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p53-Independent Regulation of p21$^{Waf1/Cip1}$ Expression and Senescence by Chk2

Cécile-Marie Aliouat-Denis,[1,2] Najoua Dendouga,[1] Ilse Van den Wyngaert,[2,3] Hinrich Goehlmann,[2,3] Ulf Steller,[1,2] Inez van de Weyer,[2,3] Nele Van Slycken,[1] Luc Andries,[4] Stefan Kass,[2,3] Walter Luyten,[2] Michel Janicot,[1] and Jorge E. Vialard[1,2]

[1]Oncology Discovery Research and Early Development, [2]Advanced BioTechnologies, and [3]Genomic Technologies, Johnson & Johnson Pharmaceutical Research and Early Development, Beerse, Belgium and [4]Histogenex, Edegem, Belgium

Abstract

The Chk2 kinase is a tumor suppressor and key component of the DNA damage checkpoint response that encompasses cell cycle arrest, apoptosis, and DNA repair. It has also been shown to have a role in replicative senescence resulting from dysfunctional telomeres. Some of these functions are at least partially exerted through activation of the p53 transcription factor. High-level expression of virally transduced Chk2 in A549 human lung carcinoma cells led to arrested proliferation, apoptosis, and senescence. These were accompanied by various molecular events, including p21$^{Waf1/Cip1}$ (p21) transcriptional induction, consistent with p53 activation. However, Chk2-dependent senescence and p21 transcriptional induction also occurred in p53-defective SK-BR-3 (breast carcinoma) and HaCaT (immortalized keratinocyte) cells. Small interfering RNA–mediated knockdown of p21 in p53-defective cells expressing Chk2 resulted in a decrease in senescent cells. These results revealed a p53-independent role for Chk2 in p21 induction and senescence that may contribute to tumor suppression and genotoxic treatment outcome.
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Introduction

The Chk2 kinase has a central role in the checkpoint response to DNA damage that leads to cell cycle arrest and apoptosis (1, 2). These components of the checkpoint response are believed to contribute to tumor suppression by curtailing inappropriate proliferation of potentially tumorigenic cells containing damaged DNA. They also contribute to treatment outcome of various genotoxic cancer therapies. Additionally, Chk2 has been described recently to be involved in replicative senescence (3, 4), a cellular process characterized by dysfunctional telomeres and associated with cellular aging that may also contribute to tumor suppression.

Senescence can be triggered by various stress signals, including DNA damage (5), through a mechanism that has been called stress or aberrant signaling-induced senescence. Although it resembles replicative senescence in many ways—they share many components in their signaling pathways and display similar cellular phenotypes—stress or aberrant signaling-induced senescence is not associated with cell replication counting or dysfunctional telomeres. At the molecular level, DNA damage–induced senescence in human cells is characterized by p53 activation and subsequent accumulation of the cyclin-dependent kinase inhibitor, p21$^{Waf1/Cip1}$ (p21), which leads to growth arrest. It shifts the balance of p53 response outcome from cell death through apoptosis or mitotic catastrophe when absent to cell cycle arrest and senescence when present (6). Although DNA damage–induced senescence can be activated in tumor cells through the p53/p21 pathway, it can also occur through alternative mechanisms (5, 7). Thus, p53 and p21 are positive but not essential regulators of replicative and stress-induced senescence (8).

Chk2 is a central component of the signal transduction pathway that is activated by DNA damage and dysfunctional telomeres resulting in apoptosis or cell cycle arrest and senescence. Signals emanating from blocked replication or DNA damage trigger the activation of the proximal checkpoint kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). When activated, these kinases phosphorylate a wide spectrum of proteins that includes Chk2 (2, 9, 10). Chk2 phosphorylation by ATM at T68 leads to its oligomerization and is followed by an autophosphorylation cascade leading to its full activation (1, 2). Activated Chk2 propagates the DNA damage signal to several effector proteins involved in cell cycle arrest, apoptosis, and DNA repair (1, 2, 10). Similarly, signals emanating from dysfunctional telomere ends result in activation of the checkpoint response, including Chk2 phosphorylation and activation, which leads to a permanent arrest in cell cycling called replicative senescence (3, 4).

Chk2-mediated apoptosis and sustained cell cycle arrest are at least partially transduced through the p53 tumor suppressor

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Note: C-M. Aliouat-Denis and N. Dendouga contributed equally to this work.
C-M. Aliouat-Denis is currently at Faculté de Pharmacie, Université de Lille 2, rue du Pr. Laguesse 3, 59 006 Lille, France. U. Steller is currently at PicoRapid Technologie GmbH, Fahrenheitstrasse 1, D-28359 Bremen, Germany. W. Luyten is currently at IriDM, Naamsestraat 59, B-3000 Leuven, Belgium.
Requests for reprints: Jorge E. Vialard, Oncology Discovery Research and Early Development, Johnson & Johnson Pharmaceutical Research and Early Development, Turnhoutseweg 30, B-2340 Beerse, Belgium. Phone: 32-14-60-3971; Fax: 32-14-60-5403. E-mail: jvialard@prdbe.jnj.com

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(1, 10). Chk2-dependent p53 phosphorylation causes its stabilization and activation, which in turn results in transcriptional modulation of various genes involved in cell cycle regulation and apoptosis, including p21 (11-13). The p21 protein is not required for the immediate and transient p53-independent cell cycle arrests following checkpoint activation. However, its accumulation contributes to maintenance of the arrests at G_1-S and G_2-M through inhibition of the cyclin-dependent kinases that regulate these cell cycle transitions (11, 12).

