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The cAMP signaling system inhibits the repair of γ-ray-induced DNA damage by promoting Epac1-mediated proteasomal degradation of XRCC1 protein in human lung cancer cells

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ABSTRACT

Cyclic AMP is involved in the regulation of metabolism, gene expression, cellular growth and proliferation. Recently, the cAMP signaling system was found to modulate DNA-damaging agent-induced apoptosis by regulating the expression of Bcl-2 family proteins and inhibitors of apoptosis. Thus, we hypothesized that the cAMP signaling may modulate DNA repair activity, and we investigated the effects of the cAMP signaling system on γ-ray-induced DNA damage repair in lung cancer cells. Transient expression of a constitutively active mutant of stimulatory G protein (GαsQL) or treatment with forskolin, an adenylyl cyclase activator, augmented radiation-induced DNA damage and inhibited repair of the damage in H1299 lung cancer cells. Expression of GαsQL or treatment with forskolin or isoproterenol inhibited the radiation-induced expression of the XRCC1 protein, and exogenous expression of XRCC1 abolished the DNA repair-inhibiting effect of forskolin. Forskolin treatment promoted the ubiquitin and proteasome-dependent degradation of the XRCC1 protein, resulting in a significant decrease in the half-life of the protein after γ-ray irradiation. The effect of forskolin on XRCC1 expression was not inhibited by PKA inhibitor, but 8-pCPT-2′-O-Me-cAMP, an Epac-selective cAMP analog, increased ubiquitination of XRCC1 protein and decreased XRCC1 expression. Knockdown of Epac1 abolished the effect of 8-pCPT-2′-O-Me-cAMP and restored XRCC1 protein level following γ-ray irradiation. From these results, we conclude that the cAMP signaling system inhibits the repair of γ-ray-induced DNA damage by promoting the ubiquitin–proteasome dependent degradation of XRCC1 in an Epac-dependent pathway in lung cancer cells.

1. Introduction

Mammalian cells are constantly subjected to their DNA damage from exogenous DNA-damaging agents, such as ionizing radiation and chemical agents, and endogenous processes, such as replication and programmed genome rearrangements [1]. The resulting DNA damage may induce mutations that cause the loss or incorrect transmission of genetic information, which in turn can cause developmental abnormalities, cell death and tumorigenesis. Thus, eukaryotic cells have evolved several mechanisms to monitor the integrity of their genome and to repair the damaged DNA [2].

The X-ray repair cross-complementing protein 1 (XRCC1) gene was the first mammalian gene isolated that affects cellular sensitivity to ionizing radiation [3]. Human XRCC1 is a 70-kDa protein that contains 633 amino acid residues. XRCC1 does not have any enzyme activity, but it has at least three discrete domains that can interact specifically with enzymes involved in DNA repair [4]. Thus, XRCC1 plays an essential role in base excision repair and single strand break repair [5]. Epidemiological association of XRCC1 polymorphisms and carcinogenesis have been studied extensively in incidences of breast cancer and lung cancer [6], and XRCC1 was reported to have potential as a predictive marker in lung, head and neck cancer patients treated with radiation and chemotherapy [7].

The cyclic AMP (cAMP) signaling system is activated by cAMP, a second messenger molecule formed from ATP by adenylyl cyclase. The activity of adenylyl cyclase is stimulated by stimulatory heterotrimeric GTP-binding proteins (G proteins), which are activated by various external signals bound to G protein-coupled receptors [8]. cAMP activates cAMP-dependent protein kinase (PKA), exchange proteins directly activated by cAMP (Epac), and cyclic nucleotide-gated ion channels, and thereby regulates a variety of cellular responses. The cAMP signaling system is involved in sensory perception, metabolic control, and the regulation of gene

Abbreviations: 8-pCPT-2′-O-Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; Epac: PKA, cAMP-dependent protein kinase; XRCC1, X-ray repair cross-complementing protein 1.

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expression, cellular growth, differentiation and proliferation [9,10]. In previous studies, we found that the cAMP signaling system modulates DNA-damaging agent-induced apoptosis by regulating the expression of Bcl-2 family proteins and inhibitors of apoptosis (IAPs) [11–13]. Thus, we hypothesized that the cAMP signaling system may modulate DNA repair activity, and we investigated the effects of the cAMP signaling system on radiation-induced DNA damage repair in H1299 lung cancer cells. We found that the cAMP signaling pathway inhibits the repair of γ-ray induced DNA damage by promoting the proteasomal degradation of XRCC1 protein, and it is mediated by EPAC1.

