Destabilization of CHK2 by a Missense Mutation Associated with Li-Fraumeni Syndrome

Sean Bong Lee, Sang Hyon Kim, Daphne W. Bell, Doke C. R. Wahrer, Taryn A. Schiripo, Melissa M. Jorczak, Dennis C. Sgroi, Judy E. Garber, Frederick P. Li, Kim E. Nichols, Jenny M. Varley, Andrew K. Godwin, Kristen M. Shannon, Ed Harlow, and Daniel A. Haber

Massachusetts General Hospital Cancer Center and Harvard Medical School, Charlestown, Massachusetts 02129 [S. B. L., S. H. K., D. W. B., D. C. R. W., T. A. S., M. M. J., K. M. S., E. H., D. A. H.]; Molecular Pathology Unit, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129 [D. C. S.]; Division of Population Sciences, Dana Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02114 [J. E. G., F. P. L.]; Division of Pediatric Oncology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania [K. E. N.]; Cancer Research Campaign, Department of Cancer Genetics, Paterson Institute for Cancer Research, Manchester M20 4BX, United Kingdom [J. M. V.]; and Medical Science Division, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [A. K. G.]

Abstract

Li Fraumeni Syndrome (LFS) is a multicancer phenotype, most commonly associated with germ-line mutations in TP53. In a kindred with LFS without an inherited TP53 mutation, we have previously reported a truncating mutation (1100delC) in CHK2, encoding a kinase that phosphorylates p53 on Ser20. Here, we describe a CHK2 missense mutation (R145W) in another LFS family. This mutation destabilizes the encoded protein, reducing its half-life from >120 min to 30 min. This effect is abrogated by treatment of cells with a proteasome inhibitor, suggesting that CHK2R145W is targeted through this degradation pathway. Both 1100delC and R145W germline mutations in CHK2 are associated with loss of the wild-type allele in the corresponding tumor specimens, and neither tumor harbors a somatic TP53 mutation. Our observations support the functional significance of CHK2 mutations in rare cases of LFS and suggest that such mutations may substitute for inactivation of TP53.

Introduction

LFS4 provides a compelling link between cancer predisposition and the inactivation of a DNA damage checkpoint (1, 2). Germ-line mutations in one allele of the tumor suppressor TP53 underlie ~80% of classic LFS families, characterized by sarcomas, carcinomas of the breast, brain, and adrenal gland, and acute leukemia. TP53 mutations are also present in ~20% of variant-LFS families, a combination of multicancer phenotypes that fails to meet the stringent criteria for classic LFS (3–5). In cases that harbor a germline mutation in one TP53 allele, the development of cancer is associated with LOH, denoting somatic inactivation of the second allele.

In screening four probands with LFS and 18 cases of variant-LFS with wild-type TP53 for germ-line mutations in other DNA damage checkpoint genes, we uncovered rare mutations in CHK2, the human homologue of Schizosaccharomyces pombe Cds1, encoding a kinase that contributes to the S-phase and G2-M checkpoints in fission yeast (6). In both yeast and mammalian cells, CHK2 is phosphorylated and activated after DNA damage or stalled replication, through both ATM-dependent and -independent pathways (7, 8). CHK2 can phosphorylate CDC25C on Ser216, leading to its cytoplasmic sequestration with 14-3-3 proteins, preventing CDC25C dephosphorylation of CDC2 on Tyr15, and resulting in a G2-M arrest (9–11). In mammalian cells, CHK2 also mediates the phosphorylation of p53 on Ser20, a critical residue that appears to be essential for the stabilization of p53 after DNA damage (12, 13). CHK2-null cells fail to activate p53 after exposure to ionizing radiation, consistent with its postulated role as an intermediate kinase in the ATM-dependent activation of p53 (14). In addition, CHK2 is capable of phosphorylating CDC25A (15), leading to activation of an S-phase checkpoint, and it has been shown to phosphorylate the breast cancer predisposition gene product BRCA1 (16). Although the relative physiological importance of these CHK2 phosphorylation targets remains to be established, its apparent function within the DNA damage response pathway is consistent with a role as a tumor suppressor gene.

