Reduced Expression and Impaired Kinase Activity of a Chk2 Mutant Identified in Human Lung Cancer

Shuhei Matsuoka, Taku Nakagawa, Akira Masuda, Nobuhiro Haruki, Stephen J. Elledge, and Takaaki Takahashi

Abstract

The checkpoint kinase Chk2 is phosphorylated and activated in response to DNA damage such as ionizing radiation. Recently, we found a somatic mutation of CHK2 with clear loss of the wild-type allele in human lung cancer. Here we show that the mutant Chk2 exhibits modestly reduced in vitro kinase activity compared with wild type, whereas it is normally phosphorylated and activated after ionizing radiation. Interestingly, this mutant Chk2 protein was found to be less stable than wild type and could be expressed in various cell types only at a significantly reduced (20%) level of wild type. These findings confirm that the DNA damage checkpoint pathway involving CHK2 is indeed inactivated in this fatal adult cancer and also suggest that reduced expression of Chk2 may also be an important inactivating mechanism, contributing to the development of lung cancer.

Introduction

The DNA damage checkpoint pathway maintains genomic integrity in part by coordinating cell cycle progression and DNA repair after DNA damage (1). Mutations in genes in the checkpoint pathway, such as p53 and ATM, result in genomic instability and cancer predisposition. The checkpoint kinase Chk2, the mammalian homologue of budding yeast Rad53 and fission yeast Cds1, is rapidly phosphorylated and activated in response to DNA damage caused by IR or UV irradiation or replication blocks by hydroxyurea (2–6). Activation of Chk2 is regulated by phosphorylation of the NH2-terminal SCD on irradiation or replication blocks by hydroxyurea (2–6). Activation of Chk2 is phosphorylated by the ATM-related kinase ataxia-telangiectasia mutated; IR, ionizing radiation; SCD, Ser-Gln/Thr-Gln cluster domain; GST, glutathione-Sepharose (Pharmacia). The kinase assay using GST-Cdc25C expressed in Escherichia coli in vitro, which interferes with the ability of Cdc25C to activate Cdc2 in vitro, also stabilizes p53 in response to IR (7–10). Chk2 phosphorylates Cdc25C on Ser216 in vitro, which interferes with the ability of Cdc25C to activate Cdc2 (2–6). Maintenance of G2 arrest and reduced Cdc2 kinase activity in response to IR is defective in Chk2+/− mouse embryonic stem cells (11). These results suggested that ATM controls G2 arrest after IR in part by activating Chk2. Chk2 also phosphorylates Ser20 on p53. Furthermore, the identification of heterogeneous germ-line mutations in CHK2 in a subset of patients with Li-Fraumeni syndrome (14, 15) supports this model.

Lung cancer is the leading cause of cancer deaths in many economically well-developed countries including the United States and Japan. Among the genetic lesions identified in lung cancer, the p53 gene, which plays an essential role in the G1 checkpoint, is the most frequently altered, suggesting an important role of this gene in the pathogenesis of lung cancer (16). Although the G2 checkpoint genes are potential targets for genetic alterations in human cancers including lung cancer, very little information had been available about such defects. We recently found an in vivo somatic mutation of CHK2 in human lung cancer, suggesting its potential involvement (17). In this study, we report detailed characterization of the mutant Chk2, which resulted in the identification of its significantly reduced protein expression and modestly impaired kinase activity in contrast with its normal phosphorylation and activation in response to IR.

Materials and Methods

Plasmid Construction. pBSSKII-CHK2-WT was constructed by ligating EcoRI-XbaI-digested pBSSKII and a normal CHK2 cDNA obtained by reverse transcription-PCR with primers FW: CCGAATTCATGTCTCGGAGTCGAGTGT and RW: GCTCTACAGTGTCAAAACCACCGGATTTCA. A fragment containing the mutation D311V, a substitution of Val for Asp311 was obtained by reverse transcription-PCR with primers F3 and R7 using total RNA from a tumor sample as a template (17). The PCR product was digested with NarI and PfuMI and then exchanged with the NarI-PfuMI fragment of pBSSKII-CHK2-WT, resulting in the construct pBSSKII-CHK2-D311V. pGST-CHK2-D311V was made by replacing the Ncol-BamHI fragment from pBSSKII-CHK2-D311V with the wild-type fragment of pGST-CHK2 (7). pcDNA3-CHA-CHK2-WT and pcDNA3-CHA-CHK2-D311V were constructed by cloning EcoRI-XbaI fragment from pBSSKII-CHK2-WT and pBSSKII-CHK2-D311V into pcDNA3-CHA, respectively. pcRet-CHA-CHK2-WT and pcRet-CHA-CHK2-D311V, retrovirus vectors encoding HA-tagged wild-type Chk2 and Chk2-D311V, respectively, were constructed in pBluescript-puro (7).

