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Breast cancer is a very heterogeneous disease. Many pathological alterations contribute to breast cancer development. Some initiate the cancer formation or accelerate its progression. Our bodies counter DNA damaging stressed form the environment, such as g-irradiation, UV, carcinogens or even from metabolic processes. If DNA damage occurs without being repaired n the genes controlling cell growth or morality, cells will acquire altered growth properties and become transformed and cancer will eventually develop. Fortunately, our bodies have natural defense mechanisms called checkpoints to keep the damaged cells in check so that the damaged genes can be repaired. In this proposal, we plan to study Chk2, one of the central components of the machinery, for its role in managing our defensive system to repair damaged DNA and to suppress cellular transformation. In our studies, we will first generate cells or animals which have no Chk2 expression for use as a model. By studying the responses to DNA damaging agents and the transformation potential, we can determine the significance of Chk2 functions in managing checkpoints and in suppressing transformation. Next, we plan to systematically identify Chk2 substrates and its associated proteins so that we can discover the entire network connecting to Chk2.

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This is a revised report. The original report detailed work we did in collaboration with Dr. Tak Mak on Chk2, the subject of our Idea Award. Our original aim was to make human cells that lacked Chk2 so we could study their properties. This did not work for technical reasons. We then resorted to deleting the same gene from mouse cells which our collaborator used to make knockout mice from. We did not house mice at Baylor and only assisted in the analysis of cells from the mouse such as MEFs(mouse embryo fibroblasts) and thymocytes. We report results from those experiments in this report and not results on the mice themselves performed by our collaborator who used our material to generate the mice. The mouse work was all funded by Dr. Mak's grants, not from my DAMD grant. We have shortened the report, removing the experiments performed with the mice by Dr. Mak and we have focused this report on the on the experiments using the Chk2-/- cells as suggested by the reviewer.

Our original SOW did describe work to be done with cells lacking Chk2. Although we wished to use human cells, mouse cells are an accepted substitute since they are of mammalian origin. Therefore, we feel this work does fall under our SOW. We have essentially completed task1,2 and 3. We are beginning tasks 4 and 5. We have not started the remaining tasks yet but plan to in the near future. We note that all future reports will adhere to the DAMD guidelines as requested and apologize for the confusion on our part.

Introduction

DNA damage activates cellular responses that promote DNA repair, arrest the cell cycle, and in some cases, induce apoptosis (56). Cell cycle arrest allows time for the repair of damaged DNA while apoptosis eliminates cells harboring abnormal DNA. It is widely believed that these DNA damage responses are required for the maintenance of genomic stability and prevention of tumor development (20). The ataxia telangiectasia (A-T) mutated (ATM) gene, which is homologous to the yeast checkpoint gene Tel1, plays a critical role in sensing DNA double strand breaks (DSBs) in mammalian DNA. ATM is a kinase involved in activating the appropriate damage response pathway, leading to either cell cycle arrest or apoptosis, and is therefore a key checkpoint molecule in regulating cell cycle responses to DNA damage (37, 45). Indeed, the majority of phosphorylation events induced by ionizing radiation (IR) are carried out by ATM. Both A-T patients and ATM-deficient mice show defective cell cycle arrest, hypersensitivity to DNA DSBs, and tumor predisposition (4, 21, 52, 53). When cells are damaged by IR (irradiation), ATM phosphorylates and activates the protein kinase Chk2 (1, 35, 36, 55). Chk2 is a homologue of the Rad53 gene in budding yeast and of the Cds1 gene in fission yeast. Once phosphorylated, activated Chk2 phosphorylates multiple Cdc25 molecules which are thought to inhibit the activation of cyclindependent kinases (7, 10, 34). However, in response to damage induced by UV-irradiation or hydroxyurea, Chk2 is phosphorylated in an ATM-independent manner, possibly by A-T and *rad3*-related (ATR) (35, 46). Notably, ATM, ATR, and Chk2 are each able to phosphorylate the tumor suppressor gene p53 (2, 9, 11, 26, 42, 49).

