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The Role of Phosphorylation in IR-induced p53 Stabilization and Activation

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It is well established that tumor suppressor p53 protein is stabilized and activated as a sequence-specific transcriptional factor in response to genotoxic stresses, including ionizing radiation (IR). Previous studies demonstrated that p53 is phosphorylated at N-terminal serines and a threonine after IR. Here, we examined the role of the phosphorylation, especially at Ser15, Thr18, and Ser20, in IR-induced p53 stabilization and activation, using alanine-substituted p53 at phosphorylation site (s). In the present study, we examined p53 stabilization and activation in two different cell lines : stabilization in HT1080-derived clone in which endogenous wild-type p53 was confirmed to be normally stabilized after IR; activation in p53-null H1299-derived clone in which ectopically-expressed p53 levels did not change before and after IR, because the increase in p53 levels affects transactivation by p53. In Western blot analysis and immunofluorescence staining, alanine-substituted p53 at Ser15, Thr18, and/or Ser20 was stabilized in the nucleus comparably with wild-type p53 2 h after 4 Gy of X-rays. However, Ser15-alanine-substituted p53 did not induce the expression of p21 protein, one of the p53-targeted gene products, after IR. These results indicate that phosphorylation at Ser15, Thr18, and Ser20 is not required for IR-induced p53 stabilization, however, Ser15-phosphorylation plays a role in IR-induced p21 induction by p53.

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Introduction

Dysfunction of p53 protein is the most common event in human cancers.[1] p53 protein is a sequence-specific transcriptional factor, and it has a potential to induce cell cycle arrest and/or apoptosis by transactivating p53-targeted genes.[2] p53 elicits its potential by stabilization and activation in response to diverse stresses, e.g. ionizing radiation (IR), though the levels and activity of p53 is usually downregulated in unperturbed proliferating cells.[2-4] The master regulator of p53 stability and activity is MDM2 oncoprotein : it induces p53 degradation by ubiquitinating p53 and masks transactivation domain of p53.[5-9] Therefore, it was expected that evasion from MDM2-binding is critical for p53 stabilization and activation. Shieh et al. reported that Ser15 of p53 is phosphorylated after exposure to IR or UV, and DNA-PK-phosphorylated p53 at Ser15 has lower affinity with MDM2 than unphosphorylated counterpart *in vitro*.[10] It was also reported that Thr18-, or Ser20-phosphorylation impairs affinity between p53 and MDM2, and Ser20-alanine-substituted p53 is not stabilized after 9 Gy of gamma-rays.[11,12] However, it remains unknown whether or which of these phosphorylations are important for p53 stabilization and/or activation *in vivo*. Therefore, in present study, we thoroughly examined the role of p53 phosphorylation at Ser15, Thr18, and Ser20 in IR-induced p53 stabilization and activation.

Materials and methods

Expression system of p53 protein

In the present study, we used ecdyson-inducible vector system (Invitrogen), in which expression of a target protein can be induced by the addition of synthetic ecdyson analog, ponasterone A (PA) to culture media. This system requires two vectors, pIND and pVgRXR.

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pIND is a expression vector, in which cDNA of a target gene can be ligated. pVgRXR expresses RXR protein and VgEcR protein, which bind PA and induce transcription of a target cDNA.

Construction of wild-type and alanine-substituted p53-inducible vectors

The pp53-EGFP vector (Clontech) was digested using *Kpn* I and *Bam* HI, and the 1.2kb fragment was ligated with the *Kpn* I-*Bam* HI-digested pIND/V5-His B vector (Invitrogen) to construct the pIND $p53^{wt}$-V5/His vector. Construction of alanine-substituted p53-V5/His-inducible vectors was performed using GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega). The pIND $p53^{wt}$-V5/His vector was used as a template for the alanine-substituted p53-V5/His-inducible plasmids. The details of these processes are described in the kit's instruction manual. To confirm mutagenesis, DNA sequence analysis was performed.

Introduction of p53-V5/His plasmids and induction of p53-V5/His protein

First, the pVgRXR vector was electroporated into H1299 (human non-small cell lung carcinoma, *p53*-null) or HT1080 (human fibrosarcoma, *p53* wild-type), and then the pIND p53-V5/His vector was electroporated. H1299-, or HT1080-derived clones containing the pVgRXR and the pIND p53-V5/His vectors were isolated after colony formation for 10~14 days in selection medium (minimum essential Eagle's MEM containing 200~400 μg/ml Zeocin (Invitrogen) and 400 μg/ml Geneticin (Life Technologies)). p53-V5/His protein was induced by an addition of PA to the culture media.