2. Materials and methods

2.1. Cell culture and reagents

Human non-small cell lung cancer cells, H1299 and A549 cells (Korea Cell Line Bank, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (JBI, Korea) and 100 units/ml penicillin/streptomycin. Cells were maintained in a 5% CO_2 incubator at 37 °C. H89, cycloheximide (CHX), dimethyl sulfoxide (DMSO), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), 8-pCPT-2′-O-Me-cAMP, and avidin-labeled 8-oxo-7,8-dihydro-2′-deoxyguanosine fluorescein isothiocyanate isomer I (avidin-8-oxo-dG-FITC) were purchased from Sigma (St. Louis, MO, USA); forskolin, isobutylmethylxanthine (IBMX), and MG-132 were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Expression constructs and transient transfection

H1299 cells were transfected with a constitutively active mutant of a long form of Gαs (GαsQ227L) in a pcDNA3 vector (Invitrogen, Paisley, UK) using the calcium phosphate method. The GαsQL mutant contains a mutation of a glutamine residue that is essential for the intrinsic GTPase activity [14]. Plasmid for expression of decahistidine-tagged wild-type XRCC1 (PCD2E-XH) was a gift from Dr. Keith W. Caldecott (University of Sussex, UK), and plasmid for expression of dominant negative PKA in MT-REVab was a gift from Dr. G. Stanley McKnight (University of Washington, WA, USA). The sequence of small hairpin RNA for Epac1 was 5′-CCG GGCAGGACTTCAACCGTATCATCTCGAGATGATACGGTTGAAGTCCTG CTTTTTG-3′.

2.3. Irradiation with γ-rays

Cells were plated in 10-cm dishes and incubated until they became 60% confluent. Cells were then exposed to γ-rays from a ^{137}Cs source at a delivering dose rate of 170.93 cGy/min.

2.4. Immunoblot analysis

Western blotting was performed as previously described [11]. Antibodies against Gαs, XRCC1, and p-CREB (S133) were purchased from Santa Cruz Biotechnology (CA, USA), an antibody against β-actin was from Sigma (St. Louis, MO, USA), and the EE-tag antibody was from Covance (Princeton, NJ, USA). The proteins on the blots were visualized by the Enhanced Chemiluminescence (ECL) reagent (Thermo scientific, Waltham, MA), and the densities of the bands were quantified using Multi Gauge v2.3 software (Fuji, Tokyo, Japan).

2.5. Real-time PCR

Real-time quantitative RT-PCR was performed as described previously [13]. The primers used were as follows: XRCC1, 5′-CGCTG GGGAGCAAGACTATG-3′ and 5′-CAAATCCAACTTCCTCTTCC-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCT GTA-3′.

2.6. Analysis of DNA damage

Confocal analysis of 8-oxo-dG: cells were fixed in 4% paraformaldehyde for 20 min, permeated with 0.5% TritonX-100 for 10 min, and blocked in 2% bovine serum albumin for 1 h. The cells were incubated with avidin-8-oxo-dG-FITC (1:100) or DAPI (0.5 μl/ml) for 1 h at room temperature. The stained cells were observed using a confocal microscope (LSM 501 META, Carl Zeiss, Inc. USA). Comet assay: a comet assay was performed using a Comet Assay kit (Trevigen, Inc. MD, USA) [15]. DNA was visualized by staining with 1 μg/ml ethidium bromide for 5 min in a refrigerator. The comet tail was captured and scored according to labeled DNA intensity using Komet software (Andor Technology, Belfast, UK).

2.7. Data analysis

At least three independent experiments were conducted for all of the analyses, and data are presented as the mean ± standard errors (SE). The nonparametric Mann–Whitney U test was used to analyze mean values, and a p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. The cAMP signaling system inhibited the repair of radiation-induced DNA damage in H1299 lung cancer cells

We assessed the effect of cAMP signaling system on DNA damage repair by expressing a constitutively active Gαs or treating with forskolin, which activates the cAMP signaling system by stimulating adenylyl cyclase. Transient expression of the constitutively active GαsQL augmented radiation-induced DNA damage and inhibited repair of the damage in H1299 lung cancer cells. Expression of GαsQL increased the green fluorescence of 8-oxo-deoxyguanosine (8-oxo-dG) by 3.27-fold over vector-transfected cells following γ-ray irradiation (Fig. 1A). Augmentation of γ-ray-induced DNA damage by GαsQL expression was confirmed by a comet assay, which also showed 1.9 ± 0.3-fold increase in tail intensity over vector control (Supplementary Fig. S1). The fluorescence of 8-oxo-dG in GαsQL-expressing cells did not return to the basal level until 3 h after irradiation, in contrast to the fluorescence in vector-transfected cells that returned to the basal level by 1 h after irradiation (Supplementary Fig. S2). Similar to the expression of GαsQL, treatment with forskolin also inhibited the repair of radiation-induced 8-oxo-dG DNA damage (Fig. 1B). This result shows that the cAMP signaling system inhibits repair of radiation-induced DNA damage in H1299 cells.

