Recovery from DNA Damage-induced G2 Arrest Requires Actin-binding Protein Filamin-A/Actin-binding Protein 280*

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Filamin-A (filamin-1) is an actin-binding protein involved in the organization of actin networks. Our previous study has shown that filamin-A interacts with BRCA2, and lack of filamin-A expression results in increased cellular sensitivity to several DNA damaging agents in melanoma cells (Yuan, Y., and Shen, Z. (2001) J. Biol. Chem. 276, 48318–48324), suggesting a role of filamin-A in DNA damage response. In this report, we demonstrated that deficiency of filamin-A results in an 8-h delay in the recovery from G2 arrest in response to ionizing radiation. However, filamin-A deficiency does not affect the initial activation of the G2/M checkpoint. We also found that filamin-A deficiency results in sustained activation of Chk1 and Chk2 after irradiation. This in turn causes a delay in the dephosphorylation of phospho-Cdc2, which is inhibitory to the G2/M transition. In addition, filamin-A-deficient M2 cells undergo mitotic catastrophe-related nuclear fragmentation after they are released from the G2 arrest. Together, these data suggest a functional role of filamin-A in the recovery from G2 arrest and subsequent mitotic cell death after DNA damage.

In response to DNA damage, cell cycle checkpoints are activated to delay or block the progression of the cell cycle. This may prevent damaged cells from progressing into the next phase of the cell cycle, thus facilitating the maintenance of genomic stability (1–4). When DNA damage is repaired, cells recover from cell cycle arrest and resume their normal cell cycle progression. Studies in yeast suggest that the transition from cell cycle arrest to cell cycle progression is not simply a passive response to the removal of DNA damage. It appears to be an active process in which cells can adapt to prolonged cell cycle arrest even when the DNA damage has not been completely repaired (5–8). However, the mechanism that regulates the recovery of cell cycle arrest is poorly understood, especially in mammalian cells.

Our previous study has shown that an actin-binding protein filamin-A (filamin-1, FLNa, or ABP-280) interacts with BRCA2, and deficiency of filamin-A renders melanoma cells more sensitive to γ-rays, bleomycin, and UV irradiation (9). In this study, we further investigated the role of filamin-A in cell cycle regulation using a filamin-A-deficient melanoma cell line (M2) and a C8161 melanoma cell line in which filamin-A expression was knocked down by RNA interference. We found that filamin-A deficiency correlated with an approximate 8-h delay in the recovery from G2 arrest after irradiation. However, filamin-A deficiency had little effect on the initiation of G2 arrest, indicating intact activation of the G2/M checkpoint. The delayed G2 recovery correlated with delayed dephosphorylation of the phospho-Cdc2 protein and sustained activation of the Chk1 and Chk2 kinases. Furthermore, we found that deficiency of filamin-A renders cells more prone to mitotic cell death. These data provide the first evidence that filamin-A plays a significant role in the recovery from radiation-induced G2 arrest and mitotic cell death in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human A7 and M2 melanoma cells were kindly provided by Drs. T. P. Stossel and Y. Ohta (Brigham and Women’s Hospital, Harvard Medical School). The M2 is a human melanoma cell line lacking filamin-A expression. The A7 cell line was derived from M2 through the stable transfection of a plasmid expressing full-length filamin-A (10). A7 and M2 cells were subcultured twice a week in minimum essential medium with Earle’s salt (EMEM) (M2) or EMEM with 0.5 mg/ml G418 (A7). The C8161 melanoma cells were kindly provided by Dr. D. R. Welch (Pennsylvania State University College of Medicine) and cultured in RPMI 1640 medium. All media contained 10% fetal bovine serum and 1% penicillin/streptomycin. To induce DNA damage, cells were irradiated with γ-rays (100 Gy) at room temperature and immediately returned to a 37 °C incubator. To inhibit Chk1 and Chk2 activities, 100 μM UCN-01 (7-hydroxystaurosporine, National Cancer Institute) was added to the cell culture 30 min before the cells were exposed to radiation and was maintained in the media until the cells were collected.

Western Blot—Cells were lysed in lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40). Cell extracts were separated by 10% SDS-PAGE. Filamin-A was detected with an anti-filamin-A antibody (Chemicon International, Temecula, CA). Cdc2 was detected with an anti-Cdc2 antibody (Cell Signaling Technology, Beverly, MA). Tyr-15 phosphorylated Cdc2 was detected with an anti-phospho-Cdc2 (Tyr-15) antibody (Cell Signaling Technology, Beverly, MA). Activated Chk1 and Chk2 kinases were detected with antibodies specific to Ser-345-phosphorylated Chk1 and Thr-68-phosphorylated Chk2, respectively (Cell Signaling Technology, Beverly, MA). α-Actin monoclonal antibody (Sigma) was used to confirm that an equal amount of protein was loaded in each sample.

