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Familial breast cancer accounts for 15 to 35% of all breast cancers. Mutations in a number of genes are now known to cause susceptibility to breast cancer; the most notorious are the *BRCA1* and *BRCA2* genes. However, it has become evident that not all (and not even the majority) of familial breast cancer families can be attributed to mutations in *BRCA1* and *BRCA2*. Recently, it was reported that germline *CHK2* mutations were found in two families with Li-Fraumeni syndrome and a third case with multiple primary cancers. The two families with Li-Fraumeni syndrome had diverse cancers, including early-onset breast cancers at ages 37, 41, and 45 years. The third proband developed breast cancer at age 47, malignant melanoma at 53 and primary lung cancer at 58, but had no family history of malignancies. These data suggest that germline *CHK2* mutations predispose to breast cancer, similar to other inherited mutations in *BRCA1*, *BRCA2*, *TP53* and perhaps *ATM*. However the extent of *CHK2* involvement in hereditary breast cancer is not fully known. Our objective was to determine the frequency of germline mutations in *CHK2/CDS1* in breast cancer-prone kindreds that have previously tested negative for mutations in *BRCA1* and *BRCA2*.

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INTRODUCTION:

Susceptibility genes presently account for only 20-25% of the hereditary risk for breast cancer (Lichtenstein et al., 2000). The majority of this risk can be attributed to the two breast cancer susceptibility genes, *BRCA1* and *BRCA2* (Miki et al., 1994; Wooster et al., 1995). Mutations in a third gene, *TP53*, appear to be responsible for a minor additional fraction of predisposition to breast cancer (reviewed in Easton, 1999). In recent studies, *TP53* changes occurred exclusively in those breast cancer families also displaying a Li-Fraumeni syndrome (LFS) or Li-Fraumeni-like syndrome (LFL)(Huusko et al., 1999). This syndrome is described by a cancer background within a family consisting of sarcomas, breast cancer, leukemia, and tumors of the central nervous system and adrenal cortex (Garber et al., 1991). These observations indicate that other breast cancer susceptibility genes must be involved to account for hereditary breast cancer risk.

Bell et al. (1999) identified germline *CHK2* mutations in *TP53*-negative LFS and LFL families (13.6%). It was suggested that mutations in *CHK2*, a gene that encodes a protein kinase that activates p53 by phosphorylation in a DNA damage dependent and ATM dependent manner (reviewed in Prives and Hall, 1999), may contribute to predisposition to sarcoma, breast and brain tumors. While there has been subsequent contradiction as to the functional significances and relevant frequencies of the original four alterations described in Bell et al. (Sodha et al., 2000; Wu et al., 2001; Allinen et al., 2001; Lee et al., 2001), several studies have emerged providing support that *CHK2* acts as a low penetrant tumor-suppressor gene making a significant contribution to familial clustering of breast cancers (Ingvarsson et al., 2002; Vahteristo et al., 2002; Meijers-Heijboer et al., 2002; Brody, LC 2002). Additionally, mutations in *CHK2* recently found among osteosarcomas is consistent with that being a defining tumor of Li-Fraumeni syndrome (Miller et al., 2002). However the preponderance of data support that germ-line mutations in *CHK2* are unlikely to account for a significant proportion of non *BRCA1*-non *BRCA2*-associated hereditary breast cancers (Sullivan et al., 2002).

The association of *CHK2* alterations with LFS/LFL families and it's identification as a regulator of BRCA1 (Lee et al., 2000) and stabilizer of p53 (Chehad et al., 2000) makes *CHK2* a valid candidate gene to contribute to hereditary breast cancer. The phenotype of germline CHK2 mutations is virtually unknown, because the discovery is new. Further evaluation of hereditary breast cancer families may confirm the present suspicion that *CHK2* alterations do not alone predispose to cancer, but are contributory on a cancer presdisposing genetic background. Recent evaluation of the sporadic colon cancer cell line HCT15 containing the R145W *CHK2* missense alteration on the selectively expressed

allele provide evidence that CHK2 and p53 have cell cycle checkpoint roles in non-overlapping pathways (Falch et al., 2001). This theory lends support to the notion that mutations of *CHK2* can provide some additional selective advantage even to cells with deleted or mutant *TP53*.