3.2. The cAMP signaling system inhibited the repair of radiation-induced DNA damage by decreasing expression of XRCC1 in lung cancer cells

To probe the mechanism how the cAMP signaling system inhibits the repair of radiation-induced DNA damage, the effect of Gαs on the expression of XRCC1 was analyzed. Expression of GαsQL increased the basal XRCC1 protein level, but decreased the expression of XRCC1 following γ-ray irradiation. In the vector-transfected control cells, the basal XRCC1 protein level was low, but the expression of XRCC1 protein increased by 4.78 ± 0.45-fold following γ-ray irradiation (Fig. 2A). The expression of XRCC1

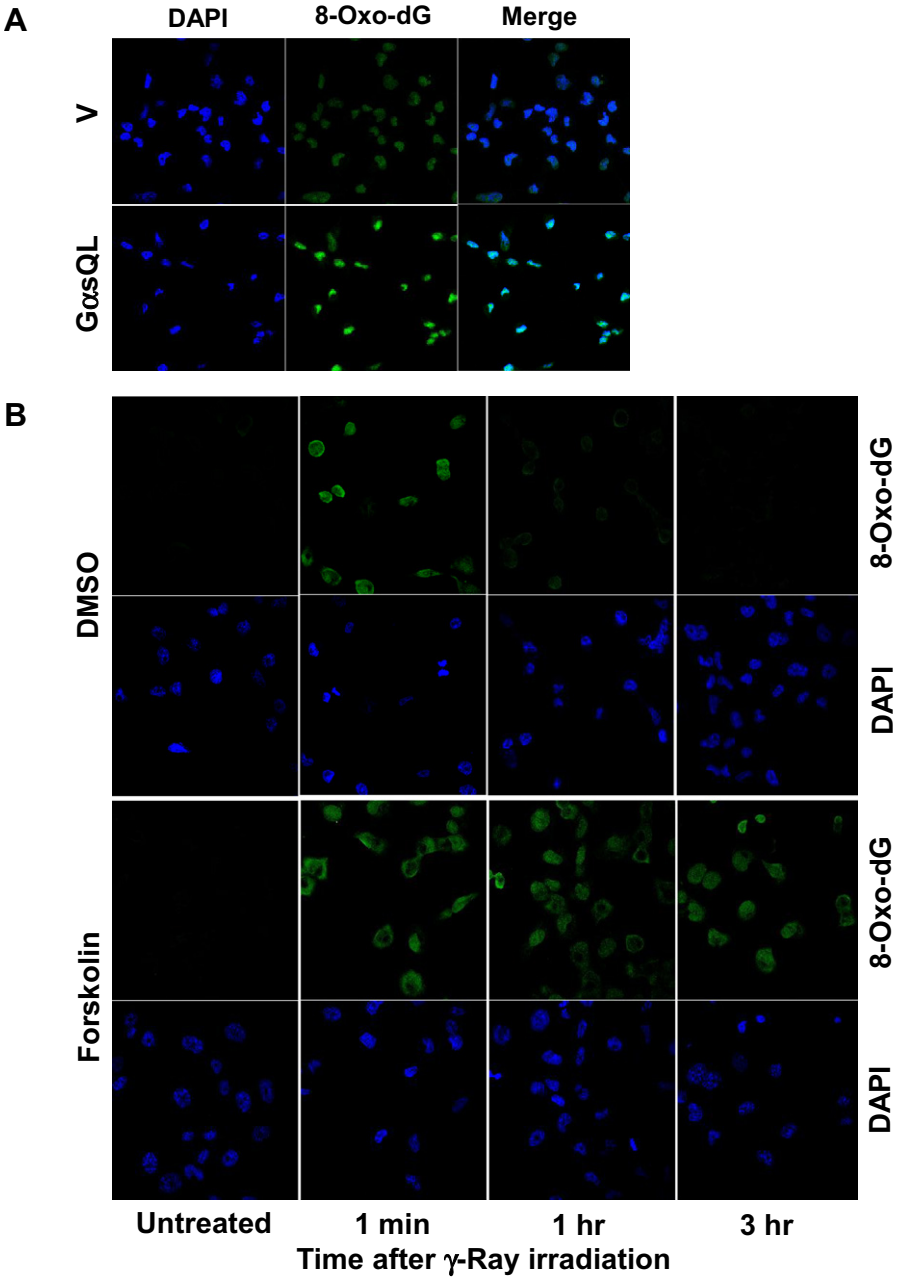

Fig. 1. The cAMP signaling system inhibited radiation-induced DNA damage in H1299 lung cancer cells. (A) Effects of GαsQL expression on the formation of 8-oxo-dG following γ-ray irradiation. H1299 cells were transfected with GαsQL or a pcDNA3 vector (V), incubated for 24 h, and irradiated with γ-rays (5 Gy). Then the resulting DNA-damage was assessed at 30 min by staining with DAPI or an avidin-8-oxo-dG-FITC and confocal microscopy. (B) Effects of forskolin on the removal of γ-ray-induced 8-oxo-dG. The H1299 cells were pre-treated with forskolin (40 μM) or DMSO for 30 min and then exposed to γ-rays (5 Gy). The resulting DNA damage was assessed at the indicated times by staining with DAPI or avidin-8-oxo-dG-FITC.