The mutational analysis of CHK2 in LFS kindreds has been complicated by partial duplications of the genomic locus (17), as well as by the observation of missense mutations (6, 18), the functional significance of which cannot be readily ascertained. Here, we describe an analysis of 10 additional cases of LFS and 49 cases of variant-LFS, using conditions that reliably distinguish the transcribed CHK2 gene on chromosome 22 from the duplicated copies that are not expressed. In addition to a previously reported premature truncation (1100delC), we observed a missense mutation within the FHA domain of CHK2 (R145W) that leads to a rapid degradation of the mutant protein via the ubiquitin-proteasome pathway. The apparent functional consequences of these two deleterious mutations in CHK2 are correlated with loss of the wild-type allele in primary tumor specimens. TP53 is wild-type in these tumors with homozygous inactivation of CHK2, suggesting that disruption of CHK2 function may relieve the requirement for mutation of TP53. We also report biallelic inactivation of CHK2 in the HCT15 sporadic colorectal cancer cell line.

Materials and Methods

Clinical Specimens and Criteria for LFS and Variant-LFS. Classic LFS families were defined by a proband with a sarcoma diagnosed under 45 years of age, with a first-degree relative having an LFS component tumor under 45 years of age, and a first- or second-degree relative with any cancer under 45 years of age or a sarcoma at any age (1). Variant LFS cases included those individuals with three or more primary tumors with the first cancer diagnosed under 45 years of age; or a family in which the proband had any childhood cancer, sarcoma, brain tumor, or adrenocortical carcinoma under 45 years of age, a first- or second-degree relative with a component LFS tumor at any age, and another first- or second-degree relative with any cancer under age 60 (3).

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2 Equal contribution.

3 To whom requests for reprints should be addressed, at Massachusetts General Hospital Cancer Center, Building 149 13th Street, Charlestown, MA 02129. Phone: (617) 726-7805; Fax: (617) 724-6919; E-mail Haber@helix.mgh.harvard.edu.

4 The abbreviations used are: LFS, Li-Fraumeni syndrome; LOH, loss of heterozygosity; FHA, forhead homology associated; CMV, cytomegalovirus; CHX, cycloheximide; tet, tetracycline.

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for LFS cases. All clinical material was collected under appropriate Institutional Review Board-approved protocols. Control EBV-immortalized lymphoblastoid cell lines were established from healthy blood donors in the Boston area. To obtain homogeneous populations of tumor cells from sections of paraffin-embedded tumors from carriers of CHK2 mutations, laser-capture microdissection was performed using a PixCell I and II LCM system from Arcturus Engineering (Mountain View, CA). Each population was estimated to be >98% “homogeneous” as determined by microscopic visualization of the captured cells.

Mutational Analysis of CHK2. Exons 1–9 of CHK2 are unique to chromosome 22 and hence could be amplified directly from genomic DNA (17). Sequences for any primer pairs and PCR conditions are provided upon request. All primers included the M13–21 tail added to the 5′ end of the forward primer or the M13–26 tail added to the 5′ end of the reverse primer to facilitate sequencing. To facilitate sequencing of CHK2 exons 10 through 14 which are duplicated, together with their intervening sequences, on multiple human chromosomes (17), we designed an initial, long-range chromosome 22-specific primary PCR spanning these exons, with subsequent nested PCRs to amplify individual exons, which were mapped to chromosome 22 using a human-rodent somatic cell hybrid panel (Coriell Cell Repositories, Camden, NJ). To analyze exon 10–14 sequences from formalin-fixed, microdissected primary tumor specimens, PCR products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and their chromosomal origin was determined, based on known intronic sequence variants between chromosome 22 and other chromosome copies. For TP53 mutational analysis, conserved exons 5–8 were PCR amplified from microdissected tumor specimens and sequenced. All uncloned PCR products were sequenced using energy transfer Dye Primer Sequencing (Amersham), using Factura and Sequence Navigator (Applied Biosystems, Foster City, CA), to mark potential heterozygous positions and display them for evaluation. Nucleotide positions at which the height of the secondary peak was >30% that of the primary peak were marked as heterozygous and were confirmed by analysis of both sense and antisense strands.