In the present study, we report that retrovirus-mediated expression of the Chk2 kinase in various cancer cells results in a cellular phenotype consistent with checkpoint activation leading to cell cycle arrest, apoptosis, and senescence. Chk2-dependent senescence occurred in p53-defective tumor cells and was dependent on p21 up-regulation, thus revealing a novel p53-independent mechanism leading to p21 accumulation and senescence.

Results

Chk2 Expression Inhibits Cell Proliferation

To examine Chk2-mediated functions, we used retroviral transduction to introduce recombinant Chk2 into modified A549 lung tumor cells that contained the tetracycline transactivator, thus allowing regulation of its expression (Tet-Off system). To facilitate identification and visualization of cells expressing recombinant Chk2, it was either coexpressed with green fluorescent protein (GFP) from a bicistronic mRNA or as a fusion with GFP (GFP-Chk2). Cells containing wild-type recombinant Chk2 ($Chk2^{WT}$ or $GFP\text{-}Chk2^{WT}$) were readily detectable within 24 or 48 hours of transduction (Fig. 1A). Strikingly, even after several days of expression, cells containing $Chk2^{WT}$ or $GFP\text{-}Chk2^{WT}$ were present primarily as single cells and sometimes doublets in the midst of normally proliferating uninfected cells (Fig. 1A), suggesting that their proliferation had been compromised. This antiproliferative effect was dependent on Chk2 kinase activity because cells expressing a kinase-defective form of Chk2 ($Chk2^{KD}$; refs. 14-16) continued dividing and formed colonies readily (Fig. 1A). $GFP\text{-}Chk2^{WT}$ and $GFP\text{-}Chk2^{KD}$ were exclusively localized in the nucleus (Fig. 1A), mirroring the subcellular distribution of endogenous Chk2 in most proliferating cell types (17, 18). Because cells expressing the $GFP\text{-}Chk2^{WT}$ fusion were indistinguishable from those expressing $Chk2^{WT}$ and GFP separately in all aspects examined, subsequent analyses were carried out using the $GFP\text{-}Chk2^{WT}$ fusion that exhibited higher fluorescence, thus facilitating identification of cells expressing recombinant Chk2.

The inhibitory effect of wild-type Chk2 expression on proliferation was confirmed in several independent cell lines established from single-cell clones as well as enriched cell populations. Growth rates of cultures expressing $GFP\text{-}Chk2^{KD}$ were indistinguishable from the parental cell line, whereas $GFP\text{-}Chk2^{WT}$ expression severely inhibited growth (Fig. 1B). This was confirmed by the inability of these cells to incorporate bromodeoxyuridine (data not shown).

Western blot analysis confirmed high-level expression of recombinant Chk2 in these cells (Fig. 1C). Additionally,

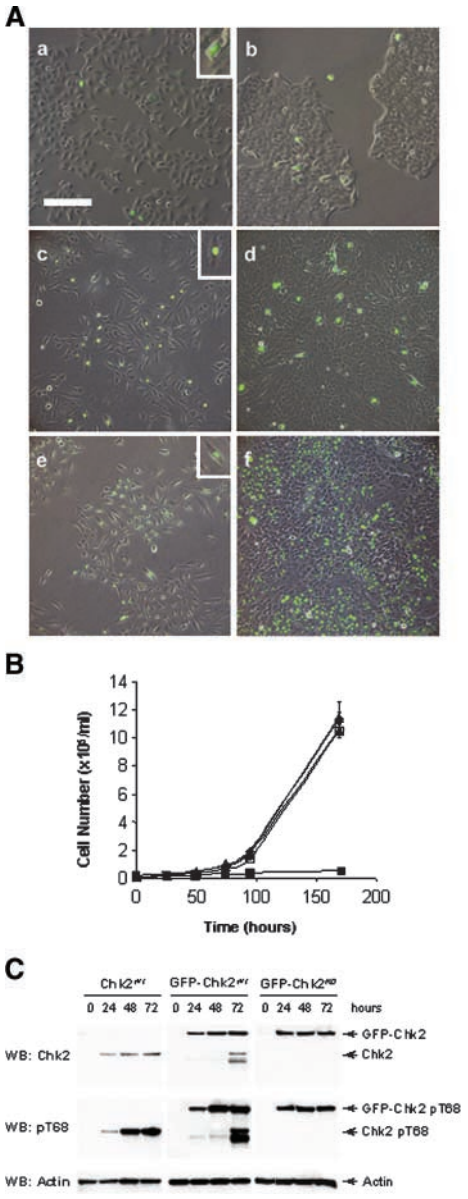

FIGURE 1. Antiproliferative effect of recombinant Chk2 expression in A549 cells. **A.** Overlays of bright-field and fluorescence microscopy images of A549 cells containing recombinant $Chk2^{WT}$ (*a* and *b*), $GFP\text{-}Chk2^{WT}$ (*c* and *d*), and $GFP\text{-}Chk2^{KD}$ (*e* and *f*) after 48 (*a*), 24 (*c* and *e*), and 120 (*b, d*, and *f*) hours of expression. Cells were transduced at a low multiplicity of infection (<1). Insets, single cells at higher magnification show nuclear localization of $GFP\text{-}Chk2^{WT}$ (*c*) and $GFP\text{-}Chk2^{KD}$ (*e*). Fluorescence in cells expressing GFP and $Chk2^{WT}$ independently (*a, inset*) is not constrained to the nucleus. Bar, 100 μm. **B.** Growth curves of A549 cells expressing recombinant Chk2. ♦, parental cells; □ and ■, clonal cell line containing $GFP\text{-}Chk2^{WT}$ under repressed and induced conditions, respectively; △ and ▲, enriched mixed population of cells containing $GFP\text{-}Chk2^{KD}$ under repressed and induced conditions, respectively. Equal numbers of cells were plated in the absence (inducing conditions) or presence (repressing conditions) of 100 ng/mL doxycycline and subsequently collected and counted at various time points. Points, mean of triplicate wells of a representative experiment; bars, SD. **C.** Western blot analysis of Chk2 protein in A549 cells at various times following induction of expression. Whole-cell lysates of a representative clone expressing $GFP\text{-}Chk2^{WT}$ and enriched mixed cell populations expressing $Chk2^{WT}$ or $GFP\text{-}Chk2^{KD}$ were probed with an antibody against Chk2 (*top*) or phosphorylated T68 Chk2 (*bottom*). Arrows, bands corresponding to Chk2 expressed separately (Chk2) or as a fusion with GFP (GFP-Chk2). Equal protein amounts of each lysate were loaded and probed.