Construction of pPUR/U6 Vector for Short Hairpin RNA (shRNA) Expression and to Confer Paromycin Resistance—The BamHI/EcoRI restriction fragment of pBS/UT6 (a gift from Y. Shi, Harvard Medical School of Medicine, Boston, MA) that contains the U6 promoter for short hairpin RNA expression was inserted into the BamHI/EcoRI sites of UCN-1, 7-hydroxystaurosporine; DAPI, 4’-6-diamidino-2-phenylindole; Me2SO, dimethyl sulfoxide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

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Published, JBC Papers in Press, December 1, 2003, DOI 10.1074/jbc.M306794200

Printed in U.S.A.
of the pPUR vector (Clontech, Palo Alto, CA). This resulted in a new vector pPUR/U6 to express the short hairpin RNA and to confer puromycin resistance in mammalian cells. The strategy to construct an shRNA expression vector was described by Sui et al. (11). Briefly, two inverted sequences of 21 bases (5′/H11032″/H11032) of the filamin-A cDNA were linked by a 9-base linker sequence and cloned downstream of the U6 promoter, resulting in the RNA interference vector pPUR/U6/filamin-A. This vector directs the synthesis of a filamin-A-specific shRNA in vivo. Vacant vector was used as the negative control.

**Analysis of DNA Content by Flow Cytometry**—Cells were trypsinized, washed with phosphate-buffered saline, and fixed with 70% ethanol. Fixed cells were then pelleted, washed with phosphate-buffered saline, and suspended in 200 µl of citrate buffer (250 mM sucrose, 0.05% Me3SO, 40 mM trisodium citrate, pH 7.6). Nine hundred µl of Solution A (0.003% trypsin in stock buffer (3.4 mM trisodium citrate, 0.1% Nonidet P-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Trizma, pH 7.6)) were added to the cells. Cells were then incubated at room temperature for 10 min with 5-6 vortexes during the incubation. Afterward, 750 µl of Solution B (0.025% trypsin inhibitor, 0.01% ribonuclease A in stock buffer) was added for another incubation of 10 min at room temperature with two vortexes during the incubation. Then, 750 µl of Solution C (0.0416% propidium iodide, 3.3 mM spermine tetrahydrochloride in stock buffer) was added to the cells on ice. Afterward, the cells were covered with aluminum foil and were ready for flow cytometry analysis. In each flow cytometry assay, 20,000 cells were collected using FACScan (BD Biosciences) (12).

**Mitotic Index Assay**—The cells were continuously treated with 1 µg/ml nocodazole for various time periods. Then the cells were harvested, swollen hypotonically for 5 min in 75 mM KCl, gently pelleted, and fixed in 0.5 ml of Carnoy’s fixative (3:1 methanol/glacial acetic acid, v/v) for 5 min. Subsequently, the cells were dropped on pre-wetted slides and allowed to air dry. Fixed cells were stained with DAPI, and mitotic cells were counted with fluorescent microscopy (Zeiss Axioskop 2) as described previously (13). For each sample, the percentage of mitotic cells was determined from 200–300 cells counted, and the experiment was repeated three to four times.

**Visualization of Nuclear Fragmentation**—To demonstrate fragmentation of the nucleus, cells were fixed with methanol for 15 min at −20°C. The cells were then stained with rabbit anti-α-tubulin antibody (Sigma) and visualized by Texas Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). DAPI was used to visualize the nucleus.

**RESULTS**

**Delayed Recovery from G2 Arrest in Filamin-A-deficient M2 Melanoma Cells**—The M2 melanoma cell line does not express filamin-A, whereas the A7 cell line is an isogenic cell line derived from M2 in which filamin-A expression is restored to its normal level (10). To investigate whether absence of filamin-A in M2 cells affects DNA damage-induced checkpoint activation, we studied the cell cycle profiles of A7 and M2 cells after irradiation. Specifically, asynchronized populations of A7 and M2 cells in the exponential growth phase were irradiated with 8 Gy of γ-rays, and their cell cycle profiles were analyzed at various time points from 4–24 h after irradiation. As shown in Fig. 1, nonirradiated control A7 and M2 cells displayed a similar pattern of heterogeneous log phase distribution throughout the 24-h period. When the cells were irradiated with γ-rays, both the A7 and M2 cells started to accumulate into the G2 phase at 4 h and peaked at 16 h. At 24 h, the percentage of A7 cells in the G2 phase decreased significantly, suggesting a release from G2 arrest. However, the percentage of M2 cells in G2 was still high at 24 h, suggesting a slower release from the G2 phase.