Expression Constructs and Generation of Inducible Cell Lines. A full-length hCHK2 cDNA was constructed by ligating a PCR-generated 5′ end to EST clone AI809500 (American Type Culture Collection). Site-directed mutagenesis, as well as reverse transcription-PCR amplification from mutant cell lines, was used to generate mutant constructs encoding the naturally occurring R145W, R31W, I157T, A247D, and 1100delC CHK2 mutations and the synthetic catalytically inactive D368N mutation. cDNAs were cloned into a CMV-driven vector either encoding (pCMV5Flag) or lacking (pCDNA3, Invitrogen) a Flag epitope and into the tetracycline-regulated vector pUHD10-3. All constructions were confirmed by nucleotide sequencing of the entire coding region. The TNT coupled transcription-translation kit (Promega) was used for in vitro translation. For transient transfection, U2OS, 293, and COS-7 cells were transfected with 5 µg of pCMV5Flag CHK2 and 0.5 µg of pEGFP-C1 (Clontech) plasmids using the calcium phosphate method. Cell lysates were analyzed 40 h after transfection by immunoblotting, using either anti-Flag (Sigma Chemical Co.) or anti-GFP antibodies (Clontech). Inducible, tetracycline-repressible expression of CHK2 constructs was achieved by co-transfecting an U2OS founder cell line with various pUHD10-3 CHK2 constructs, along with a plasmid encoding hygromycin resistance. Hygromycin-resistant clones were picked and analyzed for similar levels of inducible CHK2 expression by Northern blot analysis, and at least two independent clones were selected for measurements of protein half-life.

Kinase Assays and Measurement of Protein Stability. In vitro kinase activity of anti-Flag-immunoprecipitated CHK2 proteins from U2OS cells with inducible gene expression was determined in unirradiated cells or 1 h after γ-irradiation (10 Gy). GST-CDC25C (amino acids 200–256) was used as substrate, in a 30-min incubation using 20 mM HEPES (pH 7.4), 10 mM MgCl2, 10 mM MnCl2, 40 µM ATP, and 15 µCi [γ-32P]ATP at 30°C. The half-life of wild-type and mutant CHK2 15 mRNAs were measured by withdrawing tetracycline from U2OS cells for 20 h, adding back the drug to inhibit further CHK2 transcription, and measuring sequential levels of mRNA by Northern blotting. Two approaches were used to measure the half-life of CHK2 proteins. For pulse-chase analysis, CHK2 expression was induced for 20 h, followed by incubation with methionine-free DMEM (30 min), labeling with [35S]methionine (500 µCi/ml; 15 min), incubation with excess cold methionine, and lysis at various intervals in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% DOC, and 0.1% SDS]. Radiolabeled CHK2 was immunoprecipitated using anti-Flag antibody, resolved by 8% SDS-PAGE and quantitated using a PhosphorImager (Bio-Rad). For CHX-Western analysis, CHK2 expression was induced for 20 h, CHX (10 µg/ml) was added to inhibit protein synthesis, and steady-state CHK2 levels were measured at sequential intervals by immunoblotting using anti-Flag antibody. Anti-actin antibody (Sigma Chemical Co.) was used for loading control, and multiple exposures were analyzed to ensure that measurements were taken within the linear range. In parallel experiments, cells were treated with the proteosome inhibitor lactacystin (25 µM), 30 min prior to and for the duration of CHX treatment.

Results

CHK2 Mutational Analysis in Kindreds with Classic-LFS and Variant-LFS. We have previously screened the CHK2 coding sequence in 4 cases of LFS and 18 cases of variant-LFS, for which the availability of immortalized cell lines allowed a cDNA-based mutational analysis (6). To extend our analysis to an additional 10 cases of LFS and 49 cases of variant-LFS for which only genomic DNA was available, we developed conditions for selective amplification of the CHK2 exons on chromosome 22. The 3′ terminal exons and introns of CHK2 are duplicated on multiple chromosomes, i.e., chromosomes 2, 7, 10, 13, 15, 16, X, and Y (17), and although these genomic copies are not expressed, their high degree of sequence conservation complicates PCR-based analysis of genomic DNA. To selectively amplify the correct, transcribed CHK2 gene from chromosome 22, we used an initial long-range PCR reaction spanning from intron 9 to the 3′ untranslated region, followed by internal nested amplification of the relevant exons using intronic primers. Specific amplification of each chromosome 22 exon was verified by using somatic hybrid panels and further confirmed by analysis of sequence variants that distinguish the chromosome 22 sequences from their derivatives on other chromosomes. As noted elsewhere (17), one previously reported CHK2 mutation (1422delT) that had only been detected in genomic DNA from a variant-LFS kindred was found to be derived from the CHK2 copy on chromosome 15 and hence represents a sequence variant of no functional significance. All other previously reported sequence changes in CHK2 were confirmed as originating from the correct chromosome 22 gene.