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recombinant Chk2 was phosphorylated at T68, indicating that it was in the activated state possibly as a result of the protein reaching a concentration threshold that initiates the activation cascade (19, 20). T68 phosphorylation was presumably carried out by ATM and/or other kinases that may have included endogenous Chk2 and, when present, wild-type recombinant Chk2. Several smaller proteins sometimes detected in cells expressing GFP-Chk2^{WT} and GFP-Chk2^{KD} (Fig. 1C; data not shown) may represent truncated forms of the protein and/or endogenous Chk2.

Chk2-Dependent Cell Cycle Arrest, Apoptosis, and Senescence

To determine whether the antiproliferative effect of GFP-Chk2^{WT} expression in A549 cells was analogous to its activation in response to DNA damage or telomere dysfunction, we examined these cells with respect to various Chk2-mediated functions, including cell cycle arrest, apoptosis, and senescence. GFP-Chk2^{WT} expression resulted in accumulation of cells in G_1-G_0 and G_2-M phases of the cell cycle and a dramatic decrease in S-phase cells (Fig. 2A), consistent with the cell cycle arrests at the G_1-S and G_2-M transitions associated with the Chk2-dependent checkpoint response. The cell cycle distribution of cells expressing the GFP-Chk2^{KD} mutant was similar to that of the parental cell line and did not vary with time (data not shown).

To determine the effect of GFP-Chk2^{WT} expression on apoptosis, we employed *in situ* caspase-3 cleavage and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling. In the absence of GFP-Chk2^{WT} expression, <1% of A549 cells was positive in these apoptosis assays (Fig. 2B). After 7 days of GFP-Chk2^{WT} expression, the proportion of apoptotic cells increased to 8% as determined by caspase-3 cleavage and 21% by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. The difference in these numbers may reflect differing assay sensitivities, timing of these apoptosis related events, or an increase in necrotic death detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. Nevertheless, the increase in apoptotic cells reinforced the notion that recombinant Chk2 expression mimicked endogenous Chk2 activation in the response to DNA damage. Although apoptosis increased significantly in A549 cells expressing GFP-Chk2^{WT}, most of the cells acquired characteristics associated with senescence: they became enlarged, flattened, and remained arrested over long periods of time. We tested for senescence-associated β-galactosidase (SA-β-gal) activity at pH 6.0, an established marker for senescence (21), and found that the majority of cells expressing GFP-Chk2^{WT} were positive in this assay (Fig. 2C).

Analysis of various downstream effectors of Chk2 function also mirrored events associated with the response to DNA damage or telomere dysfunction, including activation of the p53-dependent transcriptional response. Although we did not detect a significant increase in p53 protein in whole-cell lysates from cells expressing GFP-Chk2^{WT} (data not shown), an increase in nuclear p53 protein, a subcellular localization that is necessary for its activity as a transcriptional regulator (22), was detectable within 24 hours of expression and was maintained for at least 96 hours (Fig. 2D). We also observed several additional events associated with p53 activation, such as transient accumulation of p21 protein and decreased levels of the checkpoint kinase, Chk1 (23, 24), and the antiapoptotic protein, survivin (25-27). Western blot analysis also revealed a substantial decrease in S780-phosphorylated Rb protein at 24 and 48 hours (Fig. 2D), consistent with p21-dependent cyclin-dependent kinase inhibition in cells undergoing senescence (8). These results show that Chk2 activation through overexpression mimics induction of the checkpoint response to DNA damage, including activation of the p53 pathway.

Chk2-Mediated Senescence in p53-Defective Cells

The antiproliferative effect of Chk2^{WT} (but not Chk2^{KD}) expression was not limited to A549 lung tumor cells but also occurred in cancer cell lines derived from cervix (HeLa and C33A), breast (MCF-7 and SK-BR-3), and colon (HCT-116) cancers as well as the spontaneously immortalized keratinocyte cell line, HaCaT (Fig. 3A; data not shown). Interestingly, the sustained antiproliferative effect was not dependent on p53 as some of the cell lines tested (HeLa, SK-BR-3, and HaCaT) lack functional p53. This prompted us to further examine the role of p53 in the relevant aspects of the Chk2-mediated response. Subsequent studies were carried out using SK-BR-3 and HaCaT cells, both of which contain mutant copies of p53 that are deficient in transcription factor activity (28, 29).

GFP-Chk2^{WT} expression in SK-BR-3 and HaCaT cells resulted in decreased proliferation (Fig. 3A) characterized by a decrease in S-phase cells (Fig. 3B). In SK-BR-3 cells, this was accompanied by an increase in cells in G_1-G_0 and G_2-M, similar to that seen in A549 cells. HaCaT cells did not accumulate in G_1-G_0 but exhibited a substantial increase in cells in G_2-M. In both of these cell lines, we also detected cells with a DNA content greater than 4N, suggestive of ongoing replication in the absence of cell division (Fig. 3B). Furthermore, GFP-Chk2^{WT} expression in both of these p53-defective cell lines resulted in sustained cell cycle arrest, characterized by the inability to incorporate bromodeoxyuridine (data not shown) and appearance of senescence-associated features (cell enlargement and flattening and SA-β-gal staining) in the majority of cells (Fig. 3C). Although senescence is generally associated with arrest in G_1, some of the SA-β-gal-positive cells in our study were arrested with a DNA content of at least 4N. We cannot exclude that these cells differ in other ways from senescent cells arrested in G_1.

