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KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction

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Abstract

KAP1 recruits many proteins involved in gene silencing and functions as an integral part of co-repressor complex. KAP1 was identified as Mdm2-binding protein and shown to form a complex with Mdm2 and p53 *in vivo*. We examined the role of KAP1 in p53 activation after the treatment of cells with different types of external stresses. KAP1 reduction markedly enhanced the induction of p21, a product of the p53 target gene, after treatment with actinomycin D or γ-irradiation, but not with camptothecin. Treatment with actinomycin D, but not with camptothecin, augmented the interaction of p53 with Mdm2 and KAP1. Further, KAP1 reduction in actinomycin D-treated cells facilitated cell cycle arrest and negatively affected clonal cell growth. Thus, the reduction of KAP1 levels promotes p53-dependent p21 induction and inhibits cell proliferation in actinomycin D-treated cells. KAP1 may serve as a therapeutic target against cancer in combination with actinomycin D.

Keywords: p53; Mdm2; KAP1; Actinomycin D

p53 plays a central role in tumor suppression [1]. In approximately half of the cancers, the p53 gene is deleted or harbors inactivating mutations. In remaining tumors that retain wild-type p53, p53 is often inactivated via other genetic or epigenetic alterations [2,3]. These observations indicate that the p53 pathway needs to be compromised during the development of cancer.

In non-stressed cells, p53 activity is suppressed mainly by its major regulator Mdm2 [4–6]. Once cells are exposed to external or internal stresses, p53 is activated via a coordinated p53 modification and Mdm2 inactivation [7]. Activated p53 in turn induces its downstream target genes and exerts its tumor suppressor function [8].

Several functions of Mdm2 were attributed to its activity to suppress p53 [9]. Mdm2 has intrinsic E3 ubiquitin ligase activity, and targets p53 for ubiquitin-dependent degradation and nuclear export. In addition, Mdm2 binds to the N-terminal trans-activation domains of p53 and thereby inhibits p53-dependent trans-activation. Recently, it was demonstrated that Mdm2 forms a complex with a histone deacetylase, HDAC1, and the complex thus formed suppresses p53 via its deacetylation [10]. Presumably these functions of Mdm2 collectively serve to suppress p53.

In order to gain further insight into the mechanism of Mdm2 function, we looked for novel Mdm2-binding proteins. The biochemical purification and identification of proteins co-purified with Mdm2 revealed that one of the novel Mdm2-binding proteins was KAP1/TIF1β/KRIP-1, a transcriptional co-repressor.

KAP1 was initially identified as a co-repressor for the Kruppel-associated box (KRAB)-domain-containing zinc finger proteins [11–13]. KAP1 can recruit and coordinate many components for gene silencing. KAP1-mediated gene silencing involves the recruitment of the histone deacetylase

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complex (NuRD and N-CoR1) [14,15], and binding to a histone methyltransferase (SETDB1) [16]. Thus, KAP1 orchestrates the function of these co-repressor complexes to inhibit the transcription of its target genes.

Here, we demonstrated that KAP1 forms a complex with Mdm2 and p53 in cells, and that KAP1 inhibition augmented p53-dependent induction of p21, a product of the p53 target gene. Further, we pursue the importance of KAP1 in p53 activation after exposure to several exogenous stresses. We found that the contribution of KAP1 on p53 inhibition is different, depending on the stimuli that are used to activate p53. Namely, the reduction of KAP1 promotes the p53-dependent induction of p21 after treatment with actinomycin D or γ-irradiation, but not with camptothecin. We will discuss the possibility of KAP1 as a candidate for a therapeutic target in combination with actinomycin D.

Materials and methods

Antibodies. Anti-flag antibody (M2) and anti-actin antibody were purchased from Sigma. Anti-myc-tag (9E10) antibody was purchased from Santa Cruz. The anti-p53 antibody (DO-1) was purchased from Oncogene. Anti-p53 phospho-serine 15 antibody was previously described [18]. Anti-p21 antibody was purchased from Pharmingen.

Cells. MCF-7 and HCT116 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (Sigma).