Analysis of classic-LFS and variant-LFS kindreds revealed three missense mutations. A heterozygous Arg to Trp substitution within the FHA domain (R145W) was present in the germ-line of a family with variant-LFS (Fig. 1B). This mutation had been detected previously in the sporadic colorectal cancer cell line HCT15 (6). A heterozygous Arg to Trp at codon 3 (R31W) was detected in a variant-LFS family; an Ile to Thr within the FHA domain (I157T) was found in a classic-LFS kindred. I157T was detected previously in a variant-LFS kindred (6). To test whether these missense mutations might be rare polymorphisms in the population, we screened immortalized cell lines from 200 healthy individuals (400 chromosomes), who were ethnically matched with our patient population. R145W, R31W, or I157T were not detected, suggesting that they do not constitute common polymorphisms in this control population. Combining this study with our previous analysis (6), mutational analysis of CHK2 in a total of 14 classic-LFS and 67 variant-LFS kindreds identified one premature stop codon and three independent missense mutations that were absent in a control population.

LOH in Primary Tumors. To test the potential significance of germ-line CHK2 mutations, we first analyzed primary tumor specimens for evidence of LOH. Archival, formalin-fixed, and paraffin-embedded primary tumor specimens were obtained for each of the cases harboring a germ-line CHK2 mutation. DNA was isolated, and the relevant exons were PCR amplified, cloned and sequenced. Analysis of multiple independent clones made it possible to confirm the chromosomal origin of exons derived from duplicated genomic frag-
the bona fide germ-line CHK2 allele and demonstrated, respectively, biallelic missense mutations in homozygous for 1100delC and R145W CHK2 chromosome 22. A specimen of the sarcoma from the proband could not be obtained for analysis. C, mutant peak). Tumor-derived PCR products were cloned, and 24 of 27 clones were found to contain the R145W mutation, consistent with LOH.

The proband is demonstrated by sequencing uncloned PCR products from normal cells (1265 germ-line; heterozygous peak) and microdissected tumor cells (1265 tumor; homozygous arrow 22-specific CHK2 PCR product derived from the tumor showing the 1100delC mutation and distribution of cloned products among different chromosomal origins indicating that 11 of 13 chromosome 22 from nonexpressed partial gene duplications on chromosomes 15, 16, and X. The 1100delC mutation is present on the chromosome 22 allele. Lower panel, sequence of a cloned PCR product derived from the tumor showing the 1100delC mutation and distribution of cloned products among different chromosomal origins indicating that 11 of 13 chromosome 22-specific CHK2 clones carried the 1100delC mutation. B, identification of the R145W missense CHK2 mutation in a variant LFS family. Upper panel, family pedigree, of whom only the proband (1265; arrow), diagnosed with both breast cancer and sarcoma, was available for analysis. Lower panel, loss of the wild-type CHK2 allele from the breast tumor of the proband is demonstrated by sequencing uncloned PCR products from normal cells (1265 germ-line; heterogeneous peak) and microdissected tumor cells (1265 tumor; homozygous mutant peak). Tumor-derived PCR products were cloned, and 24 of 27 clones were found to contain the R145W mutation, consistent with LOH. CHK2 cDNA 145 is unique to chromosome 22. A specimen of the sarcoma from the proband could not be obtained for analysis.