Because sustained cell cycle arrest and senescence are believed to be mediated through p53-dependent up-regulation of p21, we examined p21 protein levels in these cells. Western blot analysis confirmed the presence of activated (T68-phosphorylated) GFP-Chk2^{WT} in both SK-BR-3 and HaCaT cells (Fig. 4A). Interestingly, p21 protein levels increased dramatically in both cell lines. This was accompanied by decreased S780 phosphorylated Rb consistent with p21-mediated cyclin-dependent kinase inhibition (Fig. 4A). Because modulation of *p21* and various other genes in response to DNA damage is primarily a consequence of p53-dependent transcriptional regulation, we determined mRNA levels of several p53-responsive genes using quantification real-time reverse transcription-PCR. This revealed the expected pattern of p53-dependent modulation

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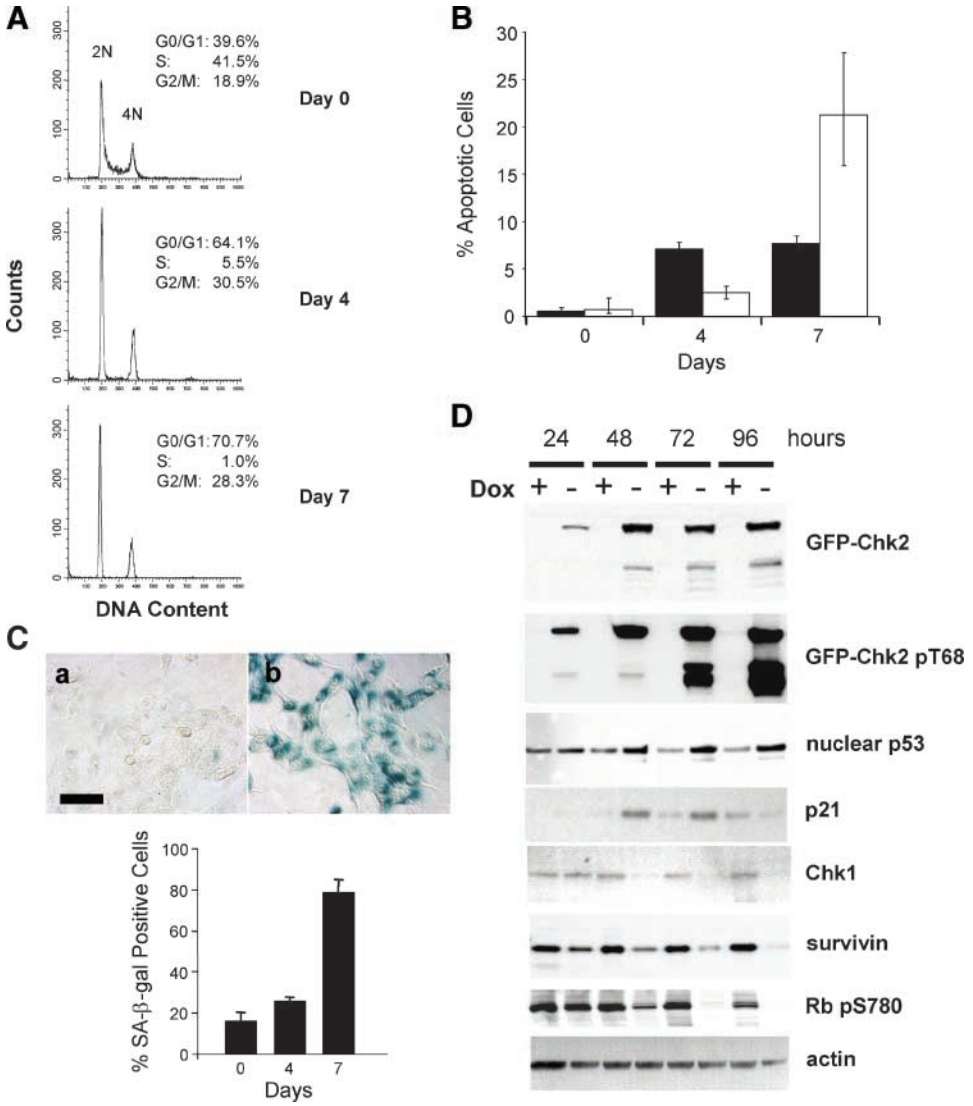