DNA transfection, immunoprecipitation, and Western blot analysis. In DNA transfection experiments using CV-1 cells, 1 μg DNA and 3 μl of Fugene6 reagent (Roche) were introduced per 1.0×10^5 cells. Cells were then incubated for 48 h before harvest. Immunoprecipitation and Western blott analysis were performed as described before [19].

siRNA transfection. The following dsRNAs (only sense strands were described) were used for KAP1 and p53 suppression: KAP1-1 (5′-aa gcgugugcaaguggauguc-3′), KAP1-2 (5′-aagcaccaggagcacauucug-3′), and p53 (5′-aagacuccagugguaaucuac-3′). KAP1-1 siRNA was used for all siRNA experiments and KAP1-2 was used only for the experiment described in Fig. 1D. Control siRNA was purchased from Dharmacon (GL2 and GFP) or Qiagen (cont1). Control siRNA purchased from Qiagen was used for all the experiments. Transfection with Hiperfect reagent (Qiagen) was performed according to the manufacturer's instructions.

Purification of Mdmx-binding proteins. Flag-tagged Mdm2 was introduced into COS-1 cells by DNA transfection. Purification of the introduced Mdm2 protein, the separation of proteins that are co-purified with Mdm2, and the identification of Mdm2-binding protein by mass spectrometry were performed as described before [19].

BrdU staining. After siRNA transfection, MCF-7 cells were incubated with 10 μM BrdU for 1hr, fixed in 4% paraformaldehyde/PBS for 10 min, and washed with 1× PBS. After permeabilization in 0.2% Nonidet P-40/PBS for 5 min, cells were treated with 4 N HCl for 10 min and used for immunostaining with the anti-BrdU antibody conjugated with FITC (Roche). Subsequently, 200 cells from each sample were counted for BrdU incorporation.

Results and discussion

KAP1 interacts with Mdm2 and p53

In an attempt to identify novel Mdm2-binding protein, we introduced Flag-tagged human Mdm2 into COS-1 cells by DNA transfection. After the purification of Flag-tagged Mdm2 on an affinity column, co-purified Mdm2-interacting proteins were separated on an SDS–PAGE gel, and the polypeptide sequences of the identified proteins were determined by mass-spectrometry (data not shown). Previously, we took a similar approach to successfully identify novel Mdmx-binding proteins [19].

One of novel Mdm2-interacting proteins identified was KAP1, a transcriptional co-repressor that is involved in repression of its specific target genes [12]. The interaction of Mdm2 and KAP1 in transfected cells was confirmed by Western blot analyses (Fig. 1A). Subsequently, the interaction of endogenous KAP1 and Mdm2 proteins was detected (Figs. 1B and 3B). Finally, we demonstrated that endogenous KAP1 and p53 form a complex in cells (Fig. 3A). These observations indicate that KAP1 forms a stable complex with Mdm2 and p53 in cells. These findings are in agreement with recent reports by Wang et al., which described the formation of the KAP1–Mdm2–p53 complex in cells [17].

KAP1 reduction augments p21 induction after treatment with actinomycin D and γ-irradiation, but not with camptothecin

In order to examine the role of KAP1 in p53 activation, we introduced KAP1 siRNA in MCF-7 cells, and determined whether the reduced levels of KAP1 affect the induction of p53 targets (p21 and Mdm2) after exposure to γ-irradiation. In agreement with recent reports [17], we found that the induction of p21 was markedly augmented in cells with reduced levels of KAP1 after γ-irradiation, while we observed mild induction of Mdm2 under the same conditions (Fig. 1C and S1).

In order to confirm that the positive effect of KAP1 siRNA on p21 reflects the inhibition of KAP1, we next examined the effect of the other KAP1 or control siRNAs. Another KAP1 siRNA (KAP1-2) enhanced p21 induction after γ-irradiation as well as the original one (KAP1-1); whereas, none of three control siRNAs significantly affected the p21 levels (Fig. 1D). These data support the initial observation that KAP1 inhibition causes the enhancement of p21 induction seen after γ-irradiation.

