cAMP signalling inhibits p53 acetylation and apoptosis via HDAC and SIRT deacetylases

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Abstract. Activation of cAMP signalling potently inhibits DNA damage-induced apoptosis in acute lymphoblastic leukemia cells by promoting the turnover of p53 protein. Recently, we showed that the cAMP-induced destabilization of p53 in DNA-damaged cells occurs as a result of enhanced interaction between p53 and HDM2. In this report, we present results showing that increased levels of cAMP in cells with DNA damage enhances the deacetylation of p53, an event that facilitates the interaction of p53 with HDM2, thus annulling the stabilizing effect of DNA damage on p53. The combined inhibition of the HDAC and SIRT1 deacetylases abolished the cAMP-mediated deacetylation of p53, implying that cAMP-mediated deacetylation of p53 is dependent on the activity of these two classes of histone deacetylases. Importantly, diminishing the activity of HDACs and SIRT1 was also found to reverse the inhibitory effect of cAMP on the DNA damage-induced p53 stabilization and apoptosis, suggesting the involvement of the p53 acetylation pathway in the anti-apoptotic effect of cAMP signalling.

Introduction

p53 is the most frequently mutated tumour suppressor gene in cancers. About 50% of tumours harbour mutations in the p53 gene, and the remainder have disruptions in the p53 pathway (1). p53 is normally a short-lived protein that is maintained at low levels in resting cells by its negative regulator HDM2. Upon DNA damage induced, for instance, by ionizing radiation (IR), p53 transiently stabilizes and accumulates in the nucleus where it acts as a transcription factor regulating multiple genes important for the regulation of cell cycle arrest, senescence

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and apoptosis. The precise mechanisms of p53 activation are not fully understood, but the activation is clearly dependent on post-translational modifications like ubiquitination, phosphorylation and acetylation (2-4). p53 was the first non-histone protein described to be acetylated (5). Upon various types of stress, acetylation of p53 is dramatically induced, indicating the importance of this specific post-translational modification. There is also a direct competition between acetylation and ubiquitination on specific lysine residues in the C-terminal domain of p53. Acetylation of p53 physically blocks the ubiquitin sites preventing ubiquitination by HDM2 and subsequent degradation (6). Acetylation is also important for recruiting CBP/p300 and PCAF to promoter regions for activation of p53-targeted genes such as p21, HDM2 and PUMA. Histone acetyl transferases (HATs) CBP/p300, PCAF and TIP60 can all acetylate p53 (5,7,8), while histone deacetylases (HDACs) can remove acetyl moieties from ε-N-acetylated lysine residues of histones and non-histone proteins such as p53. Six C-terminal residues (K305, K372, K373, K381, K382 and K386) and one DNA binding domain (DBD) residue (K164) are acetylated by CBP/p300 (5,7-10), whereas K320 is acetylated by PCAF (7,8). HDAC inhibitors are also reported to induce acetylation in non-histone proteins (5). The HDAC1 inhibitor TSA leads to acetylation of p53 on K373 primarily under conditions in which cells are subjected to IR, whereas the SIRT1 deacetylase is known to induce acetylation of p53 on K382 (8).

cAMP is a second messenger important in multiple physiological and pathological settings (11). This signal transducer is generated by adenylyl cyclases subsequent to stimulation of certain G protein-coupled receptors (GPCRs). Our lab has previously reported that elevation of cAMP in lymphoid cells leads to arrest in the G1 phase of the cell cycle (12-14), arrest in the S phase and inhibition of apoptosis by anticancer agents (15). Using B cell precursor acute lymphoblastic leukaemia (BCP-ALL) cells as a model system, we also reported that the inhibitory effect of cAMP on apoptosis is p53-dependent (16), and that cAMP antagonizes the disruption of p53-HDM2 interaction by DNA damage (17). It was therefore of interest to further investigate the mechanism by which cAMP abolishes IR-induced p53 stability and apoptosis in these cells, and in particular, reveal how cAMP signaling promotes the interaction between p53 and HDM2. Because cAMP exerted only a slight inhibitory effect on IR-induced phosphorylation of S15, T18 and

S20 on p53 (17), we directed our attention towards the effect of cAMP on p53 acetylation. Our results indicate that cAMP, through inhibition of p53 acetylation, attenuates the IR-induced dissociation of p53 from HDM2 and, thereby, prevents stabilization of p53 and apoptosis.