Our previous study showed that M2 cells are ~10 times more sensitive to irradiation than A7 cells as measured by a clonogenic survival assay (9) (see also Fig. 2A). It is possible that the delayed release from G2 arrest in the M2 cell line is because of their higher sensitivity to radiation. To verify this hypothesis, we compared the cell cycle distribution of A7 and M2 cells at
the same survival rate, namely 8 Gy for A7 and 6 Gy for M2 (pair I), as well as 12 Gy for A7 and 8 Gy for M2 (pair II) (Fig. 2A). As shown in Fig. 2B, M2 cells again displayed a much higher percentage in the G2 phase at 24 h for both pairs of iso-survival doses. This further supports the notion that the lack of filamin-A expression in M2 cells results in a slower recovery from G2 arrest, which is not necessarily because of the reduced survival in M2 cells.

To determine the difference in G2 arrest recovery between A7 and M2 cells, we conducted a nocodazole-trapping assay. Briefly, the microtubule-disrupting agent nocodazole was added to cells immediately after irradiation to block cells at the anaphase of mitosis. The accumulation of mitotic cells was determined by counting the cells that had condensed chromosomes. Under normal conditions, the mitotic index increases immediately after nocodazole treatment. However, a delayed accumulation of mitotic cells is expected if the G2/M checkpoint is activated to arrest the cells in the G2 phase. The mitotic index will increase if the G2-arrested cells recover from this arrest. As shown in Fig. 3, the mitotic indices of both A7 and M2 cells accumulated in a similar pattern as time progressed in the control groups (0 Gy), indicating that filamin-A is not necessary for normal progression of G2 cells into the M phase. However, after a prolonged mitotic blockage by nocodazole, the percentage of mitotic cells tended to decline. This might reflect an escape of mitotic cells from the nocodazole blockage, which is not unusual for many tumor cell lines. There was no mitotic accumulation in the A7 cells during the first 8 h. The mitotic index started to increase between 8–16 h post-irradiation, indicating that the G2/M checkpoint was activated upon irradiation in the A7 cells and lasted for ~8 h. However, the mitotic index in the M2 cells remained low during the first 16 h post-irradiation. It started to increase at 16–24 h, suggesting that there was normal activation of the G2/M checkpoint in the M2 cells, but the G2 arrest lasted for at least 16 h, ~8 h longer than that of the A7 cells. Therefore, data here are consistent with the data in Figs. 1 and 2. Together, these data demonstrated that a lack of filamin-A expression in M2 cells results in an ~8-h delay in the recovery from G2 arrest after irradiation.

Delayed Recovery from G2 Arrest in C8161 Melanoma Cells with Filamin-A Knockdown Using RNA Interference—To confirm the results obtained with the M2/A7 cell lines (Figs. 1–3), the filamin-A expression in the melanoma cell line C8161 was knocked down using RNA interference, resulting in the cell line C8161/FLNa-KD (Fig. 4A). The mitotic index assay was carried
Delayed recovery from G₂ arrest in filamin-A-deficient cells

Filamin-A and G₂ Arrest

Fig. 4. Delayed recovery from G₂ arrest in C8161 melanoma cells in which filamin-A expression had been knocked down by RNA interference. A, knock down of filamin-A expression by RNA interference. C8161 cells were transfected with the RNA interference vector designed for filamin-A and selected by puromycin. This resulted in C8161/FLNa-KD. The control cells (C8161) were transfected with vacant vectors. The filamin-A level (top panel) in these cells was measured by immunoblot using anti-filamin-A antibodies. The lower band in the top panel represents a proteomic degradation of the full-length filamin-A. The bottom panel shows the β-actin level in the same lanes of the top panel to demonstrate equal loading. B, the mitotic cell indices of C8161 and C8161/FLNa-KD after continuous treatment with nocodazole upon 8 Gy of irradiation. The cells were irradiated at 8 Gy, and 1 μg/ml nocodazole was added immediately. At various time points (4–48 h) after the nocodazole treatment, the percentage of mitotic cells was calculated (see “Experimental Procedures” for details). The distance between the curves of irradiated C8161 and C8161/FLNa-KD reflect the delayed recovery from G₂ arrest.