FIGURE 2. Checkpoint response in A549 cells expressing recombinant Chk2. **A.** Cell cycle profile of A549 cells expressing GFP-Chk2^{WT} (representative single-cell clone). Nuclei isolated from cells at various times following induction of GFP-Chk2^{WT} expression were stained with propidium iodide and analyzed by flow cytometry. Percentage of cells in the G_0-G_1, S, and G_2-M phases of the cell cycle (10,000 events were acquired per sample). Debris and aggregates were gated out. **B.** Apoptosis in A549 cells expressing GFP-Chk2^{WT} at various times following induction of expression. Apoptotic cells were detected by cleaved caspase-3 immunostaining (*filled columns*) or using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (*open columns*) as described in Materials and Methods. Approximately 400 cells were counted for each sample in two independent experiments using a representative single-cell clone. Bars, SD. **C.** Top, SA-β-gal staining in A549 cells harboring GFP-Chk2^{WT} (representative clone) before (*a*) and after (*b*) 7 days of expression. Bar, 20 μm. Bottom, percentage of senescent cells (SA-β-gal positive) at various times following induction of GFP-Chk2^{WT} expression. At least 1,000 cells were examined per time point in three independent experiments. Bars, SD. **D.** Western blot analysis of proteins from whole-cell lysates of A549 cells containing GFP-Chk2^{WT} at various times of expression. GFP-Chk2^{WT} expression was repressed in the presence of doxycycline (*Dox*+) and induced in its absence (*Dox*-). Right, antibodies directed against the various proteins. Loading of equal amounts of protein was confirmed by probing for actin. For p53, nuclear proteins were obtained from nuclei isolated as described in Materials and Methods.

in p53-proficient A549 cells expressing GFP-Chk2^{WT}: up-regulation of p21 and down-regulation of *Chk1, survivin, Cdc2, Cdc25C,* and *topoisomerase IIα* (Fig. 4B). In contrast, the transcriptional response of most of these genes was severely reduced or absent in the p53-defective SK-BR-3 and HaCaT cells (Fig. 4B). The notable exception was *p21,* which exhibited a substantial increase in expression in these cells, thus showing that Chk2 can positively modulate *p21* transcription selectively in the absence of p53 transactivation.

Chk2-Dependent Senescence in p53-Defective Cells Requires p21

To determine whether p53-independent p21 up-regulation contributes to Chk2-dependent senescence, we used small interfering RNA (siRNA) to knockdown p21 protein levels in p53-defective HaCaT cells expressing Chk2. GFP-Chk2^{WT} expression was induced, and 24 hours later, these cells were transfected with siRNA. The Chk2-dependent increase in p21 protein levels was not affected in cells transfected with nonspecific (control) siRNA. However, within 24 hours of

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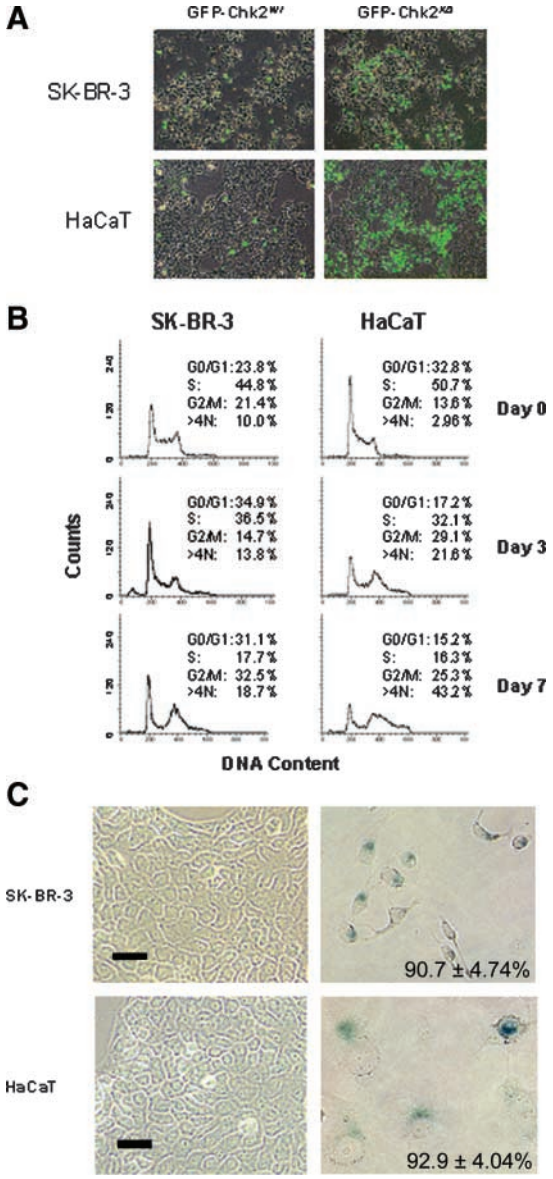

FIGURE 3. Chk2-mediated cell cycle arrest and premature senescence in p53-defective cell lines. **A.** Overlays of fluorescence and bright-field microscopy images of SK-BR-3 and HaCaT cells transduced at a low multiplicity of infection (<1) with retroviral constructs containing GFP-Chk2^{WT} and GFP-Chk2^{KD}. Cells were analyzed after 5 days of expression. **B.** Cell cycle profiles of enriched mixed cell populations of SK-BR-3 (*left*) and HaCaT (*right*) cells expressing GFP-Chk2^{WT}. Nuclei isolated from cells at various times following induction of GFP-Chk2^{WT} expression were stained with propidium iodide and analyzed by flow cytometry. Percentages of cells in the G_0-G_1, S, and G_2-M phases of the cell cycle are indicated as are the percentages of cells containing >4N DNA content (10,000 events were acquired per sample). Debris and aggregates were gated out. **C.** SA-β-gal activity (pH 6.0) in SK-BR-3 and HaCaT cells before (*left*) and after (*right*) 7 days of GFP-Chk2^{WT} expression. Bar, 10 μm. Percentage ± SE of SA-β-gal-positive cells from a representative experiment.

transfection with p21-specific siRNA, p21 protein levels were reduced to nearly undetectable levels, which were maintained for the duration of the experiment (Fig. 5A). Knockdown of p21 resulted in both a decrease in the number of remaining GFP-Chk2^{WT} cells 6 days after transfection, as a result of increased cell death at earlier time points (data not shown), and a decrease in the percentage of remaining SA-β-gal-positive cells (Fig. 5B). The combination of these effects led to a drastic reduction in the number of senescent cells at the end of the experiment. These results confirmed