Materials and methods

Reagents and antibodies. Forskolin (F), PGE2, propidium iodide (PI), cycloheximide (CHX) and nicotinamide (NIA) were obtained from Sigma-Aldrich, 8-CPT-cAMP was purchased from Biolog and Trichostatin A (TSA) was obtained from Calbiochem. Antibodies were: total p53 (DO-1, FL-393, Bp53-12), HDM2 (SMP14) and actin (C2) from Santa Cruz Biotechnology; HDM2 (IF2) from Calbiochem; HDM2 (4B2) was a kind gift from Dr A. Levine (Princeton University, NJ); acetyl-p53 (K373) and acetyl-p53 (K382) were from Epitomics and Cell Signaling, respectively.

Cell cultures, radiation treatment and cell death analysis. The BCP-ALL cell line Reh (18) was cultured as previously described (15). For treatment of cells with γ-radiation, cells were exposed to a 137 Cs source at a dose rate of 4.3 Gy/min using a Gammacell irradiator from MSD Nordion. To analyze cell death, cells were incubated with PI (20 μg/ml) at room temperature for 10 min before examination for PI uptake by flow cytometry.

Two-dimensional SDS-PAGE. For two-dimensional (2D) gel electrophoresis, cells were washed twice in saline and lysed in 7% trichloro-acetic acid (TCA) for 30 min on ice. After homogenization and centrifugation, the precipitated proteins were washed once in 5% TCA and three times in water-saturated ether to remove salts, each time followed by centrifugation at 13,000 rpm for 20 min at 4°C. The protein pellet was resuspended in sample buffer for 2D gel electrophoresis (7 M urea, 2 M thiourea, 100 mM dithiotreitol, 1.5% ampholyte 3-10, 0.5% ampholyte 5-6, 4% CHAPS). The protein concentration was measured by use of the Bradford method (19). Protein sample (100 μg) was diluted in rehydration buffer (7 M urea, 2 M thiourea, 100 mM dithiotreitol, 1.5% ampholyte 3-10, 0.5% ampholyte 5-6, 4% CHAPS, Bromophenol blue) to a final volume of 170 μl. Isoelectric focusing was performed using 7 cm pH 3.0-10.0 (Zoom Strip, Invitrogen Corp., Carlsbad, CA, USA) isoelectric focusing gel strips. Following rehydration of the strips in rehydration buffer, the strips were subjected to the electrophoresis program: 200 V for 40 min, 450 V for 30 min, 750 V for 30 min and 1,000 V for 1 h. Reduction and acetylation of sulfide bindings were performed according to the manufacturer's procedure. Second dimension gel electrophoresis was run for 1 h at 200 V. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membrane (Amersham Biosciences AB, Uppsala, Sweden) by standard electroblotting. p53 protein was detected using primary Bp53-12 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary HRP-conjugated mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA). Visualization of the proteins was performed using the Supersignal West Pico or Femto Chemiluminescent Substrate system (Pierce Biotechnology Inc., Rockford, IL, USA) and Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA).

Immunoblot analysis and immunoprecipitation. For immunoblot analysis, cells were lysed in radioimmunoprecipitation buffer [RIPA; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 50 mM NaF, 10 mM β-glycerophosphate, 0.1% SDS, 0.5% EDTA, 1 mM Na_3VO_4, 0.2 mM PMSF, 10 μg/ml leupeptin, 0.5% aprotinin). Equal amounts of protein were separated on a 10% SDS-PAGE. After transfer to a nitrocellulose membrane (GE Healthcare, Amersham, UK), proteins were detected by use of standard immunoblotting procedures.

For immunoprecipitation of HDM2 in complex with p53, cells were lysed in NP-40 lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin and 0.5% aprotinin]. Lysates containing 600 μg of protein were immunoprecipitated with the p53 antibody FL-393 followed by 50 μl of a 1:1 slurry of protein G-agarose (Upstate, Temecula, CA, USA). Beads were washed four times in lysis buffer, eluted in boiling 1X SDS buffer, and subjected to immunoblot analysis. For densitometric analysis, blots were analyzed using Genetools analysis (SynGene).

Statistical analysis. In all figures, the histograms represent the mean values, with error bars corresponding to SEM values.