Delayed Recovery from G₂ Arrest in Filamin-A-deficient Cells Correlates with Slower Dephosphorylation of Phospho-Cdc2—It is well established that the onset of mitosis is triggered by the activation of cyclin B/Cdc2, and that the activation of cyclin B/Cdc2 is absolutely required for the transition of G₂ cells into the M phase (4, 14–19). Generally, cyclin B/Cdc2 is inactivated by phosphorylations of the Thr-14 and Tyr-15 residues during the G₂ phase (4, 15–17). When the cells are ready to enter mitosis, the mitosis-promoting phosphatase Cdc25C dephosphorylates Cdc2 at Thr-14 and Tyr-15 (4, 15–17). To determine whether filamin-A-mediated recovery from G₂ arrest is regulated by Cdc2 dephosphorylation, we measured the status of Cdc2 phosphorylation in the A7 and M2 cells from 4 to 48 h after irradiation. As shown in Fig. 5, the Cdc2 triplet band (from top to bottom, panels A and C) represents double-phosphorylated, single-phosphorylated, and nonphosphorylated Cdc2, respectively. The double-phosphorylated Cdc2 is the form that sustains G₂ arrest. We confirmed that the top band in the triplet contained Tyr-15-phosphorylated Cdc2 using an antiphospho-Tyr-15-Cdc2 antibody (see Fig. 6C). In irradiated A7 cells (Fig. 5A), the relative abundance of double-phosphorylated Cdc2 (as compared with the other two forms) remained high from 8 to 16 h and started to decline after 16 h. These data are in agreement with those in Fig. 3, where the recovery from G₂-arrested A7 cells started at 8–16 h after irradiation. However, the relative abundance of the double-phosphorylated Cdc2 in M2 cells remained high during the first 24 h and declined slowly afterward. This delayed dephosphorylation of Cdc2 in M2 cells is consistent with the data in Fig. 3, where the recovery from G₂ arrest started 16–24 h after irradiation. Thus, the dephosphorylation of the double-phosphorylated Cdc2 (Fig. 5) coincides with the recovery from G₂-arrested cells (Fig. 3), suggesting that the filamin-A-dependent recovery from G₂ arrest is most likely related to the Cdc2-mediated G₂/M control mechanism.

The Slower Dephosphorylation of Cdc2 in M2 Cells Is Attributed to the Sustained Activation of Chk1 and Chk2 Kinases—Activation of Chk1 and Chk2 is the upstream event that leads to the increased phosphorylation of Tyr-15 on Cdc2. Chk1 and Chk2 (also named hCds1) are the human homologues of the fission yeast checkpoint kinases Chk1 and Cds1, respectively. Chk2 is activated mainly in response to DNA damage caused by ionizing radiation and requires phosphorylation on its Thr-68 residue by ATM kinase (20, 21). In contrast, Chk1 activation requires phosphorylation at Ser-345 by ATR kinase and is primarily elicited by DNA replication blocking agents such as hydroxyurea or nonionizing radiation such as UV light (22–24). It was recently reported that the ATR/Chk1 pathway can also be activated by ionizing radiation to elicit G₂ arrest in the absence of ATM (25). Therefore, the ATM/Chk2 and ATR/Chk1 pathways probably overlap and cooperate after DNA damage. Both activated Chk1 and Chk2 phosphorylate Cdc25C at Ser-216, preventing Cdc25C from dephosphorylating Cdc2 at Thr-14 and Tyr-15 (26). Therefore, we measured the phosphorylation of Chk1 and Chk2 in the A7 and M2 cells following irradiation to determine whether the delayed G₂ recovery in the M2 cells is mediated by these upstream kinases.

As shown in Fig. 6A, the Ser-345-phosphorylated Chk1 (Chk1 (Ser-345-P)) level was low in both the A7 and M2 cells in

out using this cell line, and its parental cell line C8161 transfected with an empty vector as the control. As shown in Fig. 4B, in the absence of irradiation, a similar accumulation of mitotic cells was observed in C8161 and C8161/FLNa-KD after nocodazole block. When the cells were irradiated, both cell lines displayed a delayed accumulation of mitotic cells when compared with nonirradiated cells, indicating an activation of the G₂/M checkpoint and subsequent G₂ arrest. Recovery from G₂ arrest in C8161/FLNa-KD was about 8 h longer than that observed in the C8161 parental cells. These data confirmed that the delayed recovery from G₂ arrest in the M2 cells was because of the lack of filamin-A expression (Fig. 3).