BODY:

Progress Report

Objective: The objective of this proposal is to determine the frequency of germline mutations in CHK2/CDS1 in breast cancer-prone kindreds that have previously tested negative for mutations in *BRCA1* and *BRCA2*.

BRCA1 and *BRCA2* negative individuals selected for *CHK2* evaluation are members of hereditary breast/ovarian cancer families recruited through the Family Risk Assessment Program (FRAP) under the direction of Dr. Mary Daly. Li-Fraumeni syndrome (LFS) families and Li-Fraumeni like (LFL) families were chosen by pedigree analysis and according to the following criteria. Clinical criteria for diagnosing a family as having LFS are the combination of (i) proband with sarcoma diagnosed under age 45, (ii) first-degree relative with an LFS component tumor (sarcoma, breast cancer, brain tumor, leukemia, or adrenal cancer) diagnosed under age 45, and (iii) first- or second-degree relative with any cancer diagnosed under age 45 or with sarcoma diagnosed at any age. Clinical criteria for LFS-variant are an individual with three separate primary cancers, with the first cancer diagnosed under age 45, or the combination of (i) proband with childhood cancer or LFS component tumor diagnosed under age 45, (ii) first- or second-degree relative with LFS component tumor diagnosed at any age, and (iii) first- or second-degree relative with any cancer diagnosed under age 60 (Birch et al., 1994; Eng et al., 1997).

A total of 58 individuals were screened for alterations in the *CHK2* gene. One individual was from a LFS classified family while the remaining individuals were from LFL families that reported a history of breast cancer. All the individuals evaluated had been diagnosed with some type of cancer; 48 individuals were diagnosed with breast cancer and 4 were diagnosed with ovarian cancer. Two of the individuals were males; one diagnosed with melanoma at age 21 and another diagnosed with sarcoma at age 33. Eight of the individuals reported to be of Ashkenazi Jewish heritage and were screened for founder mutations only. All but one of the participants had previously tested negative for *BRCA1* germline mutations, and 19 had previously tested negative for *BRCA2* germline mutations.

Evaluation of the *CHK2* gene by PCT amplification and direct sequencing is complicated by the duplication of *CHK2* exons 10, 11, 12, 13, and 14 on multiple human chromosomes. A PCR strategy was designed (Bell et al., 1999) to specifically amplify these exons from chromosome 22 only, where the intact *CHK2* gene is located, by initially performing a primary long range PCR spanning exons 10-14 (~10kb) and subsequently performing nested PCR's for each of exons 10-14. Direct sequencing of the PCR fragements failed to detect any of the previously reported *CHK2* mutations or any other mutation in the individuals screened.

In addition, in collaboration with Dr. Daniel Haber (Massachusetts General Hospital), we have recently reported the identification of *CHK2* missense mutations in three variant-LFS families (Lee et al., 2001). Ten additional cases of LFS and 49 cases of variant-LFS were screened for germline mutations in *CHK2*. Three missense mutations were detected, R145W, R3W, and I157T. None of these missense changes were detected in 400 chromosomes from healthy donors who were ethnically matched with the patient population. The R145W mutation was shown to destabilize the encoded protein, reducing its half-life from >120 min to 30 min. We also report that this effect is abrogated by treatment of cells with a proteosome inhibitor, suggesting that $CHK2^{R145W}$ is targeted through the degradation pathway. The R145W germline mutation, but not the R3W or the I157T missense variants in *CHK2* was associated with loss of the wild-type allele in the corresponding tumor specimens. Interestingly, the R145W bearing tumor did not harbor a somatic *TP53* mutation. Our observations support the functional significance of a missense *CHK2* mutation in rare cases of LSF, and suggest that such mutations may substitute for inactivation of *TP53*.