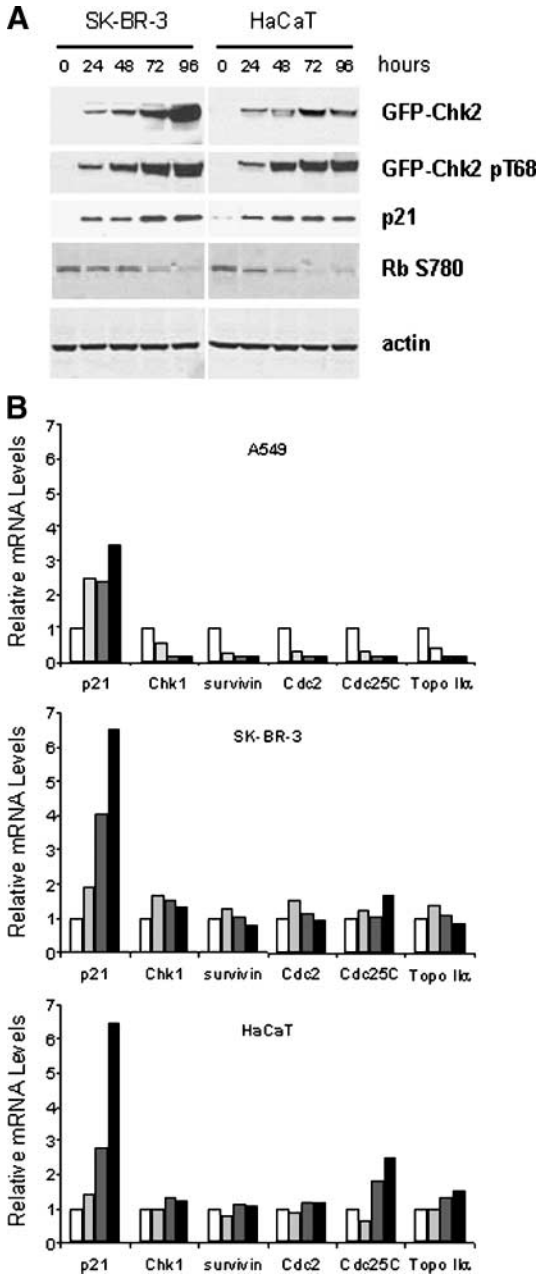

FIGURE 4. p21 modulation in p53-defective cells expressing recombinant Chk2. **A.** Western blot analysis of whole-cell lysates from SK-BR-3 and HaCaT cells collected at various times following induction of GFP-Chk2^{WT} expression. Loading of equal amounts of protein was confirmed by probing for actin. **B.** Analysis of mRNA levels of various genes from A549, SK-BR-3, and HaCaT cells expressing GFP-Chk2^{WT}. Quantification real-time reverse transcription-PCR was done on cDNA generated from total RNA at various times after GFP-Chk2^{WT} expression (0, 1, 2, and 3 days). For each gene, mRNA levels were normalized to the levels present at day 0 (equal to 1).

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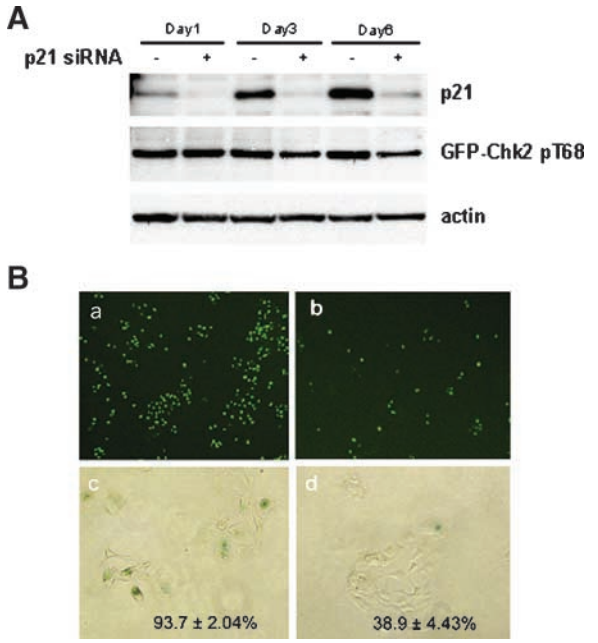

FIGURE 5. Effect of p21 knockdown on Chk2-mediated senescence in p53-deficient HaCaT cells. **A.** Western blot analysis of whole-cell lysates from HaCaT cells expressing GFP-Chk2^{WT} at various times after transfection with p21 (+) and nonspecific (-) siRNAs. Right, antibodies directed against the various proteins. Loading of equal amounts of protein was confirmed by probing for actin. **B.** Fluorescence (*a* and *b*; 25-fold magnification) and SA-β-gal staining (*c* and *d*; 100-fold magnification) in HaCaT cells expressing GFP-Chk2^{WT} 6 days after transfection of control nonspecific (*a* and *c*) and p21-specific (*b* and *d*) siRNA. Percentage ± SE of SA-β-gal-positive cells from a representative experiment.

that Chk2-induced senescence in p53-defective HaCaT cells was dependent on Chk2-mediated p21 up-regulation.