p53 is the most frequently mutated cancer-associated gene identified to date (29). In response to DNA damage, p53 undergoes phosphorylation and conformational changes which result in increased levels and activity of the protein (23). Increased p53 activity enhances the rate of transcription of numerous target genes (such as p21, Mdm2,

GADD45, and Bax) that mediate the plethora of p53-dependent functions (19, 54). These functions include the promotion of apoptosis and the induction of G1 cell cycle arrest. The p53 protein can be modified by many different protein kinases and acetylases, resulting in modulation of p53 function (39). In particular, the phosphorylation or dephosphorylation of various serine residues can have a significant impact on p53 stability. Recent studies with phospho-specific antibodies have established that serines (Ser) 6, 9, 15, 20, 33, 37, and 46 of p53 are sites of de novo phosphorylation in cells following DNA damage and that phosphorylation of different sites has different effects (2, 9, 12, 25, 38, 43, 44, 47). For example, it has been proposed that the phosphorylation of the N-terminal Ser 15, 33, and 37 residues permits subsequent modification of the distant C-terminal lysine residues of p53 through enhanced recruitment of the coactivator protein p300/CBP/PCAF (28, 41). In contrast, phosphorylation of Ser20 is required for stability of p53 in response to DNA damage (11). Ser20 comprises part of the site used by Mdm2 to bind p53 and target it for ubiquitination, and phosphorylation of Ser20 interferes with Mdm2 binding.

Previous studies have demonstrated that, in response to IR, Ser15 on p53 is phosphorylated by ATM (2, 9), whereas Ser20 is phosphorylated by Chk2 (11, 26, 42). There is abundant evidence that ATM controls p53 stabilization either directly or indirectly via Chk2, and it is also now clear that p53-mediated G1 arrest is suppressed in ATM-/- thymocytes. However, it is more controversial whether ATM is involved in p53-mediated apoptosis of damaged cells. While some laboratories have shown that ATM-/- thymocytes are resistant to IR-induced apoptosis (51, 53), others have found that these cells exhibit normal p53-mediated cell death (3, 21, 24). It appears that the pathways governing p53-dependent cell cycle arrest and apoptosis may be distinct and that ATM plays a major role in regulating only the former.

Body

Generation of a Chk2-/- knockout construct. We had proposed to delete the human Chk2 in human cells but this did not work. We then switched to mouse cells since they are easier to perform gene replacement in and since they are mammalian cells as well. The Chk2 gene was disrupted by replacing a region of the genomic sequence containing exons 8 to 11 with a neomycin resistance cassette (Fig. 1A). We generated this construct using conventional recombinant DNA methods. This construct was used by our collaborators in Toronto, Dr. Tak Mak, to disrupt ES cells Chk2 gene and to inject these ES cells into mice. They did so and the Chk2 knockout ES cell line went to the germ line. They characterized these mice and bred them together and showed that Chk2 -/- animals were viable. We did not grow the mice in our lab. All mouse growing was performed in Dr. Mak's lab. We helped with analysis of the cells from these animals.

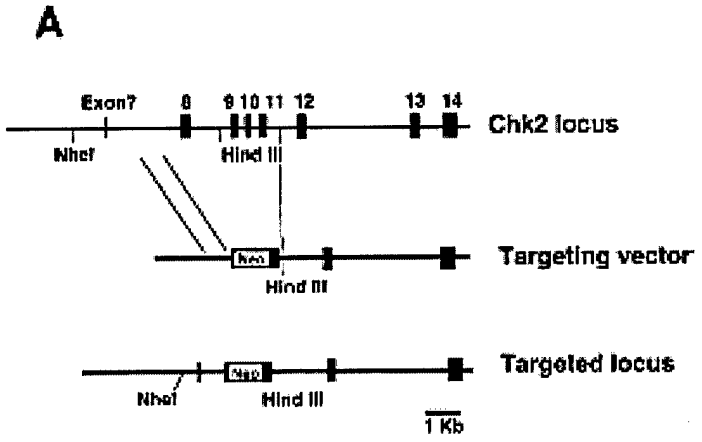


FIG. 1. Targeted disruption of the Chk2 gene in mice. (A) Targeting strategy. The genomic configuration of the germ line Chk2 locus is shown at the top. The targeting vector is shown in the center; exons 8 to 11 were replaced with a neomycin cassette (Neo). The mutated Chk2 locus is shown at the bottom.