Results

cAMP inhibits p53 accumulation and isoelectric point shift in IR-treated BCP-ALL cells on 2D-PAGE. We have previously shown that stimulation of cAMP signalling inhibits DNA damage-induced accumulation of p53 by facilitating its interaction with HDM2 (17). Because the interaction between p53 and HDM2 is known to be regulated by post-translational modifications of p53, we decided to examine whether cAMP affected the post-translational modifications of p53 in IR-treated cells. To this end, we performed 2D-immunoblotting with anti-p53 antibodies on lysates of Reh cells that were treated with IR in the absence or presence of forskolin. Reh is a BCP-ALL cell line that expresses wt p53 and forskolin is a plant-derived diterpene known to induce intracellular cAMP levels by activating adenylyl cyclase. As shown in Fig. 1, IR not only led to an increase in the abundance of p53, but it also led to accumulation of more acidic forms of p53, indicating that IR leads to modification of p53 by phosphorylation or acetylation. Interestingly, forskolin was found to inhibit the IR-induced accumulation and acidification of p53, suggesting that cAMP protects p53 from IR-induced post-translational modifications.

cAMP affects the DNA damage-induced acetylation of K373 and K382 on p53. Our recent result showing that cAMP has only a slight inhibitory effect on the IR-mediated phosphorylation of p53 (17), suggested that inhibition of acetylation might account for the ability of cAMP to reduce the IR-induced acidification of p53. To assess this assumption, Reh cells that were exposed to IR in the absence or presence of forskolin were harvested at 4 h post-IR and subjected to immunoblot analysis with antibodies specific for p53 acetylated at K373 and K382. Acetylation of p53 at these two residues is characteristically induced by IR (4). As shown in Fig. 2, IR led to an increase in acetylation of p53 at K373 and K382, and importantly, activation of the cAMP signalling pathway by forskolin inhibited this acetylation.
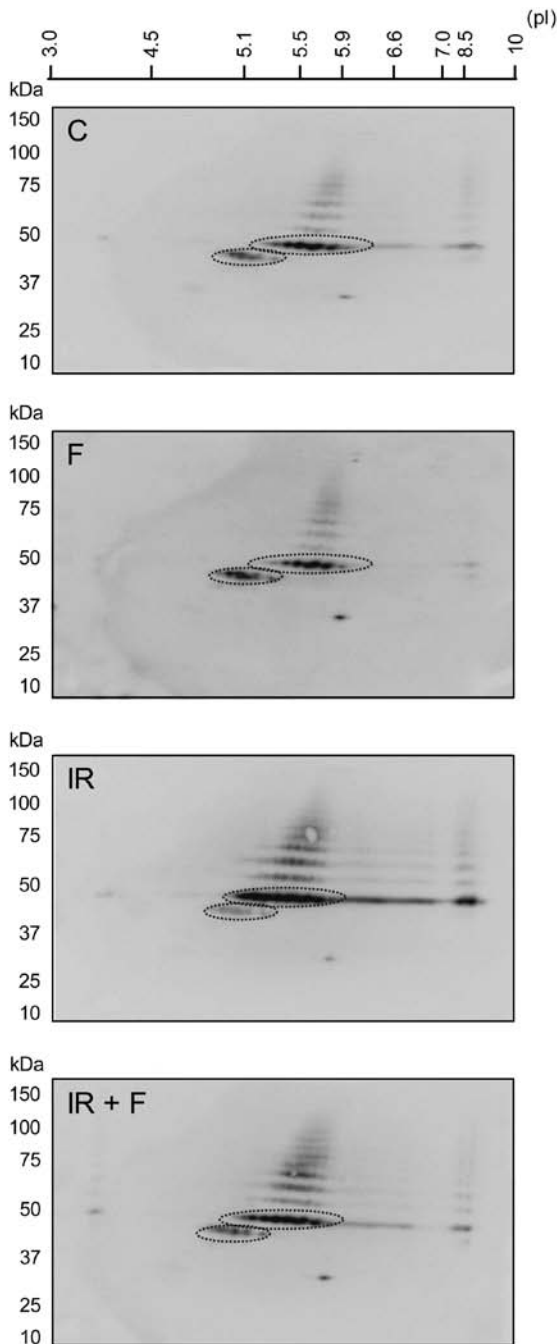
Figure 1. cAMP reverses the accumulation and isoelectric point (pI) of p53 in IR-treated BCP-ALL cells. Reh cells were pretreated with or without forskolin (60 μM) for 30 min before exposure to IR (10 Gy). Cells were harvested after 4 h before they were subjected to 2D-PAGE. The spots inside the large oval shows full length p53 while the spots in the small oval show isoforms of p53.