Finally, while screening breast tumors for mutations in *CHK2*, Bell and colleagues identified several sequence variants not previously reported (Bell, personal communication). In order to determine the significance of these sequence variants we have established a collaboration with Drs. Bell and Haber to screen for these alteration in the germline of women affected with breast cancer. Through our FRAP repository at Fox Chase, we obtained DNA from 155 women affected with breast cancer that are *BRCA1* and/or BRCA2 mutation carriers. We also have obtained and screened 147 women with breast cancer without mutations in either gene; all of these women report a family history of breast and/or ovarian cancer. Results from our samples are being combined with Drs. Bell's and Haber's data for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified LFS/LFL individuals for *CHK2* gene evaluation.
- Found that 58 of the probands tested are negative for *CHK2* mutations.
- Have reported that the R145W *CHK2* mutation functionally destabilizes the encoded protein (in collaboration with D. Haber, see below).
- Have nearly completed the evaluation of 155 *BRCA1* and/or *BRCA2* mutation carriers affected with breast cancer for sequence variants in *CHK2*.
- Have evaluated 147 women affected with breast cancer who are negative for mutations in *BRCA1* and *BRCA2* and report a family history of breast and/or ovarian cancer for sequence variants in *CHK2*.

REPORTABLE ACCOMPLISHMENTS & BIBLIOGRAPHY OF PUBLICATIONS:

Lee, S.B., Kim, S.H., Bell, D.W., Wahrer, D.C.R., Schiripo, T.A., Jorczak, M.M., Sgroi, D., Garber, J.E., Li, F.P., Nichols, K., Varley, J.M., Godwin, A.K., Shannon, K.E., Harlow, E., Haber, D.A. Destabilzation of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome. Cancer Research, 61:8062-8067, 2001.

CONCLUSIONS:

Both the association of *CHK2* with LFS and the regulatory role of CHK2 in the control of *BRCA1* make *CHK2* a candidate gene where mutation may predispose to breast cancer. We have evaluated 38 individuals for alterations in the *CHK2* gene, but no alterations were found. We are able to offer several possible explanations for these results. While *CHK2* alterations are found in Li-Fraumeni families, only one of our tested individuals was a member of a LFS family by accepted criteria. All other families were Li-Fraumeni like (LFL) only and characteristic of our center's recruited population of high-risk breast and ovarian cancer kindreds. Additionally, nineteen of our tested individuals were not fully evaluated for alterations in the *BRCA2* gene; though unlikely due to cultural heritage and family history, it is possible that mutations in that gene may account for the breast cancer burden in those untested individuals. Eight individuals were of Ashkenazi Jewish heritage and negative for the three founder alterations in *BRCA1* and *BRCA2*. Other alterations (in any gene) have not been associated with breast cancer incidence among this cultural group. Based on a 4.2% frequency of germline *CHK2* mutations reported by Bell and colleagues (Bell et al., 1999), we would expect at least 1 affected family. However, the number of carriers found will be highly dependent on understanding *CHK2* phenotypes.

Our results do support, however, growing evidence that analysis of the germline of individuals with hereditary or early onset breast cancer reveal wild-type *CHK2* sequences. Consistent with recent studies, our data indicate that germline mutations in *CHK2* are unlikely to account for a significant proportion of non *BRCA1*- non *BRCA2*-associated hereditary breast cancers. More likely, CHK2 has tumor suppressor function in a small proportion of breast tumors (Li-Fraumeni associated tumors) and represents a low penetrant allele in the majority where alterations are found in individuals with a marked family history of breast and/or ovarian cancers.