Since Chk2 activation in response to DNA DSBs is dependent on ATM (34), we anticipated that Chk2-/- cells might display overlapping phenotypes with those of ATM-/- cells. We therefore investigated several of the most obvious phenotypes of ATM-/- cells in Chk2-/- cells. While cultured ATM-/- MEFs showed extremely poor growth, cultured Chk2-/- fibroblasts did not show an obvious growth deficit (data not shown). This was consistent with what Dr. Mak observed with the Chk2 and ATM mice as ATM-/- mice are infertile due to a defect in germ cell development while, Chk2-/- male and female mice are fertile. So the cells from these animals behaved like the animals themselves phenotypically.

Several immunological abnormalities have been reported in ATM-/- cells, including a defect in T-lymphocyte maturation. However, Chk2-/- lymphoid cells from mice were normal in all respects. Again consistent with the observation that loss of Chk2 is less severe than ATM loss.

Apoptosis and cell cycle arrest induced by IR are defective in Chk2-/- cells.

Previous work, we showed that Chk2-/- were resistant to IR-induced apoptosis because of a lack of p53 stabilization. In this study, we evaluated the role of Chk2 in regulating IR-induced cell death in vivo. Our collaborator Dr. Mak, examined cells from Chk2-/- mice treated with IR. We observed that as expected, these cells failed to undergo apoptosis consistent with previous observations. The same observation was true for follicular matrical cells after 5 Gy of irradiation.

Since IR induces cell cycle arrest at several distinct cell cycle transitions, we studied the effect of the absence of Chk2 on the G1/S, S, and G2/M checkpoints. First, G1 arrest was evaluated in cultured wild-type, p53-/-, and Chk2-/- MEFs. Serum- starved cells were irradiated and stimulated to enter the cell cycle by the addition of serum. BrdU was added with the serum to allow the detection of S-phase entrance. In response to increasing doses of IR, p53-/- MEFs failed to arrest in G1, as expected (18). Wild-type MEFs arrested normally in G1 as evidenced by a dose-dependent reduction in the number of BrdU-positive cells. Interestingly, Chk2-/- MEFs were significantly defective in their ability to arrest in G1 at low IR doses but behaved like wild-type cells at higher doses. Analysis of the G2/M checkpoint in MEFs indicated that both WT and Chk2-/- MEFs arrest in response to DNA damage but that Chk2-/- cells release from the arrest earlier at 24 hrs (data not shown).

An intra-S-phase checkpoint occurs inMEFs in response to IR, and this checkpoint requires ATM function (4). We evaluated the effect of the Chk2 mutation on this checkpoint by examining the inhibition of DNA synthesis in MEFs. Both wild-type and Chk2-/- primary MEFs showed equivalent levels of DNA synthesis inhibition following IR, whereas ATM-/- cells showed a characteristic profile of radiation-resistant DNA synthesis. These findings show that the intra-S-phase checkpoint is ATM dependent but Chk2 independent.

Chk2 selectively regulates apoptosis in an ATM-independent manner. Although Chk2 acts downstream of ATM in yeast and mammals, the loss of Chk2 does not result in many of the phenotypes observed in ATM-/- cells. In fact, the only shared phenotype is defective p53 function in response to IR. This finding prompted us to explore the possibility that Chk2 and ATM might have different regulatory effects on p53 function. Whereas Chk2-/- cells have a clear defect in IR-induced apoptosis, this phenotype is variable in ATM-/- cells. We therefore carefully compared the effect of the loss of Chk2 or ATM on the regulation of p53 activation in thymocytes. p53- mediated cell cycle arrest and apoptosis have been well characterized in thymocytes, and it is possible to precisely quantify these phenotypes in this cell type. When wild-type thymocytes were subjected to 10 Gy of gamma-irradiation, the number of thymic BrdU-positive S-phase cells was reduced to 35% , 4% of that in the nonirradiated controls. The G1/S-phase checkpoint was defective in ATM-/- and p53-/- cells (ATM-/-, 132% ± 10%; p53-/-, 128% ± 11%), consistent with previous reports (3). In contrast to irradiated ATM-/- cells, irradiated Chk2-/- cells showed only a partial defect that resulted in milder G1 arrest (65% ± 5%). However, when Chk2-/- thymocytes were subjected to IR, apoptosis was dramatically impaired. ATM-/- thymocytes were more resistant than wild-type