Figure 2. cAMP affects the DNA damage-induced acetylation of K373 and K382 on p53. (A) Reh cells were preincubated with or without TSA (0.2 μM), NIA (20 mM) or a combination of both 2 h prior to treatment with forskolin (60 μM) for 30 min. Cells were then exposed to IR (10 Gy), harvested after 4 h and subjected to immunoblot analysis with antibodies against total p53 (DO-1), acetylated p53 (K373 and K382) and actin. The immunoblot shows one representative experiment of three. (B) The immunoblots represented in A were subjected to densitometry, the average densitometric values of the p53, K373 and K382 protein bands as depicted. Treated samples have been normalized to their relevant control whose values were set to 1.

Following DNA damage, p53 is acetylated through the activity of HATs such as p300/CBP and pCAF, an event that is tightly regulated by the deacetylase activity of enzymes such as HDACs and SIRT1 (4). The cAMP signalling pathway has been linked to both SIRT activation and subcellular localization of HDACs (20-23), and we therefore hypothesized that cAMP antagonizes the IR-mediated acetylation of p53 through HDACs and/or SIRT1. To test this possibility, we examined the effects of the deacetylase inhibitors trichostatin A (TSA) and
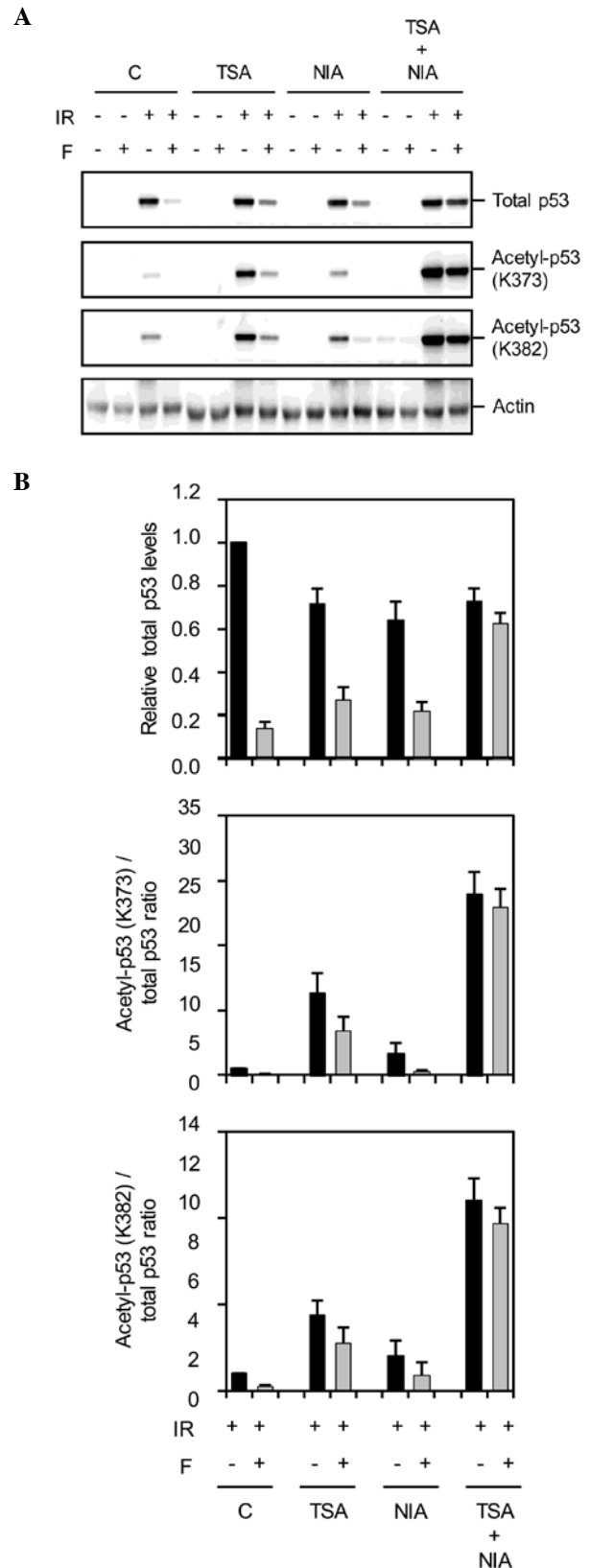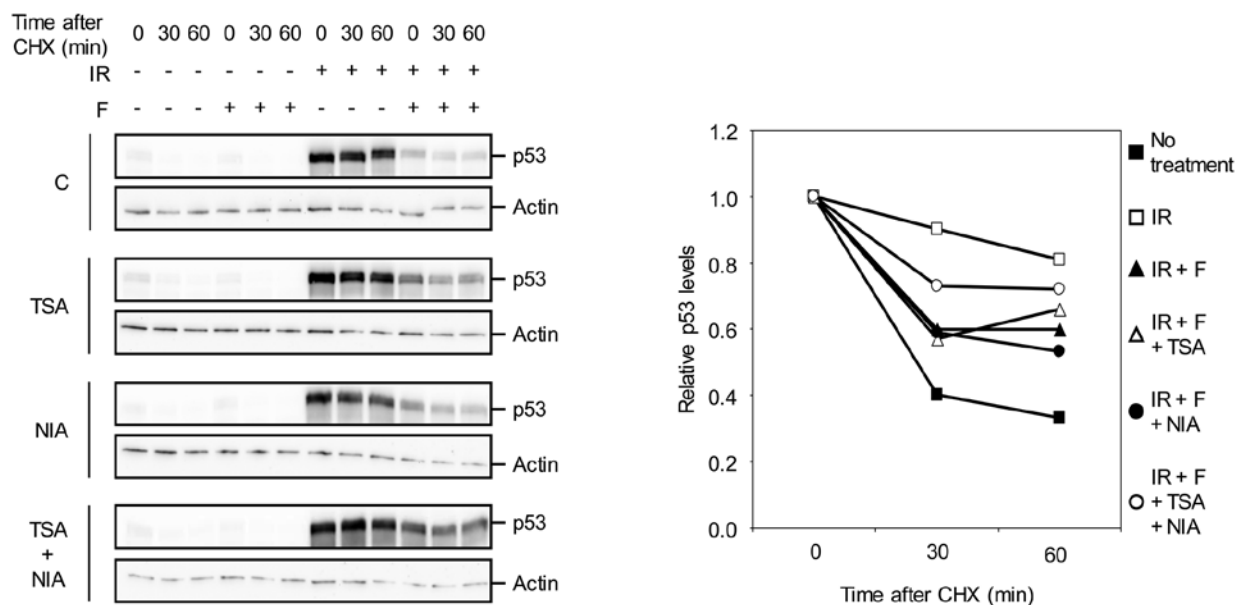
Figure 3. TSA and NIA inhibit the cAMP-induced destabilization of p53 in IR-treated cells. Reh cells were preincubated with or without TSA (0.2 μM), NIA (20 mM) or a combination of both 2 h prior to treatment with forskolin (60 μM) for 30 min. Cells were then exposed to IR (10 