In Vitro Kinase Assay. GST-Chk2-WT and GST-Chk2-D311V were expressed in the Escherichia coli strain BL21 (Stratagene) and purified with glutathione-Sepharose (Pharmacia). The kinase assay using GST-Cdc25C (200–256) as a substrate was carried out as described (2).

Protein Expression. HA-Chk2-WT or HA-Chk2-D311V was expressed in COS cells by transfection of pcDNA3-CHA-CHK2-WT or pcDNA3-CHA-CHK2-D311V with a plasmid DNA expressing β-Gal (pCMVβgal, a generous gift of T. Kiyono, Aichi Cancer Center Research Institute). HA-tagged wild type or D311V Chk2 was expressed in Chk2+/− MEF cells by retrovirus infection as reported previously (7). To generate Chk2+/− MEF cells stably expressing FLAG-tagged wild-type Chk2, Chk2+/− MEF cells were infected with retrovirus encoding FLAG-Chk2-WT (7), and 2 days after infection, cells were selected for 2 μg/ml of puromycin resistance.

Western Blot Analysis. Antibodies used in this study are anti-Chk2 (2), anti-HA (Santa-Cruz), anti-β-Gal (5 prime-3 prime), anti-FLAG (Sigma Chemical Co.), anti-Actin (Santa-Cruz), and anti-phospho-Thr68 (kindly provided by B. Zhou, SmithKline Beecham Pharmaceuticals; Ref. 8). Immuno-precipitation and Western blotting were carried out as described (2, 7).

Northern Blot Analysis. Total RNA was prepared, fractionated, and transferred onto a GeneScreen membrane (NEN Life Science Products). 32P-labeled CHK2 and β-GAL cDNA probes were prepared by random priming, and
Results

We first examined the in vitro kinase activity of the Chk2 mutant, because this mutant has an amino acid substitution of Val for Asp311 (D311V) in the kinase domain (17). Wild-type and mutant Chk2 were expressed as GST-fusion protein in E. coli, and purified GST-Chk2 proteins were incubated with [γ-32P]ATP and GST-Cdc25C (200–256) as a substrate. As shown in Fig. 1, GST-Chk2-D311V had low kinase activity compared with wild-type GST-Chk2. Direct measurements of radioactivity in GST-Cdc25C (200–256) revealed that kinase activity of GST-Chk2-D311V was 60–70% of the level observed for wild type. It was noted that the autophosphorylation ability of the D311V mutant appeared to be less affected than its ability to phosphorylate Cdc25C.

Next, we expressed wild-type and mutant Chk2 in COS cells by transient transfection. A plasmid expressing HA-tagged Chk2 wild type or the D311V mutant under the control of cytomegalovirus promoter was cotransfected into COS cells together with a plasmid encoding β-Gal. Fig. 2A shows that Chk2-D311V is expressed in cells at significantly lower levels than wild-type Chk2 in contrast with similar levels of expression of β-Gal. Similar results were also obtained when expressed in 293T cells (data not shown). Quantitative analysis indicated that the protein level of the D311V mutant was only 20% of wild type. Northern blot analysis of RNA prepared from these cells showed that both wild type and D311V were transcribed at similar levels (Fig. 2B). Furthermore, cycloheximide treatment of these cells revealed that the half-life of the mutant protein was ~6 h, whereas wild-type protein was stable over 24 h with only 10–15% reduction in protein level (Fig. 2C). These results suggest that the reduced expression of D311V is caused by instability of the mutant protein.

Phosphorylation and activation of Chk2-D311V were further examined in Chk2−/− MEF cells with and without exposure to DNA damage, because we showed previously that human Chk2 expressed in Chk2−/− MEF cells can correctly respond to IR (7). When wild type and D311V were expressed in Chk2−/− MEF cells, a significantly reduced protein level of Chk2-D311V was again observed in MEF cells (Fig. 3A), indicating that reduced expression of the mutant protein did not depend on the cell types used (COS, 293T, or MEF) and the method of expression (transfection or retrovirus infection). Mobility shift of the D311V mutant after DNA damage occurred to the extent very similar to that of wild-type Chk2 (Fig. 3B). Because we showed that mobility shift of Chk2 is parallel to its activation after DNA damage (2, 7), these results indicate that Chk2-D311V is likely to be activated in response to IR like wild-type Chk2. It was also noted that reduced expression of D311V is caused by instability of the mutant protein.