mRNA was not changed significantly in Gαs-transfected cells, γ-ray-irradiated cells, and irradiated Gαs-transfected cells ($p > 0.05$).

Pretreatment with forskolin decreased the expression of XRCC1 when cells were exposed to γ-ray irradiation, but did not significantly change the basal expression level of the XRCC1 protein (Fig. 2B). Pretreatment with isoproterenol, a Gαs-coupled receptor agonist, also decreased the γ-ray-induced expression of XRCC1 without altering the basal expression level (Supplementary Fig. S3). Similar to H1299 lung cancer cells, treatment of A549 lung cancer cells with forskolin also inhibited the γ-ray-induced expression of XRCC1 (Supplementary Fig. S4). Exogenous expression of XRCC1 abolished the inhibitory effect of forskolin and reduced 8-oxo-dG fluorescence to the basal level by 30 min (Fig. 2C). This result indicates that the cAMP signaling system inhibits repair of γ-ray-induced DNA damage by decreasing expression of XRCC1 in lung cancer cells.

3.3. The cAMP signaling system decreased XRCC1 expression by promoting the degradation of XRCC1 protein

To investigate the mechanism that decreases the radiation-induced expression of XRCC1 by the cAMP signaling system, the effect of forskolin on the degradation of XRCC1 was analyzed, because the level of XRCC1 mRNA was not changed significantly.