Discussion

Retrovirus-mediated Chk2 expression in several human cell lines resulted in cell cycle arrest, apoptosis, and senescence accompanied by molecular events associated with DNA damage checkpoint activation. This included activation of the p53 pathway as reflected by nuclear p53 accumulation and regulation of various p53-responsive genes involved in cell cycle control (*p21, Chk1, Cdc2*, and *Cdc25C*), apoptosis (*survivin*), and DNA metabolism (*topoisomerase IIα*) in cells containing wild-type p53. Considering the role of Chk2 in DNA damage–dependent p53 stabilization and attenuation of damage-induced p21 transcription observed in Chk2$^{-/-}$ cells (30), it was somewhat surprising to see a robust Chk2-dependent increase in p21 transcription and protein levels in p53-defective cells. However, although *p21* is a principal p53 target gene and a central component in a variety of p53-mediated stress responses, its expression can be positively regulated through several p53-independent mechanisms (31). It will be of interest to determine whether Chk2 regulates p21 expression through one of these or a novel p53-independent mechanism.

Although Chk2-dependent phosphorylation of various proteins, such as Cdc25A, Cdc25C, BRCA1, E2F-1, and PML contributes to several aspects of the checkpoint response (1, 10), sustained cell cycle arrest and senescence resulting from telomere dysfunction have been described to rely on p53 activation and subsequent p21 accumulation (3, 4). While this article was in preparation, another study reported that induction of Chk2 expression in p53-deficient DLD1 cells resulted in apoptosis and senescence (32). Although increased p21 protein levels were also detected in these cells, the regulatory mechanism by which this occurred and its effect on senescence were not determined. Our results show that activated Chk2 can induce p21 transcription in the absence of functional p53 and that this contributes to Chk2-mediated senescence, thus revealing a novel p53-independent mechanism that may contribute to the DNA damage checkpoint response.

Senescence has been suggested to play a role in tumor suppression by reducing the replicative potential of cells (7, 8, 33). It may also influence cancer treatment outcome, as disruption of the senescence response in lymphoma-bearing mice resulted in a poorer prognosis than disruption of apoptosis (34), thus indicating a positive role in this setting and suggesting that these responses are not redundant. In addition, the detection of senescent cells in tumors obtained from patients that had undergone genotoxic chemotherapy underscores the relevance of senescence to treatment outcome (35). However, its overall contribution to cancer and treatment outcome remains to be determined. The ability of Chk2 to trigger senescence in the absence of functional p53 suggests that it may contribute to this response in the majority of tumor cells.

Materials and Methods

Cell Culture

Human A549 cells (lung carcinoma) were maintained in F12-K Kaighn's modification medium, SK-BR-3 cells (breast carcinoma) were maintained in MEM, and HaCaT cells (immortalized human keratinocyte) were maintained in DMEM. Media (Life Technologies, Paisley, United Kingdom) were supplemented with 10% Tet System Approved fetal bovine serum (Clontech, BD Biosciences, Palo Alto, CA).

Retroviral Transduction, Cell Sorting, and Single-Cell Cloning

Wild-type (Chk2^{WT}) and kinase-defective (D347A) Chk2 mutant (Chk2^{KD}) cDNAs were introduced into the previously described TRA vectors containing the tetracycline-responsive promoter element (36). These vectors allow coexpression of Chk2 and GFP as a result of the presence of an internal ribosome entry site between the two open reading frames. Recombinant Chk2 was also introduced into a modified version of these vectors, which allows expression as a fusion with GFP. Retroviruses containing Chk2 were produced as described previously (37) and used to transduce A549, SK-BR-3, and HaCaT cells that had been modified previously by retroviral transduction to contain the tetracycline-controlled transactivator.

Mixed populations enriched for cells expressing Chk2^{KD} were obtained by fluorescence-activated cell sorting of cells grown in the absence of doxycycline (Sigma-Aldrich, Steinheim, Germany) resulting in GFP (and Chk2^{KD}) expression.

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Chk2^{WT}-enriched populations were obtained by sorting cells that had been grown under conditions that allowed fluorescence detection but did not result in cell cycle arrest (0.2 and 0.4 ng/mL doxycycline for SK-BR-3 and HaCaT cells, respectively). Single-cell cloning of A549 cells was done in the presence of 100 ng/mL doxycycline (Chk2^{WT} expression repressed). Clones exhibiting no detectable GFP-dependent fluorescence in the presence of doxycycline and high fluorescence in its absence were selected for further analysis. Expression of recombinant Chk2 was repressed by addition of 100 ng/mL (A549 cells) or 200 ng/mL (SK-BR-3 and HaCaT cells) doxycycline to the culture medium. Repression was reversed by washing the cells with PBS and addition of medium lacking doxycycline. Cell sorting was carried out on a MoFlo fluorescence-activated cell sorter (Cytomation, Fort Collins, CO).

Fluorescence Microscopy

Fluorescence microscopy was done using an Axiovert 125 microscope (Zeiss, Brussels, Belgium) equipped with a 470/520 nm excitation and 505 to 530 nm emission filter set. Images were acquired with a Magnafire camera driven by Optronics MagnaFire Camera Imaging and Control software version 1.0.A (Optronics, Goleta, CA).

Growth Curves and Cell Cycle Profiles

A549 parental cells and those containing recombinant Chk2 were seeded at low density in medium lacking doxycycline and cell numbers were determined at various times with a Z3 Coulter counter (Beckman Coulter, Mijdrecht, the Netherlands).

To analyze cell cycle distribution, subconfluent cells were collected, centrifuged, and washed in PBS. Cell pellets were resuspended in citrate buffer and nuclei were stained with propidium iodide as described previously (38). Analysis was carried out using FACScan (BD Biosciences, Erembodegen-Aalst, Belgium) and BD-LSR flow cytometers (BD Biosciences). Cell cycle profiles were analyzed using WinList 3D version 4.0 and areas under the curve were calculated using ModFit LT version 3.1 (Verity Software House, Inc., Topsham, ME).