thymocytes to IR-induced apoptosis but considerably more sensitive than either Chk2-/- or p53-/- thymocytes. It should be noted that the IR-induced apoptosis and inhibition of the G1/S transition observed in this study are p53 dependent because they are completely inhibited by the loss of p53 function. These results indicate that Chk2 acts in the pathway leading to p53- dependent apoptosis rather than in the general apoptosis program.

Our findings led us to hypothesize that Chk2 selectively regulates p53 activity leading to apoptosis. To address this question, we used Northern blotting to evaluate the transactivation of mRNA expression for known p53 target genes. Although the expression of many molecules is induced by p53 activation, p21 and Bax are the most prominent p53-responsive genes in mouse primary thymocytes (8). Loss of p21 in thymocytes leads to a clear defect of the G1 checkpoint induced by IR (18). Although Bax-/- thymocytes are not resistant to IR-induced apoptosis, Bax-/- Bak-/- thymocytes fail to die in response to IR (32). Since Bak-/- thymocytes also show normal responses to IR, the induction of Bax must be critical for IR-induced apoptosis. In wild-type thymocytes subjected to 5 Gy of IR, Bax mRNA was increased by (6.05 ± 1.44)-fold over the baseline at 3 h and by (5.68 ± 1.64)-fold at 6 h (data not shown). Strikingly, irradiated Chk2-/- thymocytes showed defective induction of Bax ([1.9 ± 0.45]-fold at 3 h and [1.80 ± 0.40]-fold at 6 h). These results represent statistically significant decreases compared to the wild type at 3 h ($P < 0.01$) and 6 h ($P < 0.01$). In contrast, irradiated ATM-/- thymocytes were slower than the wild-type to induce Bax mRNA synthesis at 3 h ($P < 0.05$) but had caught up by 6 h. p21 mRNA induction was significantly suppressed compared to that of the wild type ($P < 0.05$) in both ATM-/- and Chk2-/- irradiated thymocytes at 3 h but there were no significant differences in the level of suppression between these two genotypes. Consistent with a previous report (3), neither p21 nor Bax was induced in irradiated p53-/- thymocytes. These results suggest that thymic apoptosis induced by IR depends on p53 function and is controlled mainly by Chk2 rather than ATM. Stabilization of p53 induced by IR was suppressed in both ATM-/- and Chk2-/- thymocytes. Taken together, the data demonstrate that Chk2, rather than ATM, controls p53-mediated apoptosis, and that p53-mediated apoptosis does not correlate with stabilization of the p53 protein.

We next determined whether Chk2 phosphorylation is required for the activation of p53 leading to apoptosis. Mouse Chk2 has seven N-terminal SQ/TQ sites in the N-terminal region of the protein. In response to IR in vivo, ATM phosphorylates several of these SQ/TQ sites in Chk2, including Thr68 (1, 35, 36). Phosphorylation of Chk2 following IR is abolished by mutation of these SQ/TQ sites, and most of the endogenous Chk2 is not phosphorylated in ATM-/- cells; nevertheless, p53-mediated apoptosis can occur under these circumstances. To determine whether the phosphorylation of Chk2 SQ/TQ sites is required for Chk2-mediated regulation of p53-mediated apoptosis, we reintroduced into Chk2-/- thymocytes a mutant form of Chk2 in which all Nterminal SQ/TQ sites were replaced with AQ and analyzed apoptosis. In a previous study (35), the mutated SQ/TQ kinase had the same level of kinase activity as the wild-type enzyme when transfected into unirradiated Chk2-/- MEFs, but failed to induce apoptosis when these cells were irradiated.