It is of interest to note that in the absence of irradiation, the percentage of mitotic cells decreased after 16 h of blockage with nocodazole. This is consistent with the observation that in the absence of irradiation, the percentage of mitotic A7 and M2 cells decreased 24–32 h after nocodazole blockage (Fig. 3). The mechanism behind this phenomenon is not clear, but it is not unusual for some tumor cells to escape nocodazole-induced mitotic blockage, and it may reflect a defect of the spindle checkpoint in these cells.

Delayed Recovery from G₂ Arrest in Filamin-A-deficient Cells Correlates with Slower Dephosphorylation of Phospho-Cdc2—It is well established that the onset of mitosis is triggered by the activation of cyclin B/Cdc2, and that the activation of cyclin B/Cdc2 is absolutely required for the transition of G₂ cells into the M phase (4, 14–19). Generally, cyclin B/Cdc2 is inactivated by phosphorylations of the Thr-14 and Tyr-15 residues during the G₂ phase (4, 15–17). When the cells are ready to enter mitosis, the mitosis-promoting phosphatase Cdc25C dephosphorylates Cdc2 at Thr-14 and Tyr-15 (4, 15–17). To determine whether filamin-A-mediated recovery from G₂ arrest is regulated by Cdc2 dephosphorylation, we measured the status of Cdc2 phosphorylation in the A7 and M2 cells from 4 to 48 h after irradiation. As shown in Fig. 5, the Cdc2 triplet band (from top to bottom, panels A and C) represents double-phosphorylated, single-phosphorylated, and nonphosphorylated Cdc2, respectively. The double-phosphorylated Cdc2 is the form that sustains G₂ arrest. We confirmed that the top band in the triplet contained Tyr-15-phosphorylated Cdc2 using an antiphospho-Tyr-15-Cdc2 antibody (see Fig. 6C). In irradiated A7 cells (Fig. 5A), the relative abundance of double-phosphorylated Cdc2 (as compared with the other two forms) remained high from 8 to 16 h and started to decline after 16 h. These data are in agreement with those in Fig. 3, where the recovery from G₂-arrested A7 cells started at 8–16 h after irradiation. However, the relative abundance of the double-phosphorylated Cdc2 in M2 cells remained high during the first 24 h and declined slowly afterward. This delayed dephosphorylation of Cdc2 in M2 cells is consistent with the data in Fig. 3, where the recovery from G₂ arrest started 16–24 h after irradiation. Thus, the dephosphorylation of the double-phosphorylated Cdc2 (Fig. 5) coincides with the recovery from G₂-arrested cells (Fig. 3), suggesting that the filamin-A-dependent recovery from G₂ arrest is most likely related to the Cdc2-mediated G₂/M control mechanism.

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As shown in Fig. 6A, the Ser-345-phosphorylated Chk1 (Chk1 (Ser-345-P)) level was low in both the A7 and M2 cells in
were re-blotted with anti-M2 cells. The same membranes of
/H9252 for details) for anti-Cdc2 or anti-
prepared (see diated at 8 Gy, and protein samples were
bottom. A and C, the Cdc2 protein levels in A7 and M2 cells. The Cdc2 antibody (Cell Signaling Technology) recognized both phosphorylated and nonphosphory-
lected forms of Cdc2. From top to bottom, the triplet Cdc2 pattern represents double, single, and nonphosphorylated Cdc2, re-
spectively. B and D, β-actin level in A7 and M2 cells. The same membranes of A and C were re-blotted with anti-β-actin antibo-
dies to demonstrate equal loading for all the samples in A and C.

The dose of tyrosine phosphorylation of Cdc2 at Tyr-15. In other words, the abnormally
Chk1 and Chk2 by UCN-01 would abolish the delayed dephos-
M2 cells after irradiation, we anticipated that inhibition of
inhibitor of Chk1 (27, 28), but it also inhibits the Chk2 kinase
UCN-01, was used. UCN-01 was originally identified as an
execute two major cell death pathways, apoptosis and mitotic
derness of Tyr-15-phosphorylated Cdc2. As expected, UCN-01 treat-
ment did not change the level of Tyr-15-phosphorylated Cdc2 in nonirradiated A7 (Fig. 7A, lanes 1 and 2) and M2 (Fig. 7A, lanes 5 and 6) cells. However, the radiation-induced Tyr-15 phosphorylation on Cdc2 in A7 (Fig. 7A, lane 3) and M2 (Fig. 7A, lane 7) cells was reduced to the level of nonirradiated A7 and M2 cells after UCN-01 treatment (lanes 4 and 8). This suggests that Chk1 and Chk2 are responsible for the sustained Tyr-15 phosphorylation of Cdc2 at 24 h after irradiation in the M2 cells (Figs. 5 and 6).

