HA-Chk2 I157T)

Western: anti-HA

Figure 6:

a. Western: anti-Chk2E

b. Western: anti-Chk2B

Auto-radiography

32P-Chk2
Characterization of tumor-associated Chk2 mutations
Xianglin Wu, Shelley R. Webster and Junjie Chen

J. Biol. Chem. published online October 26, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M009727200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2000/10/26/jbc.M009727200.citation.full.html#ref-list-1