Gy), and after 4 h the cells were treated with CHX (25 μg/ml) and harvested at the indicated time points. Whole cell lysates were analyzed by immunoblot analysis using anti-p53 (DO-1) and anti-actin antibodies. The immunoblot shows one reproducible experiment of two. The immunoblots were scanned and the intensity of the p53 protein bands was quantitated and plotted with the value obtained for cells not treated with CHX set as 1.

nicotineamide (NIA) on the ability of cAMP to abrogate the IR-induced acetylation of p53. TSA is an inhibitor of HDACs whereas NIA inhibits the NAD$^+$-dependent deacetylases such as SIRT1. To this end, Reh cells were pretreated with TSA and/ or NIA before exposure to IR in the absence or presence of forskolin. Four hours post-IR, cells were harvested and subjected to immunoblot analysis sequentially with antibodies directed against p53 acetylated at K373, K382 as well as total p53. Treatment of cells with TSA or NIA alone or the combination of the two had minor effects on p53 acetylation under unstressed conditions (Fig. 2). In accordance with previous findings (16,24), exposure of cells to IR in the presence of TSA or NIA increased the IR-mediated acetylation of p53. However, pretreatment of cells with both TSA and NIA substantially further enhanced the p53 acetylation induced by IR. Interestingly, Fig. 2A and B also show that pretreatment of IR-exposed cells with both TSA and NIA almost completely abrogated the effect of forskolin on acetylated p53 levels, suggesting that the inhibitory effect of cAMP on IR-induced acetylation of p53 depends on HDACs and SIRT1 activities.