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APPENDICES:

Lee, S.B., Kim, S.H., Bell, D.W., Wahrer, D.C.R., Schiripo, T.A., Jorczak, M.M., Sgroi, D., Garber, J.E., Li, F.P., Nichols, K., Varley, J.M., Godwin, A.K., Shannon, K.E., Harlow, E., Haber, D.A. Destabilzation of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome. Cancer Research, 61:8062-8067, 2001

[CANCER RESEARCH 61, 8062–8067, November 15, 2001]

Advances in Brief

Destabilization of CHK2 by a Missense Mutation Associated with Li-Fraumeni Syndrome[1]

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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 Ser^{20}. 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 proteosome inhibitor, suggesting that $CHK2^{R145W}$ is targeted through this degradation pathway. Both 1100delC and R145W germ-line 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

LFS[4] 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 germ-line 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 G_2-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 Ser^{216}, leading to its cytoplasmic sequestration with 14-3-3 proteins, preventing CDC25C dephosphorylation of CDC2 on Tyr^{15}, and resulting in a G_2-M arrest (9–11). In mammalian cells, CHK2 also mediates the phosphorylation of p53 on Ser^{20}, 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-proteosome 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). Where possible, EBV-immortalized lymphoblastoid cell lines were established

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

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, and the M13–28 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 chromosomal 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, R3W, 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 $MgCl_2$, 10 mM $MnCl_2$, 40 μM ATP, and 15 μCi [γ-^{32}P]ATP at 30°C. The half-life of wild-type and mutant *CHK2* 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 [^{35}S]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. 1*B*). This mutation had been detected previously in the sporadic colorectal cancer cell line HCT15 (6). A heterozygous Arg to Trp at codon 3 (R3W) 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 family (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, R3W, 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-