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protein analysis.

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clear loss of the corresponding wild-type allele (17), its expression

mutation was present in the lung cancer patient in association with

expression level of the D311V mutant in the MEF cells. Interest-

impairment (data not shown); however, it was not as reproducible

D311V immunoprecipitated from the MEF cells also showed slight

of impairment was modest. We note that kinase assay using Chk2-

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missense mutation (D311V) within the kinase domain was studied

data not shown, contributing to the acquisition of genetic

DISCUSSION

It has been shown that inactivation of the p53 gene, which
disrupts the G1 checkpoint, plays a major role in the pathogenesis
of human lung cancer (16), whereas the impairment of another
important checkpoint, i.e., the G2 checkpoint, has long been ex-
pected to be present, contributing to the acquisition of genetic
instability in lung cancer cells. Indeed, we found previously that
the CHK2 gene is somatically mutated in lung cancer in vivo with
clear loss of wild-type allele (17). In the present study, this CHK2
missense mutation (D311V) within the kinase domain was studied
in detail. Although this mutated Asp311 residue is not in the
conserved kinase sub-domains I-XI (18), it is well conserved
among species, suggesting that Asp311 is functionally important
and that the substitution may affect Chk2 kinase activity. Indeed,
the mutant Chk2 exhibited impaired kinase activity in an in vitro
kinase assay with GST-Cdc25C as a substrate, although the degree
of impairment was modest. We note that kinase assay using Chk2-
D311V immunoprecipitated from the MEF cells also showed slight
impairment (data not shown); however, it was not as reproducible
as that observed with GST-Chk2-D311V, perhaps because of low
expression level of the D311V mutant in the MEF cells. Interest-
ingly, the expression experiments revealed the most apparent al-
teration in that the D311V mutant protein was less stable than wild
type and could be expressed at 20% of the level of wild-type Chk2
when the same level of mRNA was expressed. Because the D311V
mutation was present in the lung cancer patient in association with
clear loss of the corresponding wild-type allele (17), its expression
level may have been <10% of normal levels. Although the mutant
Chk2 retained considerable kinase activity, it is thus conceivable
that reduced protein expression by the mutation may have severely
affected Chk2 function in the checkpoint pathway, thereby con-
tributing lung cancer development in this case. Unfortunately, the
primary tumor specimen of this patient was not available for
protein analysis.

To date, three genuine CHK2 mutations have been identified thus
far in Li-Fraumeni syndrome as well as in a colon cancer cell line (14,
15, 19). Apparent lack of in vitro kinase activity was recently shown
in one premature truncation mutant (del(1100C)) within the kinase
domain, whereas one mutation within fork head-associated domain
was shown to have reduced kinase activity in vitro and defects in both
phosphorylation of Thr68 in the SCD and activation in response to IR
(19). The other fork head-associated domain mutant, a substitution of
Thr for Ile at amino acid 157, behaved as wild-type Chk2 in all of the
assays used (19). The present study shows clear distinctions between
the lung cancer Chk2 mutant, D311V, and the mutants reported
previously, in that D311V showed significantly reduced protein
expression with considerable retention of kinase activity and normal
response of phosphorylation on Thr68 and activation to IR, suggesting
that reduced expression of Chk2 may also be an important mecha-
nism. Although the present findings that the CHK2 mutant identified
in human lung cancer did have defects suggest its potential role in
lung cancer development, our previous genetic screen showed that
CHK2 mutation is relatively infrequent. It will therefore be interesting
to study whether epigenetic alterations might also be involved, leading
to down-regulation of Chk2 at the mRNA and/or protein levels in
human lung cancers. Further studies are also warranted to search for
additional as yet unidentified DNA damage checkpoint genes in-
volved. In this connection, studies in yeast on DNA damage check-
point points will shed light on better understanding of the molecular path-
genesis of lung cancer.

Acknowledgments

We thank B. Zhou for anti-phospho-Thr68 antibody, T. Kiyono for
pCMV/Flag, A. Osborn for helpful comments on the manuscript, K. Matsuoka
for encouragement and helping figure preparation, and C. Cherry for secretar-
ial support.

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