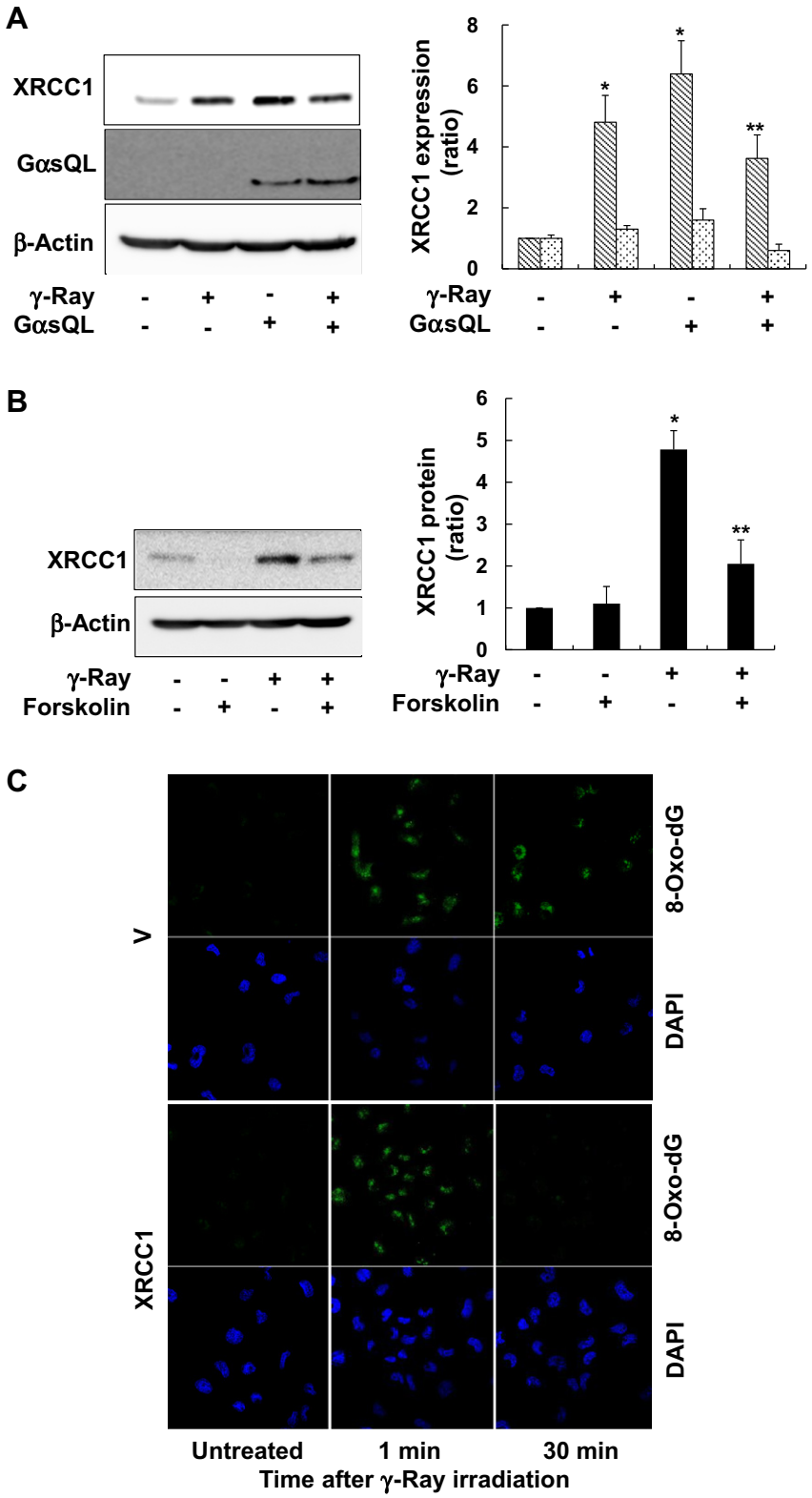

Fig. 2. The cAMP signaling system inhibited radiation-induced DNA damage by decreasing radiation-induced XRCC1 expression in H1299 lung cancer cells. (A) Effects of GαsQL on radiation-induced XRCC1 expression. Twenty-four hours after transfection with GαsQL or the vector (V), the H1299 cells were irradiated with γ-rays (5 Gy) and incubated for 30 min further before harvesting for analysis. The expression of XRCC1 and GαsQL was analyzed by western blotting. β-Actin was used as a loading control. The expression of XRCC1 mRNA was assessed by real-time PCR, and the β2-microgloblin was used as a control. The striped bars represent the XRCC1 protein, and the dot bars represent XRCC1 mRNA. (B) Effects of forskolin on radiation-induced XRCC1 expression. The H1299 cells were treated with 40 μM forskolin for 30 min and then irradiated with γ-rays (5 Gy). After incubation for 30 min, the expression of XRCC1 and β-actin was assessed by western blotting. (C) Effects of exogenous expression of XRCC1 on the removal of 8-oxo-dG in forskolin-pretreated cells. XRCC1 expression construct or a vector (V) was transfected to H1299 cells, and after 24 h incubation, the cells were with 40 μM forskolin for 30 min and then irradiated with γ-rays (5 Gy). The resulting DNA-damage was assessed at 30 min by staining with DAPI or a FITC-8-oxo-dG antibody and confocal microscopy. The histograms present the means and standard errors of at least three independent experiments, and the asterisk (*) indicates a statistically significant difference from the vector-transfected control cells or untreated cells; the double asterisks (**) represent a statistically significant difference ($p < 0.05$) from the irradiated vector-transfected control cells ($p < 0.05$, Mann–Whitney U test).

Treatment with forskolin promoted the degradation of XRCC1 following γ-ray irradiation. The half-life of the XRCC1 protein in forskolin-treated H1299 cells decreased to 13 ± 3.0 min from 31 ± 3.6 min in DMSO-treated control cells ($p < 0.05$, Fig. 3A). Because XRCC1 was reported to be degraded by proteasomal system [16], the effect of proteasomal inhibitor on the forskolin-promoted degradation of XRCC1 was analyzed. Treatment with a proteasomal inhibitor, MG132, completely blocked the forskolin-promoted degradation of XRCC1 protein, resulting in an increase in the protein amount more than the control level (Fig. 3B). Then the effect of forskolin on ubiquitination of XRCC1 was analyzed. Forskolin pretreatment increased the ubiquitination of XRCC1 following γ-ray irradiation (Fig. 3C). This result indicates that the cAMP signaling system decreases XRCC1 expression by promoting the ubiquitin proteasome-dependent degradation of XRCC1 protein.

3.4. EPAC1 mediated the promotion of XRCC1 degradation by the cAMP signaling system

To determine which effector molecule mediates cAMP-promoted degradation of XRCC1, we analyzed the role of PKA and EPAC. Treatment with H89, a PKA inhibitor, had no effect on the forskolin-promoted degradation of XRCC1 (Fig. 4A), but pretreatment with 8-pCPT-2′-O-Me-cAMP, an EPAC-selective cAMP analog, decreased expression of XRCC1 following γ-ray irradiation, which was neither blocked by treatment with H-89 nor by expression of dominant negative PKA (Fig. 4B). Knockdown of EPAC1 by shRNA abolished the effect of 8-pCPT-2′-O-Me-cAMP and restored the radiation-induced expression of XRCC1 (Fig. 4C), and pretreatment with 8-pCPT-2′-O-Me-cAMP increased ubiquitination of XRCC1 protein (Fig. 4D). This result indicates that Epac mediates the promotion of XRCC1 degradation induced by the cAMP signaling system.