Fig. 1. CHK2 LOH and TP53 mutational analysis in primary tumors of CHK2 mutation carriers. A, somatic loss of the wild-type CHK2 allele from the sarcoma of LFS patient MA81, harboring a heterozygous germ-line 1100delC mutation (pedigree and cosegregation of the mutation with cancer predisposition reported previously; Ref. 6). Upper panel, partial genomic structure of CHK2 flanking the intron 9/exon 10 junction, indicating the intronic polymorphism that is used to distinguish the transcribed CHK2 sequence on chromosome 22 from nonexpressed partial gene duplications on chromosomes 15, 16, and X. The 1100delC mutation is present on the chromosome 22 allele. Lower panel, sequence of a cloned PCR product derived from the tumor showing the 1100delC mutation and distribution of cloned products among different chromosomal origins indicating that 11 of 13 chromosome 22-specific CHK2 clones carried the 1100delC mutation. B, identification of the R145W missense CHK2 mutation in a variant LFS family. Upper panel, family pedigree, of whom only the proband (1265; arrow), diagnosed with both breast cancer and sarcoma, was available for analysis. Lower panel, loss of the wild-type CHK2 allele from the breast tumor of the proband is demonstrated by sequencing uncloned PCR products from normal cells (1265 germ-line; heterogeneous peak) and microdissected tumor cells (1265 tumor; homozygous mutant peak). Tumor-derived PCR products were cloned, and 24 of 27 clones were found to contain the R145W mutation, consistent with LOH. CHK2 cDNA 145 is unique to chromosome 22. A specimen of the sarcoma from the proband could not be obtained for analysis. C, mutational analysis of TP53 in sporadic tumors derived from LFS patients with the bona fide germ-line CHK2 mutations 1100delC and R145W and the missense variants of unknown significance, I157T and R3W. No TP53 mutations were identified in the tumors homozygous for 1100delC and R145W CHK2 mutations. Tumors from cases with heterozygous I157T and R3W germ-line CHK2 mutations did not show somatic loss of the wild-type CHK2 allele and demonstrated, respectively, biallelic missense mutations in TP53 or a homozygous missense mutation.

The observation of LOH in tumors from carriers of the 1100delC and R145W germ-line mutations primarily supports the functional significance of these two alterations in the CHK2 transcript.

**Destabilization of CHK2 by the R145W Mutation.** To directly test their functional properties, full-length cDNAs encoding either wild-type or mutant CHK2 were cloned into expression plasmids either containing or lacking a 5′ Flag epitope. Transfection of a synthetic dominant-negative CHK2 mutant into U2OS cells demonstrated a modest disruption in the G1 checkpoint (12), but this approach could not be used to test potential loss-of-function mutations. We therefore tested the baseline and γ-irradiation induced kinase activity of immunoprecipitated CHK2 proteins, using a domain of CDC25C (amino acids 200–256) as substrate. CHK21100delC and CHK2I157T demonstrated kinase activity comparable with that of the wild-type protein (Fig. 2A). As expected, no kinase activity was detected for CHK21100delC, consistent with disruption of the kinase domain by the premature termination codon, or for the synthetic mutant CHK2D368N with a disrupted catalytic domain. Surprisingly, transfection experiments demonstrated dramatically reduced expression of the CHK2R145W product, with a corresponding reduction in kinase activity.

To ensure that all CHK2 cDNA constructs were capable of producing equal amounts of protein in vitro, translation of wild-type and variant cDNAs were compared. In vitro translation of CHK21100delC produced a protein of expected quantity and size by SDS-PAGE, compared with the wild-type product (Fig. 2B). However, transient transfection of expression constructs into either COS, U2OS, or 293 cells resulted in minimal steady-state expression of CHK2R145W, as detected by Western blot analysis (Fig. 2C and data not shown). To allow studies of mRNA and protein half-life, we generated a panel of U2OS cells with tightly regulated, tet-repressible expression of the...
wild-type and mutant CHK2 alleles. In these cells, no difference in mRNA half-life was detected after rapid termination of CHK2 transcription by readadition of tet (Fig. 3A). Despite comparable expression of the inducible transcript, the CHK2R145W product was consistently expressed at a fraction of the wild-type protein. Measurement of protein turnover using pulse-chase analysis demonstrated a $t_{1/2}$ of $>120$ min for wild-type CHK2 and for CHK2R3W and CHK2I157T (Fig. 3B). Remarkably, in multiple cell lines with inducible CHK2, translation ($IVT$) of $^{35}$S-labeled CHK2 proteins, demonstrating comparable synthesis of wild-type (WT) protein and the three missense variants. C, steady-state protein expression of CHK2 variants, 40 h after transient transfection into COS-7 cells (5 μg of pCMV5Flag constructs). Immunoblotting analysis using anti-Flag antibody to identify transfected CHK2 and anti-GFP antibody (control for transfection efficiency).