cAMP destabilizes IR-induced p53 through acetylation inhibition. The immunoblot shown in Fig. 2A revealed that in addition to reversal of forskolin-induced deacetylation of p53, the combination of TSA and NIA also abrogated the inhibitory effect of forskolin on IR-induced accumulation of p53. Given our previous finding that cAMP facilitated the degradation of p53, we wished to examine whether TSA and NIA abolished the inhibitory effect of cAMP on IR-mediated accumulation of p53 by increasing the stability of p53. To this end, Reh cells treated with IR in the absence or presence of forskolin were exposed to the protein synthesis inhibitor cycloheximide (CHX) and then analyzed for the level of p53 by western blot analysis.

In agreement with our previous finding, forskolin substantially decreased the half-life of p53 in IR-treated cells (Fig. 3). Interestingly, combination of TSA and NIA alleviated the inhibitory effect of forskolin on p53 stability in IR-treated cells. This result indicates that simultaneous inhibition of HDACs and SIRT1 antagonizes the destabilizing effect of cAMP on p53, and implicates the modulation of p53 acetylation as the means by which cAMP regulates the stability of p53.

cAMP facilitates p53-HDM2 binding through p53 acetylation. We have shown that cAMP abrogates the DNA damage-induced stabilization of p53 by promoting its interaction with HDM2 (17). This, together with the finding that p53 acetylation has an inhibitory effect on its association with HDM2 (6,25) suggested deacetylation of p53 as the mechanism by which cAMP enhances the p53-HDM2 interaction in IR-treated cells. To assess this hypothesis, we examined the formation of p53-HDM2 complexes under conditions in which the inhibitory effect of cAMP on IR-induced acetylation of p53 is blocked. For this purpose, Reh cells were pretreated with TSA or NIA alone or in combination before exposure to IR in the absence or presence of forskolin. Cells were harvested at 4 h after IR, and the lysates were subjected to immunoprecipitation