In addition, the nocodazole-trapping assay was carried out to
demonstrate that inhibition of Chk1 and Chk2 also abolishes
G2 arrest in M2 cells. Briefly, 100 nM UCN-01 was added to the
cell culture and maintained in medium until the cells were
collected. Cells were then irradiated, and 1 μg/ml nocodazole
was added immediately. As seen in Fig. 3, the biggest differ-
ence in mitotic indices between A7 and M2 cells occurred at
24 h after irradiation. Therefore, the mitotic cells were scored
at 24 h, and the mitotic index of each group was calculated. As
expected (Fig. 7B), the addition of UCN-01 did not affect the
mitotic indices of the A7 cells and nonirradiated M2 cells. As
shown previously, in irradiated M2 cells there was a significant
decrease in the percentage of mitotic cells (Fig. 7B), reflecting a delayed release of G2-arrested cells into mitosis as shown in
Fig. 3. This delayed release was abolished by the addition of
UCN-01 (Fig. 7B). These data further demonstrate that the G2
arrest in M2 cells is controlled by the pathway involving Chk1
and Chk2 kinases, which are upstream effectors of Cdc25C and
Cdc2.

**Mitic Catastrophe Followed the Delayed Recovery from G2
Arrest in Filamin-A-deficient M2 Cells after Irradiation**—When
mammalian cells cannot repair their DNA damage, they can
execute two major cell death pathways, apoptosis and mitotic
catastrophe (30–34). If apoptosis is the major cause of cell
death, a sub-G1 population of cells would be observed in a DNA
content-based cell cycle analysis. Our data in Figs. 1 and 2 did not
not exhibit a sub-G1 population of M2 cells, suggesting that
apoptosis was not the cause of increased cell death. In addition,
we used terminal deoxynucleotidyltransferase-mediated dUTP
nick end labeling (TUNEL) assay to visualize potential apop-
totic cells, but no positive TUNEL staining was observed (data

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**Fig. 5. Relative abundance of phospho-Cdc2 proteins at various time points after 8 Gy of irradiation in A7 and M2 cells.** Log phase cells were irradiated at 8 Gy, and protein samples were prepared (see “Experimental Procedures” for details) for anti-Cdc2 or anti-β-actin immunoblot as indicated on the right. Cell lines are labeled on the left. The dose of irradiation is labeled on the top, and the time points post-irradiation are labeled on the bottom. A and C, the Cdc2 protein levels in A7 and M2 cells. The Cdc2 antibody (Cell Signaling Technology) recognized both phosphorylated and nonphosphory-
lected forms of Cdc2. From top to bottom, the triplet Cdc2 pattern represents double, single, and nonphosphorylated Cdc2, re-
spectively. B and D, β-actin level in A7 and M2 cells. The same membranes of A and C were re-blotted with anti-β-actin antibo-
dies to demonstrate equal loading for all the samples in A and C.

| Cell line:       | A7 (Filamin-A) | M2 (Filamin-A) |
|------------------|----------------|----------------|
| Dose of γ-rays(Gy): | 0 0 0 0 0 | 0 0 0 0 0 |
| Hours after IR:  | 8 16 24 32 | 8 16 24 32 |
| Chk1 (Ser345-P)   | A       | A              |
| Chk2 (Thr68-P)    | B       | B              |
| Cdc2(Tyr15-P)     | C       | C              |
| β-actin           | D       | D              |

**Fig. 6. Phosphorylation of Chk1, Chk2, and Cdc2 upon irradiation.** A7 and M2 cells were irradiated with 8 Gy γ-rays. At various times after the irradiation, Western blots were performed to detect the phosphorylated forms of Chk1, Chk2, and Cdc2. Phospho-Chk1(Ser-345)(A), phospho-Chk2(Thr-68)(B), and phospho-Cdc2(Tyr-15)(C)-specific antibodies were used. The type of cell lines, radiation doses, and time after irradiation are labeled on the top. The protein sample of nonirradiated cells at 32 h was not loaded because of a limitation of gel space (only 14 specimens can be loaded on the same gel). β-Actin was used as a loading control (D). IR, irradiation.