Fig. 3. Instability of CHK2 protein encoding R145W mutation. A, comparable stability of variant CHK2 transcripts. CHK2 expression was induced for 20 h in multiple independent clones of U2OS cells, with regulated expression of wild-type transcript (WT; clones 2 and 21), or missense mutations R145W (clones 7 and 10), and I157T (clone 7). Expression of CHK2 mRNA was measured by Northern blot at various intervals after readdition of tet to suppress new CHK2 transcription ($EtBr$, ethidium bromide staining loading control). B, instability of CHK2R145W, demonstrated by pulse-chase analysis. Expression of CHK2 protein was induced for 20 h in U2OS cells with comparable induction of the transcripts encoding wild-type and missense mutation, followed by pulse-labeling with $^{35}$S methionine (15 min), and chase with excess cold methionine for indicated times. Cell lysates were immunoprecipitated using anti-Flag antibody and analyzed by SDS-PAGE and autoradiography. Half-lives ($t_{1/2}$) of the CHK2 proteins were quantitated using Phosphorimager analysis (Bio-Rad). C, suppression of CHK2R145W degradation by the proteasome inhibitor lactacystin and measured protein half-life using the CHX-Western assay. Treatment with lactacystin had no effect on turnover of wild-type CHK2, but it restored stable expression of CHK2R145W (Fig. 3C).
TP53 in the primary sarcoma specimen homozygous for the 1100delC CHK2 mutation or in the primary breast tumor homozygous for the R145W mutation (Fig. 1C). In contrast, the breast tumor harboring a heterozygous 1157T CHK2 mutation demonstrated inactivation of TP53 by two independent point mutations within the conserved domain in exon 6: a novel mutation F212V, and a previously reported CHK2 alanine at codon 247 (A247D) in the HCT15 colorectal cancer cell line (6). Given the detection of LOH in a tumor arising in a patient with variant LFS and a germ-line R145W mutation, we speculated that the second CHK2 allele in HCT15 cells might also harbor a mutation. Reanalysis of the CHK2 sequence confirmed the heterozygous R145W mutation and also identified a second, previously undetected, missense mutation, a C to A at nucleotide 740, resulting in a substitution of aspartic acid for a conserved alanine residue at codon 247 within the kinase domain (Fig. 4A). The A247D mutation disrupts a residue in the catalytic motif II, which is invariant among CHK2 homologues in vertebrates, Drosophila, and yeast, and also shows a high degree of conservation among other classes of kinases (22). The A247D CHK2 mutation was not detected in 187 control individuals, indicating that it is not a common sequence variant in the population. Analysis of cloned PCR products indicated that the R145W and A247D mutations are present on different CHK2 alleles in HCT15 cells, consistent with biallelic inactivation of the gene. To test its functional properties, we generated an expression construct encoding CHK2A247D, which was transiently transfected into COS and U2OS cells (Fig. 4C; data not shown). Similar to CHK2R145W, steady-state expression of transfected CHK2A247D was minimally detectable by Western blotting (Fig. 4C). Thus, the presence of biallelic point mutations leading to grossly unstable proteins explains the absence of detectable endogenous CHK2 in HCT15 cells (23).

Discussion

Although most cases of classic-LFS and a subset of variant-LFS harbor germ-line mutations in TP53, a small number of cases have mutations in CHK2 (6). The similar phenotype conferred by germ-line mutations in TP53 and CHK2, and the fact that tumors arising in CHK2-mutant kindreds may not carry somatic TP53 mutations, are consistent with CHK2 encoding a kinase responsible for the phosphorylation of p53 on Ser20 and its stabilization after DNA damage. Analysis of additional cases of LFS linked to CHK2 mutations will be required to confirm whether somatic inactivation of TP53 and CHK2 are truly redundant, and whether the clinical tumor spectrum associated with germ-line mutations in these two genes can be distinguished. The partial duplication of the CHK2 genomic locus on seven different chromosomes has greatly complicated mutational analysis (17) and prevented demonstration of LOH in archival tumor specimens that were not amenable to cDNA-based analysis. By developing PCR conditions capable of distinguishing the expressed CHK2 gene on chromosome 22 from the duplicated genomic fragments, we were able to show definitive evidence of LOH for two LFS-associated germ-line mutations. The functional consequences of the premature truncation within the kinase domain of CHK2 (1100delC) are evident, but analysis of germ-line missense mutations is notoriously difficult in the absence of reliable functional assays. To this end, the presence of LOH in the primary tumor specimen and gross instability of the protein encoded by the R145W missense mutation support its characterization as a deleterious mutation.