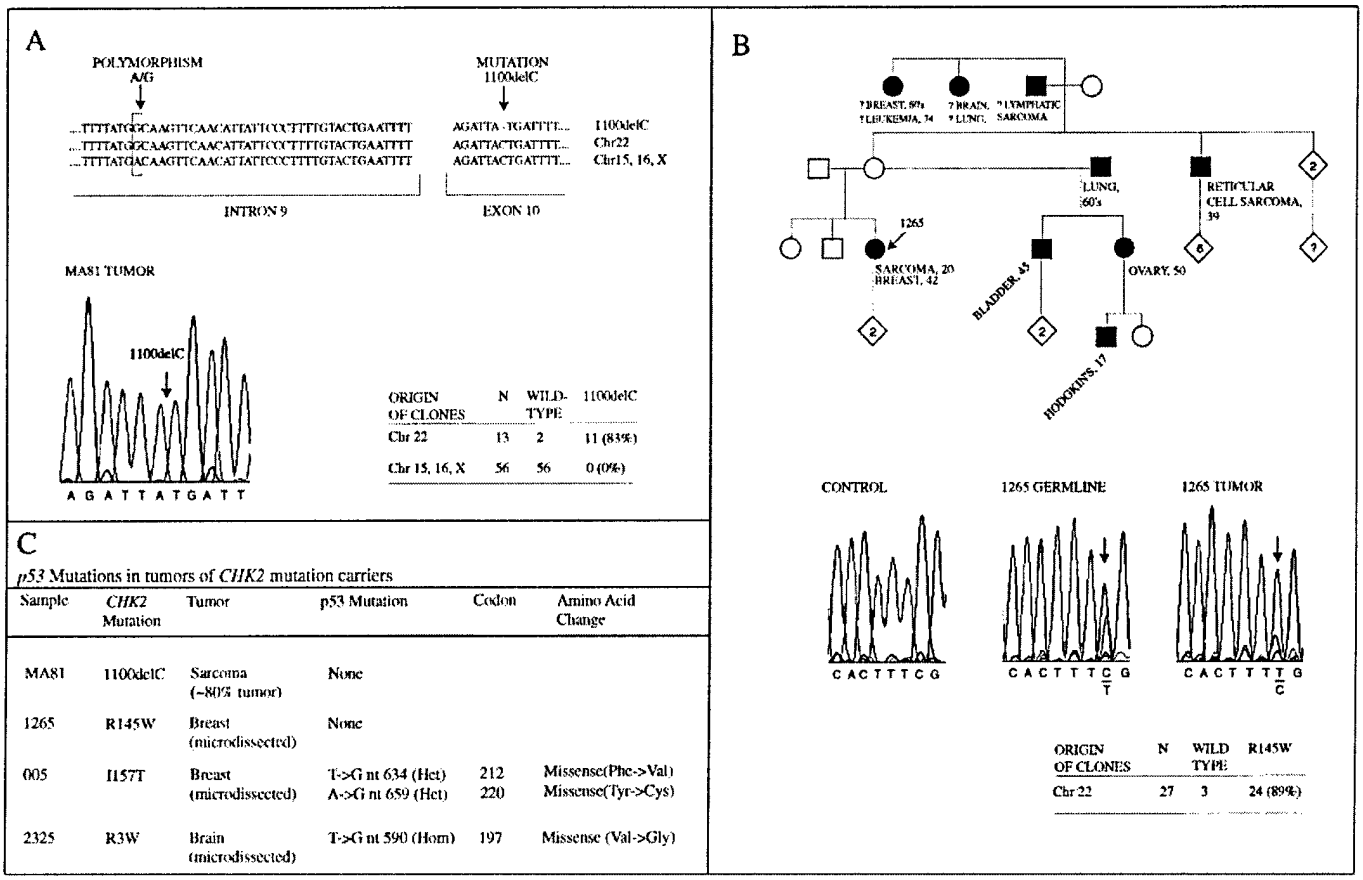

Fig. 1. CHK2 LOH and TP53 mutational analysis in primary tumors of CHK2 mutation carriers. *A*, somatic loss of the wild-type *CHK2* allele from the sarcoma of LFS patient MA81, harboring a heterozygous germ-line 1100delC mutation (pedigree and cosegregation of the mutation with cancer predisposition 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; heterozygous 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* codon 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.

ments of *CHK2*, using sequence variations that distinguish between these copies (17). For 1100delC, 11 of 13 (85%) clones derived from the chromosome 22 gene contained the mutant allele, indicating LOH (Fig. 1*A*). The R145W mutation occurs in a nonduplicated exon; sequencing analysis also demonstrated "reduction to homozygosity" for the mutant allele (24 of 27 clones with mutant sequence; Fig. 1*B*). In contrast, neither R3W nor I157T showed LOH in primary tumor specimens. Because our analysis investigated only those exons harboring identified *CHK2* mutations, it does not preclude the occurrence of an independent somatic point mutation within the second *CHK2* allele, rather than hemizygosity for the germ-line mutation. Nonetheless, 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 G_1 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. $CHK2^{R3W}$ and $CHK2^{I157T}$ demonstrated kinase activity comparable with that of the wild-type protein (Fig. 2*A*). As expected, no kinase activity was detected for $CHK2^{1100delC}$, consistent with disruption of the kinase domain by the premature termination codon, or for the synthetic mutant $CHK2^{D368N}$ with a disrupted catalytic domain. Surprisingly, transfection experiments demonstrated dramatically reduced expression of the $CHK2^{R145W}$ 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 $CHK2^{R145W}$ produced a protein of expected quantity and size by SDS-PAGE, compared with the wild-type product (Fig. 2*B*). However, transient transfection of expression constructs into either COS, U2OS, or 293 cells resulted in minimal steady-state expression of $CHK2^{R145W}$, as detected by Western blot analysis (Fig. 2*C* 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