4. Discussion

This study was performed to determine whether the cAMP signaling system can modulate the repair of γ-ray-induced DNA damage in lung cancer cells and, if so, to elucidate the underlying molecular mechanism. This study shows that the cAMP signaling system inhibits the repair of γ-ray-induced DNA damage by decreasing the expression of XRCC1 protein in lung cancer cells and that it decreases XRCC1 expression by promoting EPAC-mediated ubiquitin–proteasome dependent degradation of XRCC1.

The finding that the cAMP signaling system inhibits radiation-induced DNA damage in lung cancer cells is evidenced by the result that the expression of constitutively active Gαs or pretreatment with forskolin, an adenylyl cyclase activator that results in the formation of cAMP and the activation of the cAMP signaling system, augmented radiation-induced DNA damage, and that such activation of the cAMP signaling system inhibited the repair of γ-ray-induced DNA damage. This finding corresponds with previous reports that the cAMP signaling system is involved in the DNA damage response and cellular resistance to DNA damaging agents. For instance, the activity of PKA is involved in resistance to cisplatin and its associated DNA repair capacity [17], and high cAMP levels reduce the double-strand break (DSB)-rejoining fidelity [18].

Then, we found that the cAMP signaling system inhibits radiation-induced DNA damage by decreasing the expression of (the DNA repair protein), XRCC1. This finding is supported by the fact that the expression of constitutively active Gαs or pretreatment with either forskolin or isoproterenol decreased the radiation-induced expression of XRCC1 and inhibited DNA repair, and that exogenous expression of XRCC1 abolished the DNA repair inhibiting