The R145W missense mutation was initially reported in the HCT15 colorectal cancer cell line, where it is present in one of the two alleles (6). We have now detected a second missense mutation in this cell line, A247D, which is present on the second allele and also appears to encode a grossly unstable protein. Taken together, these observations explain the absence of detectable CHK2 expression in this sporadic colon cancer cell line (23) and suggest that it represents a functionally null cell line for CHK2.

The instability of both CHK2R145W and CHK2A247D raises the possibility that both mutations may be associated with significant disruption of protein folding. CHK2R145W, which arises within the FHA domain, is of particular interest given the crystal structure of this domain derived from the Saccharomyces cerevisiae homologue Rad53 (24). Cocrystallization of this domain with an optimal phosphothreonine-containing peptide revealed 11 anti-parallel β-sheet strands with three loops generated by six β-strands with three loops that are not conserved, and where a nonconservative mutation might disrupt the β-sheet strand formation, inducing misfolding of the protein. In fact, computer modeling of the secondary structure of the CHK2 FHA domain containing the R145W mutation

![Fig. 4. Biallelic inactivation of endogenous CHK2 in HCT15 cells. A. heterozygous C to A missense mutation at nucleotide 740, resulting in substitution of aspartic acid for alanine at codon 247 (A247D) in the CHK2 transcript from the sporadic colon cancer cell line HCT15. The alanine at codon 247 is a highly conserved residue within the CHK2 kinase domain. B. schematic representation of the A247D mutation and the previously reported R145W mutation within the FHA domain (6) in HCT15 cells. Analysis of cloned reverse transcription-PCR products indicated that the two mutations are present on transcripts encoded by different alleles (A1 and A2), consistent with biallelic point mutations. C. CHK2A247D encodes an unstable protein. Immunoblotting analysis of COS-7 cells, 40 h after transfection with constructs encoding either wild-type (WT) CHK2, or the A247D mutation (5 μg of pCMV5Flag). Cotransfected green fluorescent protein (GFP) was used as control for transfection efficiency. CONTROL lane, untransfected Cos-7 lysate.](https://cancerres.aacrjournals.org)
predicts formation of an extra β-sheet strand (Chow-Fasman algorithm, data not shown), which might disrupt the critical loop structures that constitute the binding surface for the phosphoprotein substrates. Identification of binding partners for the FHA domain of CHK2 will allow more direct analysis of the altered protein interactions that result from disruptions of this domain.

The instability of CHK2R145W also has implications for the interpretation of functional results reported with this mutant protein. Using a stably transfected cell line with detectable expression of CHK2R145W, Wu et al. (23) demonstrated that this protein had decreased in vitro kinase activity, reduced ATM kinase, and migrates within a larger protein complex than native CHK2 (23). Our finding that CHK2R145W is predicted to be misfolded, grossly unstable, and degraded by the proteosome pathway provides a potential explanation for these observations. More recent data indicate that the CHK2R145W mutation also disrupts the ability of the protein to undergo autophosphorylation at amino acids T383 and T387 (25) and to phosphorylate CDC25A (15), which is also consistent with a grossly abnormal protein.

In contrast to R145W, we did not detect LOH in primary tumors derived from individuals with the germ-line R3W or I157T CHK2 mutations. As noted above, LOH analysis does not address the possibility that the second CHK2 allele is disrupted by an independent somatic point mutation, but rather by a chromosomal event that leads to homozygosity for the germ-line mutation. Nonetheless, we could not identify gross abnormalities in the proteins encoded by these two missense mutations and would therefore classify them as mutations of unknown significance. The I157T mutation, however, may be of some interest. We did not detect this mutation in 200 control individuals from the Boston area, although I157T has been observed in ~5% of healthy controls in Finland (26). Recently, Falck et al. (15) reported that CHK2R145W, along with CHK2R145W, are deficient in both binding and phosphorylation of CDC25A, a novel function for CHK2 associated with regulation of S-phase entry. Whether I157T represents a possibility that the second CHK2 is deficient in both binding and phosphorylation of p53, and whether CHK2-mediated phosphorylation of other targets may be differentially modulated by its sequence variants.

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References
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