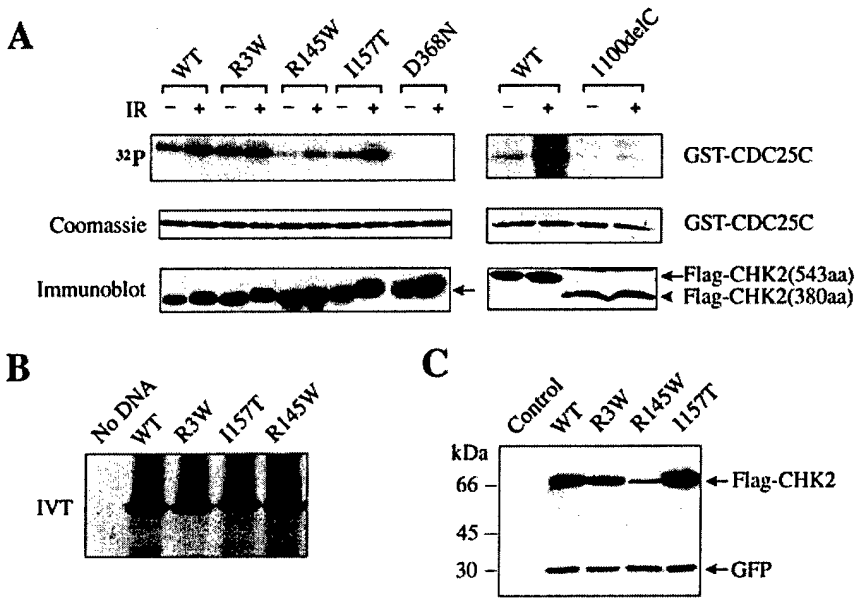

Fig. 2. Kinase activity and steady-state expression of CHK2 mutation products. *A, in vitro* kinase activity of CHK2 proteins immunoprecipitated using anti-Flag-antibodies from U2OS cells with tet-regulated gene expression, at baseline or 1 h after 10 Gy of ionizing radiation (*IR*). Comparable levels of inducible *CHK2* transcript was present in all cells, 20 h after tetracycline withdrawal. CHK2 kinase activity was measured by *in vitro* phosphorylation of GST-CDC25C substrate (total amount per reaction stained with Coomassie). *CHK2* constructs tested encode wild-type protein (*WT*), naturally occurring missense mutations R3W, R145W, and I157T, premature termination 1100delC, and a synthetic kinase-inactive mutant, D368N. Total levels of CHK2 in the immunoprecipitates were shown by immunoblotting with anti-Flag antibody. *Arrows,* full-length CHK2; *arrowheads,* truncated mutant form of CHK2. *B, in vitro* 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).

wild-type and mutant *CHK2* alleles. In these cells, no difference in mRNA half life was detected after rapid termination of CHK2 transcription by readdition of tet (Fig. 3*A*). Despite comparable expression of the inducible transcript, the CHK2^{R145W} 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 CHK2^{R3W} and CHK2^{I157T} (Fig. 3*B*). Remarkably, in multiple cell lines with inducible CHK2^{R145W}, this mutant protein had a half-life of only 30 min (Fig. 3*B*). This reduction in half-life for CHK2^{R145W} was confirmed using a second approach: inhibition of protein synthesis using CHX, followed by sequential measurement of steady-state protein levels by Western blotting. A $t_{1/2}$ of 30 min was again calculated for CHK2^{R145W}, compared with >6 h for the wild-type protein (Fig. 3*C*). To determine the mechanism underlying the rapid degradation of CHK2^{R145W}, we treated cells expressing inducible constructs with the proteosome 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 CHK2^{R145W} (Fig. 3*C*).