the absence of irradiation. Irradiation significantly increased
Chk1 phosphorylation at Ser-345 in both cell lines. In the A7
cells, the level of Ser-345-phosphorylated Chk1 peaked at 16 h
and returned to normal by 24 h. However, in the M2 cells the
level of phosphorylated Chk1 peaked at 8 h and returned to
normal at 32 h. This suggests a more sustained phosphoryla-
tion of Chk1 at Ser-345 in M2 cells than in A7 cells. Chk2 was
also activated by irradiation in both cell lines as shown in Fig.
6B. However, the activation was greater in M2 cells than in A7
cells (Fig. 6C). Prolonged phosphorylation of Chk1 and Chk2 in
M2 cells agrees with the observed change of Tyr-15 phosphor-
ylation of Cdc2 (Figs. 5 and 6C) and the recovery from G2
arrest shown in Fig. 3. These data suggest that the delayed
recovery from G2 arrest in filamin-A-deficient M2 cells is be-
cause of the sustained activation of the G2/M checkpoint path-
way involving the Chk1/Chk2-dependent signal transduction
cascades.

To further confirm this notion, a Chk1 and Chk2 inhibitor,
UCN-01, was used. UCN-01 was originally identified as an
inhibitor of Chk1 (27, 28), but it also inhibits the Chk2 kinase
activity as shown recently (29). Because sustained activation of
both Chk1 and Chk2 was observed in the filamin-A-deficient
M2 cells after irradiation, we anticipated that inhibition of
Chk1 and Chk2 by UCN-01 would abolish the delayed dephos-
phorylation of Cdc2 at Tyr-15. In other words, the abnormally
high level of Tyr-15-phosphorylated Cdc2 in M2 cells would be
reduced to a level comparable with that observed in A7 cells. To
examine this possibility, 100 nM UCN-01 or Me2SO (as a con-
trol) was added to the cells 30 min before irradiation and kept
in the medium until the cells were harvested. Twenty-four h
after irradiation, the cells were harvested to measure the level
of Tyr-15-phosphorylated Cdc2. As expected, UCN-01 treat-
ment did not change the level of Tyr-15-phosphorylated Cdc2 in
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decrease in the percentage of mitotic cells (Fig. 7B), reflecting a delayed release of G2-arrested cells into mitosis as shown in
Fig. 3. This delayed release was abolished by the addition of
UCN-01 (Fig. 7B). These data further demonstrate that the G2
arrest in M2 cells is controlled by the pathway involving Chk1
and Chk2 kinases, which are upstream effectors of Cdc25C and
Cdc2.
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Figure 7. UCN-01 abolishes the prolonged phosphorylation of Cdc2 and delayed recovery from G₂ arrest in irradiated M2 cells. A, UCN-01 abolishes the sustained phosphorylation of Cdc2 in M2 cells. UCN-01 was added 30 min before irradiation and was kept in media until cells were harvested. Twenty-four h after irradiation, the Try-15-phosphorylated Cdc2 levels were detected by Western blot using phospho-Cdc2(Tyr-15) antibody. The dose of irradiation and treatment of UCN-01 are labeled on top of the figure. β-Actin was used as the loading control. B, UCN-01 abolishes the extended G₂ arrest in M2 cells. Thirty min before irradiation, UCN-01 was added and was kept in the culture until cells were collected (Me2SO was added in control groups). Immediately after 8 Gy of irradiation, nocodazole was added to block the cells at mitosis, and the mitotic cells were scored at 24 h. Plotted are the mitotic indices of A7 and M2 cells at 0 and 8 Gy irradiation and in the absence (−) or presence (+) of UCN-01. IR, irradiation.

Figure 8. Mitotic catastrophe in filamin-A deficient M2 cells after 8 Gy irradiation. A, morphology of nuclear fragmentation in M2 cells. Representative nuclei in nonirradiated A7 and M2 cells (panels I and II) and irradiated A7 and M2 cells (panels III and IV) at 48 h after irradiation are shown. Nuclei were stained with DAPI, and α-tubulin was stained to visualize the whole cell. Shown in panel IV is an M2 cell with a fragmented nucleus after irradiation. B, enhanced nuclear fragmentation in M2 cells after irradiation. The percentage of cells with fragmented nuclei was calculated out of at least 300 total cells at various time periods after 8 Gy of irradiation. M2 and A7 indicate M2 and A7 cells without UCN-01 treatment at the time of irradiation, M2/UCN-01 and A7/UCN-01 indicate M2 and A7 cells treated with UCN-01 at the time of irradiation.