with anti-p53 antibodies followed by immunoblot analysis with antibodies against p53 and HDM2. In accordance with our previous results, pretreatment of cells with forskolin prevented the IR-induced dissociation of p53 from HDM2 (Fig. 4). Importantly, TSA and NIA together alleviated the facilitating effect of forskolin on the p53-HDM2 interaction and reduced the level of HDM2 in complex with p53 to a level comparable to that found in IR-only treated cells. This result suggests that cAMP counteracts the IR-induced p53-HDM2 dissociation through inhibition of p53 acetylation.
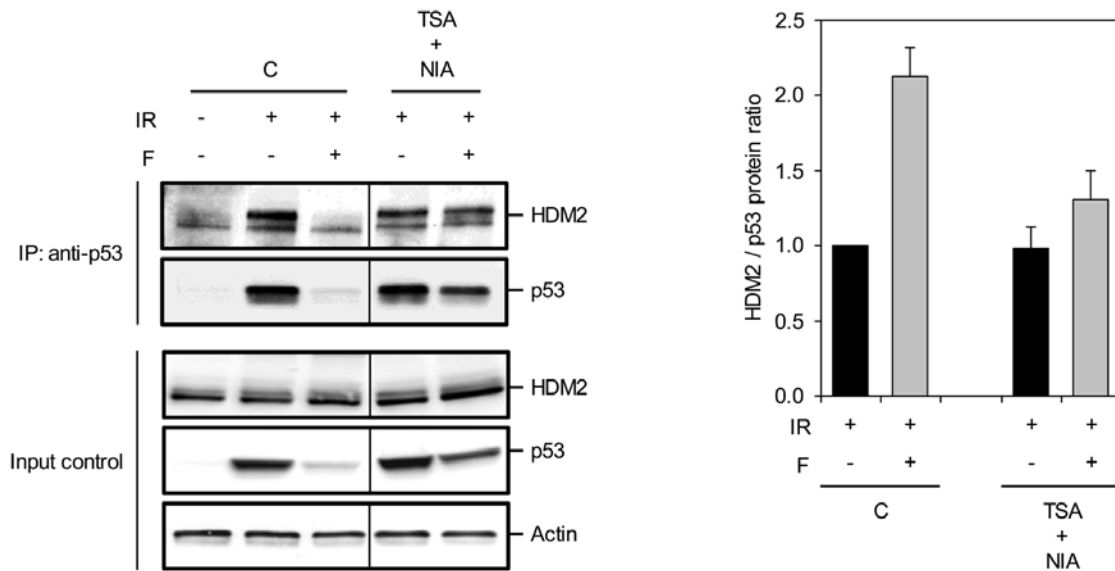
Figure 4. TSA and NIA inhibit the facilitating effect of cAMP on the interaction of p53 with HDM2 in IR-treated cells. Reh cells were pretreated with or without the combination of TSA (0.2 μM) and NIA (20 mM) for 2 h before addition of forskolin (60 μM). After 30 min, the cells were exposed to IR (10 Gy) and harvested after 4 h. Whole cell extracts were prepared and subjected to immunoprecipitation with anti-p53 (FL-393). The recovered proteins were resolved on SDS-PAGE and subjected to immunoblot analysis using anti-HDM2 (a combination of SMP14, IF2 and 4B2) and anti-p53 (DO-1) antibodies. The immunoblot in the left panel shows one representative experiment of three. In the right panel, the intensity of protein bands was quantified densitometrically and the ratio of HDM2 relative to p53 was calculated and plotted with the value obtained for cells not treated with forskolin, TSA and NIA set as 1 (n=3).
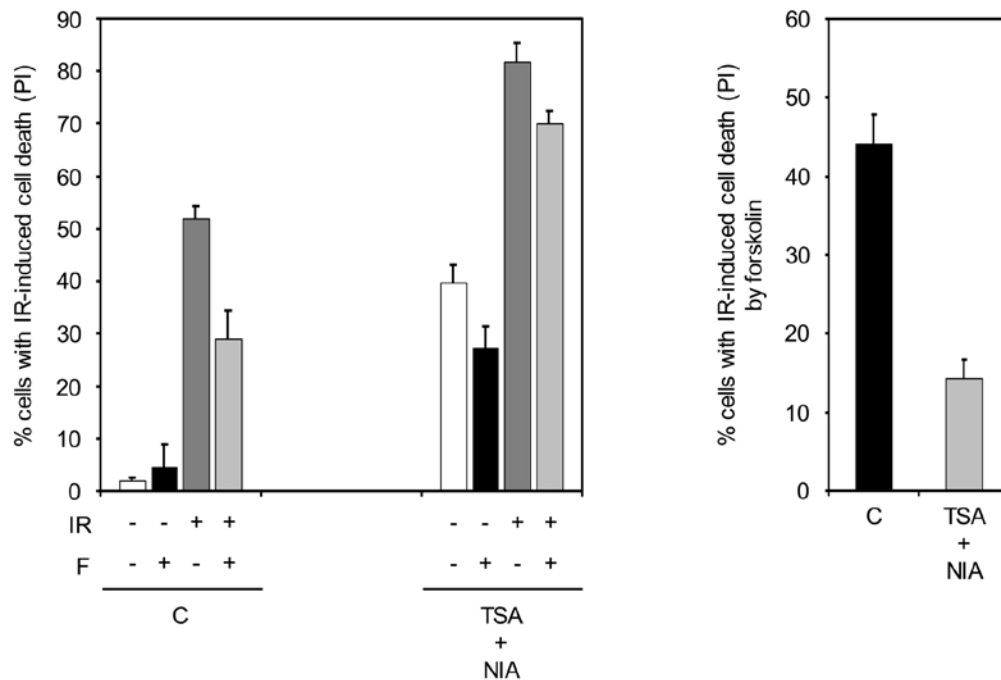

Figure 5. Inhibition of deacetylases attenuates the inhibitory effect of cAMP on IR-induced cell death. Reh cells were pretreated with or without the combination of TSA (0.2 μM) and NIA (20 mM) for 2 h before addition of forskolin (60 μM). Cells were then incubated for 20 h before PI staining and FACS analysis (n=4). The histogram in the right panel depicts percent inhibition of IR-induced cell death by forskolin in the absence or presence of TSA and NIA.