Wild-type *TP53* in Primary Tumors with Homozygous CHK2 Mutations. Functional analysis, together with the observation of LOH in primary tumors, suggested that *CHK2^{1100delC}* and *CHK2^{R145W}* are likely to encode inactive proteins, whereas *CHK2^{R3W}* and *CHK2^{I157T}* are missense alleles of unknown significance. To obtain further genetic evidence indicative of functional significance, we searched for somatic mutations in *TP53* in primary tumor specimens associated with these germ-line mutations. Given the ability of CHK2 to phosphorylate p53 on Ser^{20} (12, 13) and the failure of p53 stabilization after γ-irradiation of *Chk2*-null thymocytes (14), we reasoned that inactivation of these two proteins might be functionally redundant in cancer cells. Indeed, we did not identify mutations in

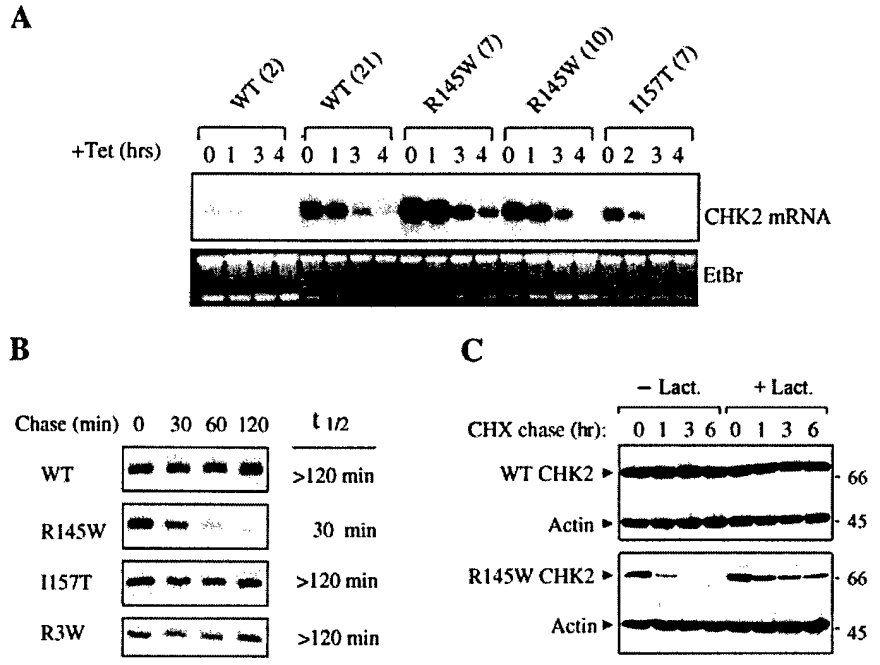

Fig. 3. Instability of CHK2 protein encoding R145W mutation. *A,* comparable stability of variant *CHK2* transcripts. *CHK2* expression was induced for 20 h in multiple independent clones of U2OS cells, with regulated expression of wild-type transcript (*WT*; clones 2 and 21), or missense mutations R145W (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 CHK2^{R145W}, 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 CHK2^{R145W} degradation by the proteosome inhibitor lactacystin. U2OS were induced to express either wild-type CHK2 or the R145W mutation for 20 h and then treated with CHX to block new protein synthesis, followed by immunoblotting analysis at sequential intervals to measure steady-state CHK2 levels using anti-Flag antibody (*Actin* control). Duplicate experiments were performed in the presence of lactacystin (+*Lact*) to inhibit proteosome degradation pathways.

TP53 in the primary sarcoma specimen homozygous for the 1100delC *CHK2* mutation or in the primary breast tumor homozygous for the R145W mutation (Fig. 1*C*). In contrast, the breast tumor harboring a heterozygous I157T *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 mutation Y220C (Ref. 19; Fig. 1*C*). The breast tumor that was heterozygous for the R3W *CHK2* mutation was homozygous for a mutant *TP53* allele, V197G (Fig. 1*C*), which has been reported in both sporadic cancers and LFS (20). Thus, the two tumors arising in patients with bona fide *CHK2* mutations contained wild-type *TP53* alleles, whereas those derived from patients with missense mutations of uncertain significance demonstrated homozygous inactivation of p53.