We next examined whether KAP1 reduction affects p21 induction after treatment of the cells with various exogenous stimuli that are known to activate p53. After KAP1 inhibition with siRNA, we treated the cells either with γ-irradiation, actinomycin D, or camptothecin, all of which are known to activate p53. Subsequently, we determined whether decreased levels of KAP1 have any impact on p21 induction. In cells with reduced levels of KAP1, we observed the striking enhancement of p21 induction after exposure to actinomycin D (Fig. 2A, lanes 7 and 8). As we described above, KAP1 reduction also stimulated p53 induction after γ-irradiation (Fig. 2A, lanes 3 and 4) [17]. In contrast, when camptothecin was used to activate p53, KAP1 inhibition did not cause a significant increase of p21 expression (Fig. 2A, lanes 5 and 6). Thus, reduced

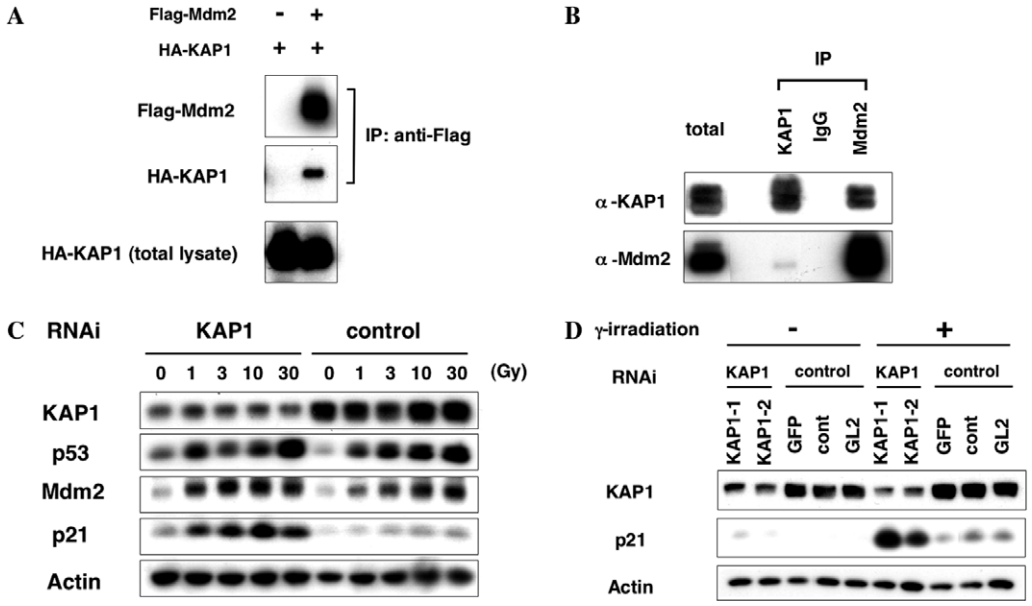

Fig. 1. KAP1 associates with Mdm2 *in vivo*, and KAP1 inhibition augments p21 induction after γ-irradiation. (A) CV-1 cells were transfected with HA-tagged KAP1 alone or together with Flag-tagged Mdm2, and lysates prepared from transfected cells were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were then used for Western blot analyses with anti-Flag or anti-HA antibody. (B) Lysates prepared from MCF-7 cells were immunoprecipitated with the indicated antibodies and the immunoprecipitates were used for Western blot analyses with the anti-KAP1 or the anti-Mdm2 antibody. (C) MCF-7 cells were transfected with KAP1 or control siRNA and treated with the indicated doses of γ-irradiation for 4 h. Subsequently, the lysates prepared from the treated cells were used for Western blot analyses with the indicated antibodies. (D) MCF cells were transfected with control (GFP, cont, and GL2) or KAP-1 siRNAs (KAP1-1, KAP1-2), and exposed to γ-irradiation or left untreated. Western blot analyses were performed as described in (C).

levels of KAP1 have varying impacts on p21 expression, depending on the types of exogenous stimuli.

p53 mediates enhanced p21 induction in cells with reduced levels of KAP1

In order to determine whether enhanced p21 induction after such treatment with actinomycin D or γ-irradiation is dependent on p53, we examined the p21 levels in cells after p53 inhibition. MCF-7 were transfected with KAP1 siRNA alone or together with p53 siRNA, and the transfected cells were exposed to γ-irradiation to determine whether the reduction of p53 affects the KAP1-mediated increase of p21 levels after the damage. In cells in which p53 was knocked down, p21 induction after γ-irradiation treatment was almost completely abolished both in KAP1-reduced cells as well as in control cells (Fig. 2B). These results indicate that the positive effect of KAP1 reduction on p21 induction is mediated by p53 activation.