Key research Accomplishments

A) Generationof Chk2 -/- cells

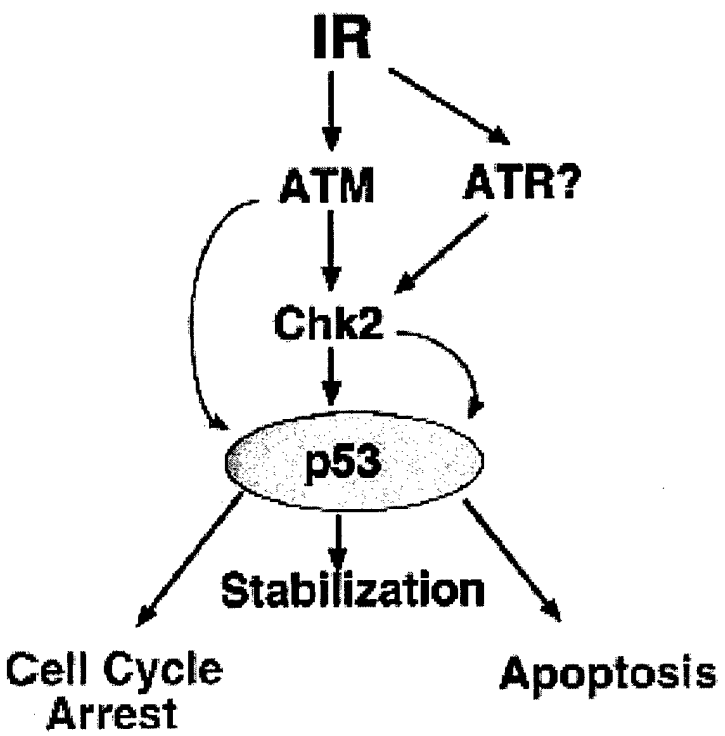
B) Discovery that Chk2 controls apoptosis in ATM-dependent and independent ways.

Reportable Outcomes

Hirao et al. *Mol. Cell Biol.* 22:6521-32.

Conclusions

We have shown in this study that there are differences between Chk2-/- and ATM-/- cells in the IR-induced activation of checkpoint and apoptotic responses, despite the fact that ATM regulates IR-induced Chk2 activity. ATM and Chk2 both modify p53 and activate it; however, our results show that at least some p53 phosphorylation is Chk2 dependent and ATM independent. Moreover, Chk2-dependent and ATM-dependent p53 phosphorylation events may differentially affect downstream p53-dependent



Model of the regulation of p53 activation by Chk2 in response to IR. Chk2-mediated stabilization of p53 induced by IR and leading to apoptosis is controlled independently of ATM, possibly by ATR. ATM appears to stabilize p53, leading to cell cycle arrest without involving Chk2.

transactivation targets. Specifically, we have demonstrated that Chk2 predominantly regulates IR-induced apoptosis rather than the G1/S checkpoint in thymocytes, whereas ATM is predominantly involved in the regulation of the G1/S checkpoint. Contradictory results have been reported regarding the effect of ATM on p53-mediated apoptosis (3, 21, 24, 53). We find that ATM-/- thymocytes were more resistant than the wild type to IR but significantly less resistant than Chk2-/- thymocytes. Consistent with the results of the apoptosis assay, the induction of Bax mRNA was more profoundly impaired in Chk2-/- thymocytes than in ATM-/- cells. This tight correlation between the induction of apoptosis and the transactivation of p53 downstream molecules suggests that the inhibition of apoptosis induced by the loss of Chk2 is caused by the suppression of p53 activation itself and not by effects on molecules further downstream in the apoptosis pathway.