Cell culture

H1299-, or HT1080-derived clones, which contain the pVgRXR and the pIND p53-V5/His vectors were cultured with minimum essential Eagle's MEM containing 200~400 μg/ml Zeocin (Invitrogen) and 400 μg/ml Geneticin (Life Technologies). In order to maintain exponential cell growth, 3×10^5 cells were seeded in T25 flasks (25 cm^2) and subcultured every 3 days.

X-irradiation

Exponentially growing cells were irradiated by X-rays from an X-ray generator at 150 kVp and 5 mA with a 0.1-mm copper filter at a dose rate of 0.492 Gy / min. Twenty-four hours before irradiation, PA was added to the media in order to induce p53-V5/His protein.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, hydrochloride (AEBSF, Wako). The cell lysate was cleared by centrifugation at 15,000 rpm for 10 min at 4 ºC, and the supernatant was used as the total cellular protein. Protein concentration was determined using a BCA protein assay kit (Pierce). Sixteen μg of total proteins were electrophoresed on SDS-polyacrylamide gels. The proteins were electrophoretically transferred to a polyvinyl difluoride membrane in transfer buffer (100 mM Tris, 192 mM glycine), and the membrane was incubated with blocking solution (10% skim milk) overnight. The membrane was then incubated with anti-p53 monoclonal antibody (clone BP-53-12 or DO-1, Neomarkers), or anti-V5 epitope monoclonal antibody (Invitrogen) for 2 hr. After this treatment, the membrane was incubated with biotinylated secondary antibody followed by incubation with streptavidin-alkaline phosphatase. The band was visualized after the addition of nitroblue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate as a substrate.

Immunofluorescence staining

2×10^5 cells were seeded onto a coverslip in a 35-mm dish. Two days later, cells were fixed using methanol. Twenty-four hours before irradiation, PA was added to media in order to induce p53-V5/His protein. Two hours after irradiation, the cells were fixed using 100% methanol. After fixation and washing with $1xPBS^-$, the cells were incubated with anti-V5 epitope monoclonal antibody (the same as that used in Western blotting) for 2 hours. The cells were then washed with $1xPBS^-$ and incubated with secondary antibody conjugated with Alexa 488 (Molecular Probes) for an hour. After washing by $1xPBS^-$, the coverslip was mounted on a slideglass. Nuclei were stained with propidium iodide (PI).

Results

Phosphorylation effect on IR-induced p53 stabilization was examined using HT1080-derived clones, because endogenous p53 is stabilized normally after IR. To distinguish endogenous and ectopically-expressed p53, anti-V5 epitope antibody was used. Two hours after 4 Gy of X-rays, wild-type p53-V5/His proteins were stabilized in every 5 clone, but degree of accumulation differed between the clones, suggesting clone's intrinsic p53-accumulating ability affects stabilization of even wild-type p53. Alanine-substituted p53-V5/His at Ser15, Thr18, or Ser20 was also stabilized in every clone, comparably to wild-type p53. It was reported that p53 is degraded by cytoplasmic proteasome and Ser15-, and Ser20-phosphorylation inhibits p53 export to cytoplasm.[15] Therefore, we examined localization of alanine-substituted p53-V5/His after 4 Gy of X-rays. All alanine-substituted p53-V5/His was accumulated in the nucleus in a similar manner to wild-type p53-V5/His. We also examined stabilization and localization of double-, and triple-alanine-substituted p53 at these three sites, and similar results were obtained.

Phosphorylation effect on IR-induced p53 activation was examined using p53-null H1299-derived clone. In H1299, the levels of ectopically-expressed p53 does not change before and after IR. This

is convenient to examine p53 activation, because the increase in p53 levels affects transactivation by p53. As an indicator of p53 activation, the expression of p21 protein, that is one of the p53-targeted gene products, was examined. After 4 Gy of X-rays, p21 induction was significantly impaired in Ser15-alanine-substituted p53-V5/His-expressing clone compared to wild-type p53-V5/His-expressing clone. Furthermore, we examined p21 induction in 6 independent clones that express Ser15-alanine-substituted p53-V5/His. In every clone, p21 induction was not observed 4 hours after 4 Gy of X-rays.