In an attempt to further confirm the role of p53 in KAP-1-dependent p21 regulation, we knocked down KAP1 in HCT116 cells that harbor wild-type p53 (HCT +/+) or in its derivative, in which both p53 alleles were deleted (HCT −/−), and determined whether the positive effect of KAP1 inhibition on p21 induction after actinomycin D treatment is mediated by p53. As expected, the reduction of KAP1 significantly augmented the p21 induction after actinomycin D treatment in HCT +/+ cells (Fig. 2C, lanes 3 and 4). In contrast, p21 induction after the drug treatment was completely abolished in the p53-deficient derivatives, even if levels of KAP1 were reduced (Fig. 2C, lanes 7 and 8). Taken together, these results indicate that enhanced p21 induction in KAP1-reduced cells is mediated via p53 activation. Further, KAP1 reduction augments p21 expression at RNA levels after treatment with actinomycin D or γ-irradiation (Fig. S2). Combined together, we conclude that KAP1 inhibits p21 induction at least in part by inhibiting the transcriptional activity of p53.

Interaction of p53 with Mdm2 and KAP1 increases after treatment with actinomycin D or γ-irradiation, but not with camptothecin

The data presented in Fig. 2A indicate that levels of KAP1 have differentiating effects on p21 induction, depending on the type of exogenous stimuli used to activate p53. In order to obtain insight into the molecular mechanism of how such differentiating effects are achieved, we examined whether these stresses differentially affect the physical interaction among p53, Mdm2, and KAP1.

First, we examined the p53–Mdm2 and the p53–KAP interaction in MCF-7 cells. In order to avoid potential degradation of p53 via Mdm2-dependent ubiquitination, cells were treated with MG132, a proteasome inhibitor, before the interaction was examined by immunoprecipitation-Western blot analyses (Fig. 3A). Actinomycin D treatment caused a marked increase of association of p53 with KAP1 (Fig. 3A, lane 8). The p53–Mdm2 interaction was also