We and others have proposed a model of IR-induced phosphorylation leading to apoptosis via Chk2. Mutation of the SQ/TQ sites on Chk2 abolished p53 activation leading to apoptosis, demonstrating that the phosphorylation of the SQ/TQ sites in the N-terminal region of Chk2 is essential for this process. Most of the phosphorylation of Chk2 induced by IR is abolished if ATM is absent. However, our data suggest that there is some ATM-independent phosphorylation at SQ/TQ sites of Chk2 in response to IR. It is possible that the very low level of ATM-independent phosphorylation of Chk2 that occurs is insufficient to alter the mobility of the protein. The most likely agent of ATM-independent Chk2 phosphorylation is ATR, although other members of the phosphatidylinositol 3-kinase family are also possibilities. ATR is a phosphatidylinositol 3-kinase-related kinase which contains a protein kinase domain similar in sequence to a region of *Schizosaccharomyces pombe rad3* (6, 13). Matsuoka et al. have reported that ATR phosphorylates Thr26, Ser50, and Thr68 in the SQ/TQ cluster domain of human Chk2 in vitro (35). Although ATR is believed to act primarily in response to a DNA replication block or UV-irradiation, it is also involved in responses to IR. Cells lacking ATR die within several days of exposure to IR; however, prior to their deaths, a profound defect in the IR-induced G2/M checkpoint can be demonstrated (16). Other studies have shown that overexpression of a kinase-dead mutation of ATR causes increased sensitivity to IR and a defect in the G2/M arrest and S-phase checkpoints (14). Furthermore, ATR mutation also abolishes DNA damage- induced phosphorylation of Ser15 on p53 (49). These data suggest a potential functional overlap between ATM and ATR with respect to IR responses, consistent with our hypothesis that ATM and ATR cooperate in regulating p53 activity. We theorize that ATR selectively regulates p53 activation leading to apoptosis via Chk2, while ATM governs cell cycle arrest in a Chk2-independent manner.
In addition to phosphorylating p53, Chk2 is known to phosphorylate Cdc25. It has been reported that ATM and Chk2 are required for the S-phase checkpoint induced by IR and that this induction depends on Chk2-dependent phosphorylation of Cdc25A (22). In our study, we confirm that ATM is required for the S-phase checkpoint in MEFs but we also demonstrate that Chk2 is dispensable for this checkpoint in this cell type. In experiments with immortalized MEFs, we have observed a slightly slower onset and shorter duration of the S-phase checkpoint in the absence of Chk2 (unpublished data). On balance, however, we believe that Chk2 is not essential for S-phase arrest in normal cells. It is possible that Chk1 can substitute for Chk2 in the phosphorylation of Cdc25 and that Chk2 thus has a redundant function in the intra-S-phase checkpoint. Previous reports concluding that Chk2 was required for the intra-S-phase checkpoint utilized overexpression constructs containing mutated Chk2 genes identified in sporadic colon cancer and as a germ line mutation in Li-Fraumeni syndrome (LFS). The data showed that these mutations had a dominant-negative impact in wild-type cells. Constructs containing such mutations could therefore have an inhibitory effect on a downstream component of the S-phase checkpoint (such as Cdc25), thereby preventing the operation of any compensatory Chk1-dependent process. Alternatively, differences in Chk2 dependency in different tissues or between mice and humans may underlie the discrepancy between the previous reports and our data. Failures in the transcriptional response to damage, cell cycle arrest and apoptosis induced by IR should lead to a higher incidence of tumor development.

Unexpectedly, however, our Collaborator Dr. Mak found that Chk2-/- mice do not have obvious tumors, unlike ATM-/- mice, which die within 4 months of birth with thymic lymphomas. Interestingly, the thymic lymphomas in ATM-/- mice are critically

dependent on V(D)J recombination, whereas thymic lymphomas in p53-/- mice arise independent of V(D)J recombination (30). These observations indicate that at least two different mechanisms of lymphoma development are at work in these mutant animals, such that ATM-mediated p53 activation may not be required for the development of lymphomas. ATM phosphorylates many target substrates in addition to Chk2 and p53, including Nbs1 and Brca1 (17, 31). These molecules, which act downstream of ATM, may contribute in an unknown way to the prevention of spontaneous lymphoma development. Mutations of Chk2 are found more frequently in patients with variant LFS, which has a moderate phenotype, than in patients with classical LFS (5). In contrast, mutations of p53 have been reported in 70% of classical LFS cases and 20% of variant LFS patients and certain mutants alleles cause a 2-fold increase in breast cancer (50).

In conclusion, the results of this study have shown that p53 activation leading to cell cycle arrest is regulated differently from that leading to apoptosis. Chk2 is required for p53-mediated apoptosis and must undergo phosphorylation in order to function, but this phosphorylation is not carried out by ATM. We propose a new model for the IR pathway that emphasizes the independent effects of Chk2 and ATM.

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