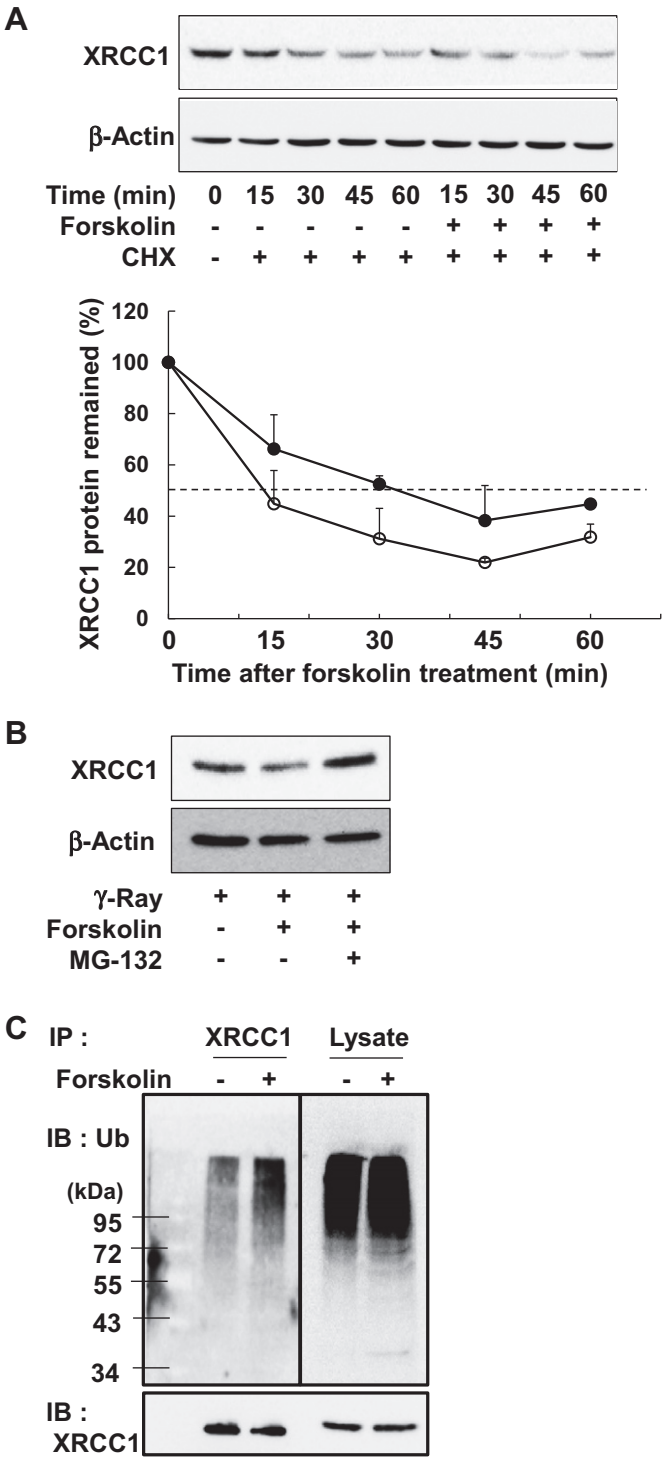

Fig. 3. Forskolin promoted ubiquitin–proteasome dependent degradation of XRCC1 following γ-ray irradiation in H1299 cells. (A) Effects of forskolin on the degradation of XRCC1 proteins. H1299 cells were pre-treated with 10 μg/ml cycloheximide (CHX) and 40 μM forskolin or DMSO for 30 min, and then cells were irradiated with γ-rays (5 Gy). The cells were harvested at the indicated times, and XRCC1 protein was analyzed by western blotting. The graph shows the average densities of XRCC1 from three independent analyses. (B) Effects of MG-132 on the forskolin-promoted degradation of XRCC1. The cells were pretreated with 40 μM MG-132, and the remaining XRCC1 protein levels were quantified 30 min after irradiation by Western blotting. (C) Effects of forskolin on the ubiquitination of XRCC1 following γ-ray irradiation. The cells were treated with 40 μM forskolin or DMSO for 30 min in the presence of 40 μM MG-132 before the γ-ray exposure. Ubiquitinated XRCC1 was immunoprecipitated with an antibody against either ubiquitin or XRCC1, and then analyzed by western blotting.

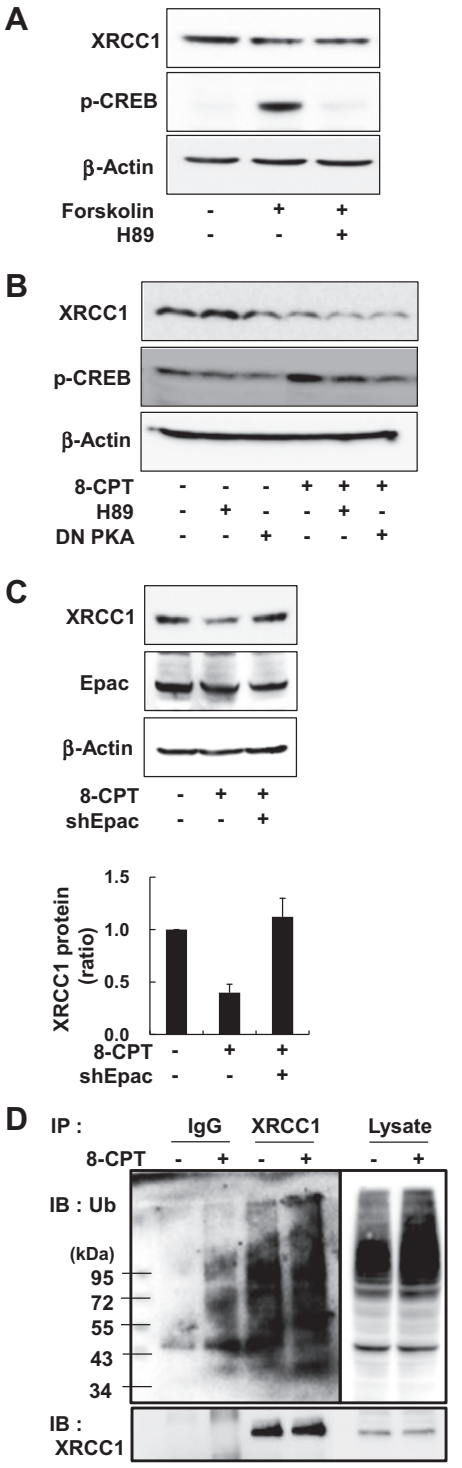

Fig. 4. Epac1 mediated XRCC1 degradation in H1299 cells. (A) Effects of H89 on XRCC1 expression in forskolin-pretreated cells. The cells were treated with 20 μM for 30 min, and irradiated with γ-ray (5 Gy). (B) Effect of 8-pCPT-2′-O-Me-cAMP on radiation-induced XRCC1 expression. The cells were treated with 20 μM 8-pCPT-2′-O-Me-cAMP (8-CTP) in the presence of 20 μM H89 or dominant negative PKA (DN-PKA). (C) Effects of Epac1 knockdown on the radiation-induced XRCC1 expression. H1299 cells were transfected with shEpac1, and after 24 h, they were treated with 20 μM 8-pCPT-2′-O-Me-cAMP for 30 min. Then the cells were irradiated with γ-rays (5 Gy), and after 30 min, the expression of XRCC1 was assessed by western blotting. The histograms present the means and standard errors of at least three independent experiments, and the asterisk (*) indicates a statistically significant difference from the vector-transfected control cells ($p < 0.05$, Mann–Whitney U test). (D) Effects of 8-pCPT-2′-O-Me-cAMP on radiation-induced XRCC1 ubiquitination.

effect of forskolin. Isoproterenol is an agonist for the β-adrenergic receptor, which is coupled with the Gαs protein to stimulate adenylyl cyclase. Furthermore, a similar decrease in XRCC1 expression following γ-ray irradiation was observed in another lung cancer cell line, forskolin-pretreated A549 cells, suggesting that the cAMP signaling system may have the similar effect on XRCC1 expression in other lung cancer cells.