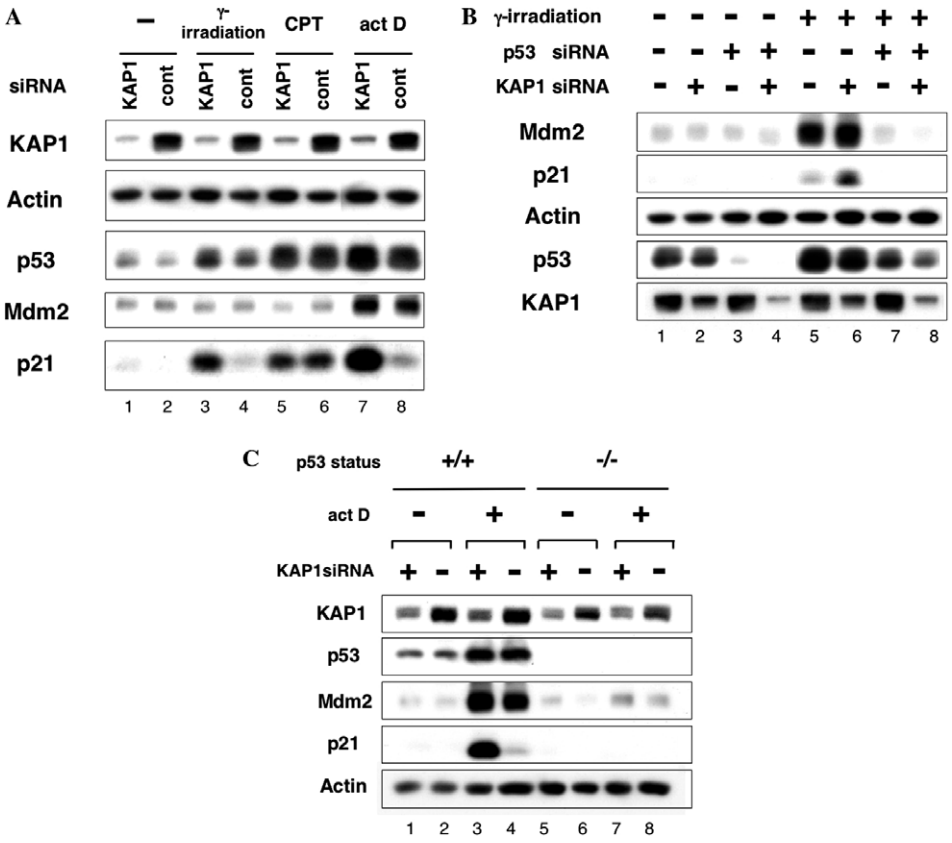

Fig. 2. Effects of KAP1 reduction on p53-dependent p21 activation after exposure to various external stimuli. Western blot analyses of the treated cells were performed as described in Fig. 1C. (A) MCF-7 cells were transfected with control or KAP1 siRNA, as described in Fig. 1C, and exposed to 10Gy γ-irradiation, 0.3 μM camptothecin or 5 nM actinomycin D for 12 h. (B) MCF-7 cells were transfected with p53 and/or KAP1 siRNA and exposed to 10Gy γ-irradiation for 4 h. (C) HCT116 cells with wild-type p53 or without p53 were transfected with control or KAP1 siRNA and treated with 5 nM actinomycin D for 12 h.

augmented in the presence of Actinomycin D (Fig. 3A, lane 8). In contrast, camptothecin treatment failed to increase the interaction of p53 with both Mdm2 and KAP1 (Fig. 3A, lane 7). Thus, these stresses differentially affect both the p53–KAP1 and the p53–Mdm2 interaction. On the other hand, any of these stresses did not significantly affect the levels of the KAP1–Mdm2 complex (Fig. 3B, bottom column). Because Mdm2 bridges the interaction between KAP1 and p53 [17], our results indicate that the regulation of the p53–KAP1 interaction by these stimuli mainly reflects their effects on the p53–Mdm2 interaction.

Remarkably, the levels of the p53–Mdm2–KAP1 complex formed after the external stresses (Fig. 3A) show good correlation with the extent of KAP1-dependent inhibition of p21 activation under these stimuli (Fig. 2A). Therefore, it is likely that differentiating effects of these stresses on p53 activation at least in part depend on how they impact on the p53–Mdm2–KAP1 interaction.

Cellular stresses such as DNA damage induce phosphorylation of the N-terminal domain of p53, which blocks interaction between p53 and Mdm2 [5]. Therefore, we examined whether the stimuli affects the phosphorylation status of the pivotal phosphorylation site, serine 15 of p53. Indeed, camptothecin treatment, but not treatment with actinomycin D or γ-irradiation, elicited the drastic induction of phosphorylation at serine 15 (Fig. 3C). These results suggest that strong phosphorylation of the N-terminal domain of p53 in camptothecin-treated cells contributes to the lack of strong interaction of p53 with Mdm2 and KAP1 (Fig. 3A).

KAP1 reduction promotes cell cycle arrest and inhibits cell growth after treatment with actinomycin D

Strong induction of p21 after actinomycin D treatment in KAP1-reduced cells prompted us to investigate whether cell proliferation was inhibited via KAP1 down-regulation in such cells. Therefore, we examined the cell cycle progression of the control or the KAP1-reduced celsl after actinomycin D treatment. KAP1 inhibition causes the reduced entry of cells in S phase after actinomycin D treatment (Fig. 4A). Decreased S phase entry in KAP1-reduced cells was further confirmed by the decreased number of cells that incorporate BrdU (Fig. 4B).

Subsequently, the effect of KAP1 reduction after actinomycin D treatment was examined by colony formation assay. Clonal cell growth of KAP-1 reduced cells was decreased after treatment with actinomycin D (Fig. 4C).