DISCUSSION

The activation of cell cycle checkpoints plays a significant role in the DNA damage response. It prevents damaged cells from entering the next phase of the cell cycle. Although much is known about the genes that activate the G₂/M checkpoint, little is known about the genes that control the recovery from G₂ arrest in mammalian cells. The data presented here shed new light on this important aspect. We showed that filamin-A deficiency in the melanoma cell lines M2 and C8161/FLNa-KD caused a delayed recovery from G₂ arrest after irradiation. This correlated with the sustained activation of Chk1 and Chk2 kinases and delayed dephosphorylation of the phospho-Cdc2. However, deficiency of filamin-A in M2 and C8161/FLNa-KD cells did not affect the initiation of the G₂/M checkpoint. Although the molecular mechanism by which filamin-A may affect the Cdc2-dependent recovery from G₂ arrest is not clear, several possible scenarios are worthy of further discussion. First, a certain level of filamin-A and a fragment of filamin-A normally reside in the nucleus (9, 38). The nuclear filamin-A and the actin network may serve as a supporting matrix for the BRCA2-related DNA damage response process via an interaction between filamin-A and BRCA2. Thus, lack of filamin-A may result in delayed DNA repair that in turn leads to delayed recovery from G₂ arrest. Second, it is possible that filamin-A directly regulates the Cdc2-dependent recovery from G₂ arrest.
Protein shuffling between the nucleus and the cytoplasm is required for precise regulation of Cdc2 phosphorylation and dephosphorylation (39–41), and it has been recognized that filamin-A plays a significant role in protein translocation between the cytoplasm and the nucleus (42). Last but not least, disruption of actin organization in yeast cells activates a morphogenesis checkpoint that arrests cells at the G2/M border (43–46). It is possible that filamin-A and its associated actin network contribute to G2/M regulation in a way mechanistically resembling the morphogenesis checkpoint observed in yeast cells. Nevertheless, our data present an important link between the DNA damage response and the cellular cytoskeletal components.

It has been controversial whether an abnormal G2/M checkpoint contributes directly to radiation sensitivity (for a review, see Ref. 4). Some reports suggest that the activation of the G2/M checkpoint in response to DNA damage enhances cell survival, because chemicals that abolish the G2/M checkpoint reduce clonogenic cell survival (47, 48). Others have suggested that prolonged G2 arrest contributes to the ability of the cells to survive radiation (49–51), whereas Xu et al. (52) suggest that the G2/M checkpoint status has no direct influence on the cellular sensitivity to DNA damage. Previously, we reported an interaction between BRCA2 and filamin-A. We found that deficiency of filamin-A in M2 melanoma cells renders them more sensitive to γ-radiation, bleomycin, and UV-C radiation (9). In the work reported here, we found that filamin-A deficiency does not affect the activation of the G2/M checkpoint, but rather it delays recovery from G2 arrest. Our data suggest that delayed recovery from G2 arrest may also be associated with sensitivity to DNA damage. It has been recognized that mitotic catastrophe after irradiation in M2 cells compared with A7 cells increases more dramatically than in the first 48 h (Fig. 8B). This time point coincides with the release of G2-arrested cells (24–32 h for A7 cells and 32–48 h for M2 cells, as indicated in Fig. 3). These data suggest that release (or adaptation) from a sustained G2 arrest may be associated with mitotic catastrophe, an idea supported by others (54, 56, 57). However, it is currently unclear whether the delayed recovery of G2 arrest in M2 cells is the cause of increased cell death in M2 cells or is simply a correlation between them. Because filamin-A interacts with BRCA2, it is possible that the DNA repair process was much slower in filamin-A-deficient M2 cells than in filamin-A-proficient A7 cells, which is supported by our data suggesting that a less efficient RAD51 focus formation in response to radiation in M2 cells than in A7 cells. Therefore, it is plausible to suggest that the delayed recovery from G2 arrest in M2 cells may be a result of the slowed DNA repair process, which results in a more sustained DNA damage signal to the Chk1/Chk2 signal transduction pathway. This in turn causes a delayed recovery from G2 arrest in M2 cells, a scenario that warrants further investigation.

Acknowledgments—We thank Dr. T. Stossel (Harvard University) for providing A7 and M2 melanoma cells, Dr. D. Welch (Pennsylvania State University College of Medicine) for providing C8161 melanoma cells, Dr. S. Yang (Harvard University) for providing the pBlS6 plasmid, Dr. Y. Wang (Thomas Jefferson University) for providing UCN-01, and the flow cytometry and fluorescent microscope facilities at University of New Mexico Cancer Research and Treatment Center for providing facility support.

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