Immunoblot Analysis

Cells were disrupted by sonication in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% NP40, 1 mmol/L DTT] supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L Na_3VO_4, 60 mmol/L sodium β-glycerophosphate, Sigma-Aldrich). Nuclear protein extracts were prepared by disruption of cells in nuclear isolation buffer [0.5% NP40, 10 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl, 2 mmol/L $MgCl_2$, 3 mmol/L $CaCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride] containing protease and phosphatase inhibitors and by subsequent centrifugation for 5 minutes at 420 $\times$ g. The nuclei in the resulting pellet were washed in nuclear isolation buffer lacking NP40 and disrupted by sonication in lysis buffer. Whole-cell and nuclear proteins were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes for immunoblotting.

Primary antibodies directed against p21 were obtained from BD Transduction Laboratories (San Jose, CA); Chk2 (C-18), Chk2 phosphorylated T68, p53 (FL-393), Chk1 (G-4), Rb (IF-8), and actin (I-19) were from Santa Cruz Biotechnology (Santa Cruz, CA); Rb phosphorylated S780 was from Cell Signaling Technology (Beverly, MA); survivin (ab469) was from Novus-Biologicals (Cambridge, United Kingdom); and actin (CP01) was from Oncogene (San Diego, CA).

Apoptosis Assays

Subconfluent adherent and detached A549 cells expressing Chk2^{WT} were collected from 175-cm^2 flasks, centrifuged, and captured in artificial tissues as described previously (39). Paraffin sections were subjected to a microwave citrate pretreatment and subsequently stained for cleaved caspase-3 (BD PharMingen, Erembodegen-Aalst, Belgium). A secondary antibody conjugated to peroxidase was incubated with 3-amino-9-ethylcarbazole as a substrate. Hematoxylin was used for nuclear counterstaining. For detection of DNA fragmentation, the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling technique was used as described previously (40).

Table 1. Sequences of Primers Used in Quantification Real-time Reverse Transcription-PCR

Gene ID	Sequence	
p21	CAGCGACCTTCCTCATCCA	Forward primer
	CCCATCCCTCCCCAGTTCATTGC	5′[6-FAM]-3′ [TAMRA]-probe
	GACTCCTTGTTCCGCTGCTAA	Reverse primer
Cdc2	CATTTGGAGTATAGGCACCATATTTG	Forward primer
	TGAACTAGCAACTAAGAAACCACTTTTCCATGGG	5′[6-FAM]-3′ [TAMRA]-probe
	TCTGAAAATCCTGAAGAGTTGATCA	Reverse primer
Cdc25C	GCTACTGCCCTATGCATCATCA	Forward primer
	TGGCTTCGACACCTCAGCAACTCAGTCT	5′[6-FAM]-3′ [TAMRA]-probe
	CCCCTTCCTGCACTTTGCT	Reverse primer
Chk1	TGGTTGACTTCCGGCTTTCT	Forward primer
	AAGTGTCTCTTGAACTCCAATCCATCACCCT	5′[6-FAM]-3′ [TAMRA]-probe
	TCAATCAGCTTCCCTTTAATCTTCA	Reverse primer
Topoisomerase IIα	GAGTAGTTATGTGATTATTTCAGCTCTTGAC	Forward primer
	TGTCCCCTCTGGCTGCCTCTGAGTC	5′[6-FAM]-3′ [TAMRA]-probe
	TTGGTTTCTCTCTTTGGGAGATTC	Reverse primer
Survivin	GGAGTCTGGGAAGGGTTGTG	Forward primer
	CCTCACCCATAGCCCAGAAGCCTCAT	5′[6-FAM]-3′ [TAMRA]-probe
	GGCTCTAACCTGCCATTGGA	Reverse primer

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SA-β-gal Staining (pH 6.0)

Cells were assessed for senescence by staining for β-galactosidase activity at pH 6.0 as described previously (21). Briefly, cells were washed in PBS, fixed for 15 minutes at room temperature in 0.2% glutaraldehyde-2% formaldehyde, rinsed with PBS, and incubated overnight at 37°C in SA-β-gal staining solution (40 mmol/L citric acid, 40 mmol/L $H_2NaPO_4{\cdot}2H_2O$, 5 mmol/L $K_4Fe(CN)_6{\cdot}3H_2O$, 5 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$, 150 mmol/L NaCl) at pH 6.0 containing 1 mg/mL X-gal (U.S. Biochemical, Cleveland, OH).

Quantification Real-time Reverse Transcription-PCR

Total RNA was isolated from cells containing GFP-Chk2^{WT} at various times following expression using a RNeasy Mini kit as described by the manufacturer (Qiagen, Venlo, the Netherlands). Reverse transcription was carried out from 1.0 μg purified RNA using SuperScript III Reverse Transcriptase kit as described by the manufacturer (Invitrogen, Merelbeke, Belgium). The sequences of the TaqMan FAM and TAMRA probes and primers for the genes in this study are described in Table 1. Real-time quantification was carried out using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as described by the manufacturer.

siRNA Treatment

siRNA duplexes corresponding to p21 sequence (GCCTTAGTCTCAGTTTGTGTGTCTT) and a nonspecific (control) sequence (TTCGTAAGAGACCGTGGATCCTGTC) were prepared as described by the manufacturer (Sequitur, Natick, MA). Cells containing GFP-Chk2^{WT} were grown in medium lacking doxycycline, and 24 hours later, these were transfected with the siRNAs (25 μmol/L) using Oligofectamine according to the manufacturer's instructions (Invitrogen).

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p53-Independent Regulation of p21$^{Waf1/Cip1}$ Expression and Senescence by Chk2

Cécile-Marie Aliouat-Denis, Najoua Dendouga, Ilse Van den Wyngaert, et al.

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