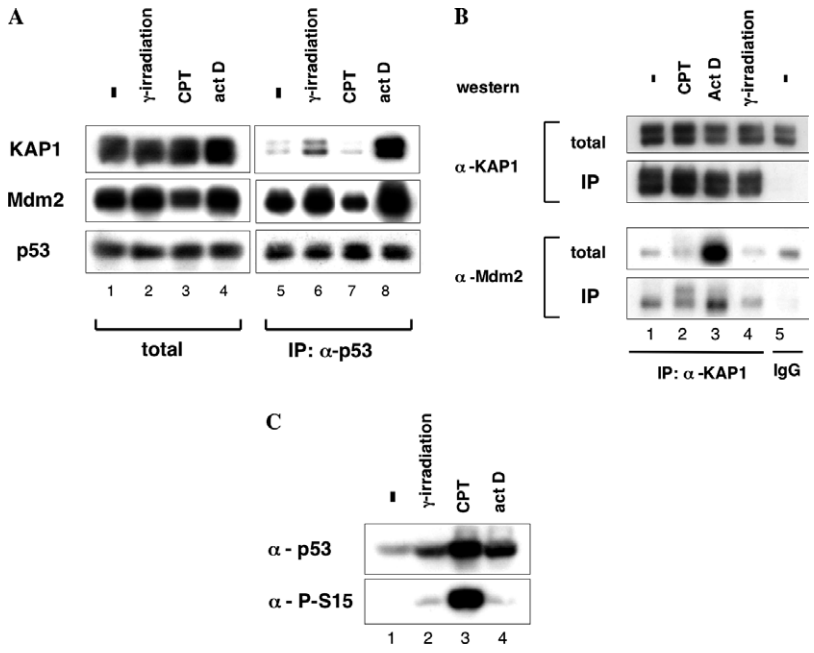

Fig. 3. Differential effects of external stimuli on the p53–Mdm2–KAP1 complex formation and on p53 phosphorylation at serine 15. MCF-7 cells were treated with the indicated stimuli for 12 h, as described in Fig. 2A. (A) After treatment with a proteasome inhibitor (50 μM MG132) for 3 h, lysates prepared from the treated cells were immunoprecipitated with anti-53 antibody. The immunoprecipitates were then used for Western blot analyses with the indicated antibodies. (B) Lysates prepared from the treated cells were immunoprecipitated with anti-KAP antibody or control IgG. The immunoprecipitates and total lysates were then used for Western blot analyses with anti-KAP1 or anti-Mdm2 antibody. (C) Lysates prepared from the treated cells were used for Western blot analyses with anti-p53 antibody (DO-1) or anti-phospho-S15 antibody.