Homozygous Inactivation of CHK2 in the HCT15 Sporadic Colon Carcinoma Cell Line. We previously screened 49 cancer-derived cell lines for mutations in *CHK2*, using denaturing high-performance liquid chromatography (21) to detect sequence mismatches followed by nucleotide sequencing, leading to the identification of a heterozygous R145W mutation 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. 4*A*). 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 $CHK2^{A247D}$, which was transiently transfected into COS and U2OS cells (Fig. 4*C*; data not shown). Similar to $CHK2^{R145W}$, steady-state expression of transfected $CHK2^{A247D}$ was minimally detectable by Western blotting (Fig. 4*C*). Thus, the presence of biallelic point mutations leading to grossly unstable proteins explains the absence of detectable endogenous CHK2 in HCT15 cells (23).

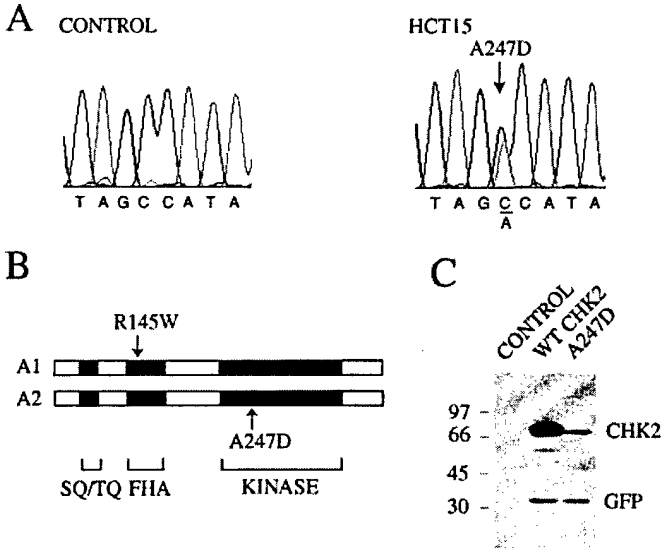

Fig. 4. Biallelic inactivation of endogenous CHK2 in HCT15 cells. *A*, heterozygous C to A missense mutation at nucleotide 740, resulting in substitution of aspartic acid for alanine at codon 247 (A247D) in the *CHK2* transcript from the sporadic colon cancer cell line HCT15. The alanine at codon 247 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*, $CHK2^{A247D}$ 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.

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 Ser^{20} 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 $CHK2^{R145W}$ and $CHK2^{A247D}$ raises the possibility that both mutations may be associated with significant disruption of protein folding. $CHK2^{R145W}$, 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). Cocrystalization of this domain with an optimal phosphothreonine-containing peptide revealed 11 anti-parallel β-sheet strands with three loops generated by six β-sheet structures that forms the phosphothreonine peptide binding surface. Remarkably, only 6 of the 11 β-strands are conserved with those of other FHA domains. The mutated Arg residue at codon 145 lies in the middle of the third β-strand, a sequence that is not conserved, but 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

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 $CHK2^{R145W}$ also has implications for the interpretation of functional results reported with this mutant protein. Using a stably transfected cell line with detectable expression of $CHK2^{R145W}$, Wu *et al.* (23) demonstrated that this protein had decreased *in vitro* kinase activity, reduced phosphorylation by ATM kinase, and migrates within a larger protein complex than native CHK2 (23). Our finding that $CHK2^{R145W}$ 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 $CHK2^{R145W}$ 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, rather than 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 $CHK2^{I157T}$, along with $CHK2^{R145W}$, 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 Finnish founder mutation with potentially compromised CHK2 function remains to be determined. In summary, the germ-line and somatic inactivation of *CHK2* points to a rare genetic mechanism for disruption of a cell cycle checkpoint important in tumor suppression. Functional assays will be essential to determine whether this effect is entirely derived from the role of CHK2 in the phosphorylation of p53, and whether CHK2-mediated phosphorylation of other targets may be differentially modulated by its sequence variants.

Acknowledgments

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