To our knowledge, this study is the first report that shows the regulation of XRCC1 expression by the cAMP signaling system, except for one paper that described the promoter region of baboon XRCC1 as having a putative cAMP response element, the mutation of which did not significantly change the promoter activity [19]. XRCC1 is a scaffold protein that interacts with several DNA repair proteins and thereby coordinates and facilitates DNA base excision repair and repair of DNA single strand breaks [4,5]. Therefore, this study presents a novel mechanism, which involves the regulation of XRCC1 expression, for modulation of DNA repair by the cAMP signaling system, and this mechanism is considered to mediate the potential DNA repair modulating effect of various molecules acting on the cAMP signaling system. Furthermore, XRCC1 is assumed to involve in carcinogenesis and cancer cell death, because it facilitates DNA repair. The expression level of XRCC1 was reported to predict cancer-specific survival after various cancer treatments [7]. The polymorphisms of XRCC1 gene has been intensively studied for the association with the variability in toxicological response to environmental agents [6], the risk of developing cancer of lung, breast, stomach and liver [20], and the clinical response of various cancers to chemotherapy and radiotherapy [21,22]. Therefore, the regulation of XRCC1 expression by the cAMP signaling system might influence on carcinogenesis and responses to cancer treatments, so the cAMP signaling system can be used as a potential target for prevention of carcinogenesis or improvement of the efficiency of various cancer treatments.

From the study on how the cAMP signaling system decreases γ-ray-induced expression of XRCC1, we found the cAMP signaling system decreases γ-ray-induced expression of XRCC1 by promoting the proteasomal degradation of XRCC1, which is mediated by Epac. This conclusion is corroborated by the result that forskolin pretreatment promoted the ubiquitination and degradation of XRCC1 protein following γ-ray irradiation, which was completely inhibited by a proteasome inhibitor, MG132. Furthermore, the effect of forskolin on XRCC1 expression was not inhibited by PKA inhibitor, whereas 8-pCPT-2′-O-Me-cAMP, an Epac-selective cAMP analog [23], increased ubiquitination of XRCC1 protein and decreased XRCC1 expression. Knockdown of Epac1 abolished the effect of 8-pCPT-2′-O-Me-cAMP and restored XRCC1 protein level following γ-ray irradiation. XRCC1 acts as a scaffold for the formation of DNA-repair complexes on damaged DNA to stabilize the BER proteins, and therefore, when XRCC1 is no longer needed for the repair complex, it is ubiquitinated by the E3 ubiquitin ligase CHIP and degraded by the proteasome [16]. The cytoplasmic form of the casein kinase 2 phosphorylates XRCC1 to prevent its ubiquitination and proteasomal degradation [24,25]. In addition, XRCC1 is ubiquitinated by a poly (ADP-ribose)-dependent E3 ligase, iduna that regulates cell survival and DNA repair [26]. Our study shows that the ubiquitin–proteasome dependent degradation of XRCC1 is promoted by the cAMP signaling system, suggesting the ubiquitination of XRCC1 and possibly other proteins may be modulated by various signals acting on the cAMP signaling system.

Epac proteins form one family of cAMP effectors, and play a role as cAMP-dependent guanine nucleotide exchange factors (GEFs) for both Rap1 and Rap2, which belong to the Ras family of small G proteins. Epac proteins have been shown to be involved in a large number of cellular functions such as cell division, differentiation,

secretion and growth [27,28]. This study shows a novel role of Epac1, the mediation of cAMP-induced degradation of XRCC1, which leads to inhibition of DNA damage repair. Thus, Epac proteins are suggested to be involved in carcinogenesis, cancer progression, and cell death induced by anticancer drugs and radiation, in which DNA damage repair plays an important role. However, the mechanism how Epac proteins regulate ubiquitination of XRCC1 and the responsible signaling pathway need to be investigated in the future study.

From these results, we conclude that the cAMP signaling system inhibits the repair of by γ-ray-induced DNA damage by promoting the Epac-mediated ubiquitin–proteasome dependent degradation of XRCC1 in lung cancer cells. This finding suggests that the cAMP signaling system may play important roles in modulation of DNA damage repair pathways.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.139.

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