cAMP attenuates the IR-induced apoptosis by inhibiting p53 acetylation. Initiation of a p53-dependent apoptotic program is dependent on acetylation of p53 (10). Given that inhibition of HDACs and SIRT1 abrogates the ability of cAMP to suppress the IR-induced acetylation of p53, we wished to examine whether blocking the HDACs and SIRT1 activities could alle-viate the inhibitory effect of cAMP on IR-mediated apoptosis. To this end, Reh cells were pretreated with TSA and NIA before exposure to IR in the absence or presence of forskolin and then examined for cell death. As shown in Fig. 5, the combined inhi-bition of HDACs and SIRT1 opposed the inhibiting effect of forskolin on IR-induced cell death, suggesting that inhibition of

p53 acetylation plays an important role in the ability of cAMP to inhibit IR-mediated cell death.

Discussion

Elevation of intracellular cAMP levels has an inhibitory effect on DNA damage-induced apoptosis in normal lymphoid cells as well as BCP-ALL cells (16). We have previously shown that this inhibitory effect of cAMP is mediated through its ability to abrogate the DNA damage-induced p53-HDM2 dissociation, leading to restoration of p53 degradation in DNA-damaged cells (17). Here, we provide evidence for the mechanism by which cAMP promotes the interaction of p53 with HDM2 and thereby prevents DNA damage-induced cell death. We show that activation of cAMP signalling attenuates the acetylation of p53 after DNA damage, thus facilitating the p53-HDM2 association.

The p53 levels are tightly regulated by its HDM2-mediated ubiquitination and the ensuing proteasomal degradation. Because ubiquitnation and acetylation of p53 occur at the same lysine residues, the acetylation of p53 at lysine residues in its C-terminus inhibits the HDM2-mediated ubiquitination and degradation of p53 (6,26). Furthermore, p53 acetylation has also been shown to block its interaction with HDM2 (10). Thus, acetylation of p53 plays a central role in regulation of p53 stability and activity following DNA damage by modulating its interaction with HDM2. Acetylation of p53 is carried out by a number of acetyl transferases, among which p300/CBP is responsible for acetylating the C-terminal lysine residues of p53 (5,7-9). The steady-state level of acetylated p53 is achieved by HDAC1 and SIRT1 deacetylases. Indeed, deacetylation of p53 by these two enzymes is required for restraining hyper-activation of p53 after DNA damage (4). Our finding that cAMP inhibits DNA damage-induced acetylation of p53 provides a mechanistic explanation for the ability of cAMP to abrogate a p53-dependent response following DNA damage. By inducing the deacetylation of p53, cAMP favours p53-HDM2 interaction and thus abrogates the DNA damage-mediated stabilization of p53. This ability of cAMP requires deacetylation of p53 at both HDAC- and SIRT1-targeted sites, because only simultaneous inhibition of both HDACs and SIRT1 reverses the destabilizing effect of cAMP on p53. In addition, through induction of p53 deacetylation, cAMP attenuates DNA damage-induced events downstream of p53. This conclusion is supported by the observation that inhibition of HDACs and SIRT1 reverses the inhibitory effect of cAMP on the DNA damage-mediated apoptosis. Thus, cAMP, through modulation of p53 acetylation, inhibits stabilization of p53 and prevents subsequent cell death induced by DNA damage.

In theory, cAMP could inhibit DNA damage-induced acetylation of p53 by two distinct mechanisms: (i) by inhibiting the enzymes that acetylate p53 or (ii) by stimulating the deacetylases that target p53. Supported by studies showing that cAMP signalling stimulates the deacetylase activity of SIRT1 and promotes the nuclear retention of HDACs (20-23), we favour the second possibility and suggest that cAMP, by utilizing HDACs and SIRT1, maintains p53 in a hypoacetylated state, thus leading to its HDM2-dependent degradation even in the face of DNA damage. Given the cytotoxic effect of histone deacetylase inhibition (27,28), we are tempted to suggest that combination of inhibitors of cAMP signalling with histone deacetylase inhibitors might prove beneficial for increasing the antitumour activity of histone deacetylase inhibitors.

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