Discussion

Here we demonstrated that Ala-substituted p53 proteins at Ser15, Thr18, and/or Ser20 were stabilized significantly in response to X-rays. There were no remarkable differences in the maximum levels and localization of p53 between Ala-substituted mutants and wild-type p53 after irradiation. As the induced levels of p53-V5/His were similar to those of endogenous p53 in HT1080 cells, modification and accumulation of p53-V5/His protein should occur physiologically, and in fact, the kinetics of p53-V5/His accumulation are almost the same as those of endogenous p53. Therefore, from these results, it can be concluded that the phosphorylation of Ser15, Thr18, and Ser20 is not always necessary for stabilizing p53 in response to DNA-damaging agents. This is strongly supported by the fact that Ala-substituted p53 at all N-terminal serines and threonine was accumulated in response to UV or actinomycin D.[13]

Previously, Chehab et al. reported that Ser20-Ala-substituted HA-tagged-p53 was not accumulated after 9 Gy of gamma-rays in U2OS cells.[12] The differences between their results and ours can be explained by the phosphorylation levels at Ser20. In U2OS cells, the phosphorylation levels of p53 at Ser20 after IR are higher than that of HT1080 cells.[14] Therefore it is possible that Ser20-phosphorylation plays a more important role in IR-induced p53 stabilization in U2OS cells.

With regard to Thr18-phosphorylation, Sakaguchi et al. reported that phosphate at Thr18 significantly increased the dissociation constant (*Kd*) between p53-phosphopeptide and MDM2 *in vitro*.[11] However, the role of this phosphorylation *in vivo* has not been examined. Although anti-Thr18-phosphorylated p53 antibody showed phosphorylation at Thr18 in response to ionizing radiation in some tumor cells, such as A549, it is possible that the Thr18-phosphorylation of p53 is less significant in HT1080 cells.[11]

Zhang et al. reported that phosphorylation of p53 at Ser15 and Ser20 inhibits the function of N-terminal nuclear export signal and protects p53 from degradation by cytoplasmic proteasome in U2OS cells.[15] However, in our results, no difference was observed in the localization between wild-type p53 and alanine-substituted p53 at Ser15 and/or Ser20 after IR. Therefore, it can be concluded that the impact of Ser15-, and Ser20-phosphorylation on p53 stability depends on the cell type, and the importance of these phosphorylations cannot be applied to all types of cells.

Then, what is the mechanism that stabilizes Ala-substituted p53 protein at Ser15, Thr18, and Ser20? Several studies have reported that ATM kinase is involved in p53 stabilization because IR-induced p53 accumulation is significantly reduced in cells derived from ataxia-telangiectasia patients, in which the *ATM* gene is mutated.[16] ATM phosphorylates many proteins other than p53, such as histone H2AX, 53BP1, BRCA1, NBS1, CHK2, MDM2, and so on.[17-22] Thus, it is possible that the phosphorylation of these proteins alter their interaction with p53, which results in stabilization of p53. Among them, the phosphorylation of MDM2 is the most possible candidate. Khosravi et al. reported that MDM2 is rapidly phosphorylated at Ser395 in an ATM-dependent manner, and becomes unrecognized by phosphorylation-sensitive anti-MDM2 antibody, 2A10 after IR.[22,23] As 2A10-recognized MDM2 was slightly reduced 30 min after 4 Gy of X-rays in HT1080 cells, phosphorylation of MDM2 at Ser395 may be partially involved in accumulation of Ala-substituted p53-V5/His.

Blattner et al. demonstrated that MDM2 is dephosphorylated at serines within central conserved region II after IR and alanine-substituted MDM2 at these serines fails to promote p53 degradation.[24] Although the dependence of this IR-induced MDM2 hypophosphorylation on ATM has not been determined, it may occur in HT1080 cells.

Ser15-phosphorylation seems to have different impact on stabilization and activation of p53. However, we cannot simply conclude that evasion from MDM2 is required for p53 activation, and not for stabilization, because Thr18-, and Ser20-phosphorylation, that also inhibits p53-MDM2 binding, has no effect on p53 activation. It can be speculated that conformational change of p53 by Ser15-phosphorylation is necessary for p53 activation, in addition to evasion from MDM2. In fact, it is reported that Ser15-phosphorylation induces change of cleavage pattern of p53 by calpain.[10] Therefore, it is possible that conformational change by Ser15-phosphorylation makes p53 more susceptible to coactivator recruitment, such as p300.[25]

There are a number of reports regarding the mechanisms that stabilize and activate p53. However, many of those reports are conflicting, and the contradictions remain unresolved. Thus, comprehensive study is needed to clarify how this versatile molecule works as a potent tumor suppressor.

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