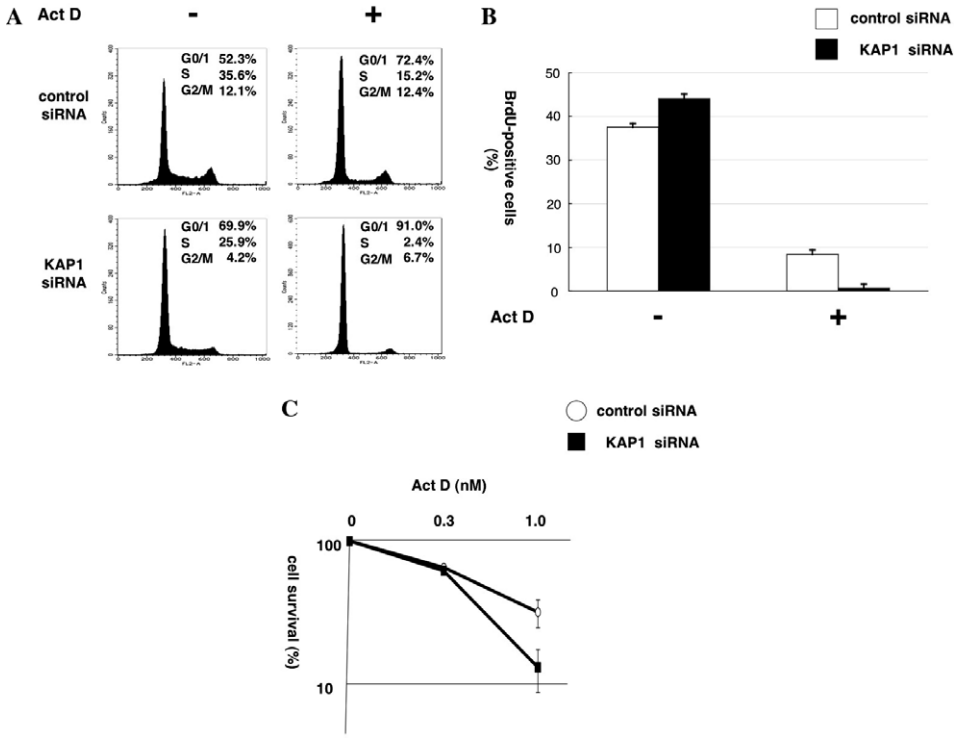

Fig. 4. KAP1 inhibition causes cell cycle arrest and inhibits clonal cell growth. (A) KAP1 inhibition facilitates cell cycle arrest after actinomycin D treatment. MCF-7 cells were transfected with control or KAP1 siRNA, as described in Fig. 1C, and exposed to 1 nM actinomycin D for 24 h. The DNA contents of the treated cells were then analyzed by flow cytometry. The number of BrdU-positive cells was scored for 200 cells in three independent experiments. The average percentage of BrdU-positive cells is shown. (C) KAP1 inhibition inhibits clonal cell growth after actinomycin D treatment. MCF-7 cells were transfected with control or KAP1 siRNA, as described in Fig. 1C, and exposed to actinomycin D at the indicated concentration for 24 h. Subsequently, the effect of actinomycin D on colony formation was determined by calculating the relative survival ratio in comparison to the control cells.

Thus, KAP1 reduction not only augments p53 activation, but also promotes the inhibition of cell growth after actinomycin D treatment.

Actinomycin D possesses a potent anti-tumorigenic effect, and has been used as part of chemotherapeutic regimens for the treatment of subsets of cancer, such as Wilms' tumor, rhabdomyosarcoma, and Ewing's sarcoma [20–22]. Although the precise mechanism of how actinomycin D blocks the growth of cancer cells is not known, it was shown that the treatment of cells with low dose actinomycin D facilitates the interaction of Mdm2 with several ribosomal proteins, and the inhibition of Mdm2's ability to suppress p53 [23–25]. Therefore, actinomycin D may suppress the growth of cancer cells, at least in part by activating p53 via inhibition of Mdm2 function.

Although actinomycin D treatment suppresses Mdm2's activity to ubiquitinate p53 [24,25], the down-regulation of the Mdm2 ubiquitinase activity by the drug causes the inhibition of Mdm2 auto-ubiquitination and contributes to the increased levels of Mdm2 [24,26]. Increased levels of Mdm2 after actinomycin D treatment, combined with the lack of inhibitory phosphorylation at the N-terminal p53, presumably facilitate the formation of the p53–Mdm2–KAP1 complex and KAP1-dependent suppression of p53, providing the molecular basis for the striking enhancement of p53 induction in KAP1-reduced cells.

Interestingly, it was recently demonstrated that KAP1 itself is phosphorylated and inactivated by ATM kinase after DNA damage [27]. It is possible that DNA damage caused by camptothecin inactivates KAP1 via phosphorylation. Thus, phosphorylation of both p53 and KAP1 after DNA damage may serve to release p53 from inhibition by KAP1.

Our observation that p53 activation after actinomycin D treatment is augmented in KAP1-reduced cells suggests that KAP1 may serve as a target for the re-activation of p53 in cancer cells. By treating cells with actinomycin D, together with compounds that inhibit KAP1–Mdm2 interaction or reduce KAP1 levels, Mdm2 function may be effectively suppressed, resulting in strong p53 activation. In future, it will be of considerable interest to develop a specific inhibitor of KAP1 as a part of cancer chemotherapy in combination with actinomycin D.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.022.

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