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Roscovitine-Activated HIP2 Kinase Induces Phosphorylation of wt p53 at Ser-46 in Human MCF-7 Breast Cancer Cells

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Abstract Human MCF-7 breast cancer cells are relatively resistant to conventional chemotherapy due to the lack of caspase-3 activity. We reported recently that roscovitine (ROSC), a potent cyclin-dependent kinase 2 inhibitor, arrests human MCF-7 breast cancer cells in the G_2 phase of the cell cycle and concomitantly induces apoptosis. Exposure of MCF-7 cells to ROSC also strongly activates the wt p53 tumor suppressor protein in a time- and dose-dependent manner. The p53 level increased despite upregulation of Hdm-2 protein and was attributable to the site-specific phosphorylation at Ser-46. The p53 protein phosphorylated at serine 46 causes the up-regulation of the p53AIP1 protein, a component of mitochondria. In the present study we identified the pathway mediating ROSC-induced p53 activation. Exposure of MCF-7 cells to ROSC activated homeodomain-intereacting protein kinase-2 (HIPK2). The overexpression of wild-type but not kinase inactive HIPK2 increased the basal and ROSC-induced level of p53 phosphorylation at Ser-46 and strongly enhanced the rate of apoptosis in cells exposed to ROSC. We show that HIPK2 is activated by ROSC and mediates ROSC-induced P-Ser-46-p53, thereby stabilizing wt p53 and increasing the efficacy of drug-induced apoptosis in MCF-7 cells. These results identify HIPK2 as a component of the ROSC-induced signaling pathway leading to the stabilization and activation of wt p53 protein. J. Cell. Biochem. 100: 865–874, 2007.

Key words: G_2 cell cycle arrest; apoptosis; depolarization of mitochondria; CDK-inhibitors; p53 phosphorylation; PML protein; PML bodies

The p53 protein, a product of a tumor suppressor gene, is a key molecule controlling cell cycle progression and induction of apoptosis and is therefore a good target for anti-cancer therapy (for reviews, see Blagosklonny [2002]). New approaches based on p53 gene therapy were proposed and are currently under evaluation [Roth, 2006]. However, it has also been recognized that the (re)activation of the endogenous wild-type (wt) p53 in cancer cells may substantially contribute to the positive outcome of chemotherapy [Wesierska-Gadek et al., 2002; Lane, 2004]. Wt p53, usually maintained in cells at low levels, is upregulated by a variety of anti-cancer drugs what is most frequently attributable to DNA damage generated by these agents [Maltzman and Czyzyk, 1984; Fritsche et al., 1993]. Interestingly, a number of recently developed inhibitors of cyclin dependent kinases such as flavopiridol, UCN-01, or substituted

Abbreviations used: AIF, apoptosis inducing factor; CDKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; Hdm-2, human double minute-2; HIPK2, homeodomain-interacting protein kinase-2; IP, immunoprecipitation; KA, kinase assay; Mdm-2, mouse double minute-2; mt, mutant; NOC, nocodazole; p53AIP-1, p53 apoptosis inducing protein 1; OLO, olomoucine; PARP-1, poly(ADP-ribose) polymerase-1; PML, promyelocytic leukemia; PD, Petri dish; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WB, Western blotting; WCL, whole cell lysate; wt, wild-type.

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purines, additionally to the CDK2 inhibition also positively affect the levels of p53 protein in cancer cells expressing wt p53 [Ljungman and Paulsen, 2001; Blagosklonny et al., 2002]. Interestingly, substituted purines, for example, roscovitine (ROSC) and olomoucine mimicking the ATP molecule [De Azevedo et al., 1997; Meijer et al., 1997] exert very low, if any, direct cytoxicity and they activate p53 protein in a way independent of DNA damage.

Human MCF-7 breast cancer cells are relatively resistant to conventional chemotherapy presumably due to the lack of caspase-3 activity. It became evident that ROSC is a very efficient drug towards human MCF-7 cells and it inhibits proliferation of MCF-7 cells at lower concentrations than cisplatin [Wesierska-Gadek et al., 2003]. ROSC blocked asynchronously growing MCF-7 cells in the G_2 phase of the cell cycle, and after longer exposure it induced apoptosis. ROSC strongly upregulated the cellular expression of p53 protein [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005]. ROSC markedly extended the half-life of p53 protein in MCF-7 cells [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005]. It seems that site-specific phosphorylation of p53 protein was essential for its stabilization. Unlike other agents, ROSC did not induce phosphorylation of p53 protein at serine 15 and 20 but modified p53 at the serine residue in position 46 [Wesierska-Gadek et al., 2005]. The onset of phosphorylation of serine 46 preceded the appearance of p53AIP1 protein, a specific downstream target of site-specifically phosphorylated p53 transcription factor [Wesierska-Gadek et al., 2005]. p53AIP1 protein upregulated by the p53 isoform phosphorylated at serine 46 accumulated after *de novo* synthesis in the cytosol and then translocated into the mitochondria [Wesierska-Gadek et al., 2005].

It was surprising that ROSC, a strong inhibitor of CDKs, activated another cellular kinase that specifically modified p53. Therefore, we raised the question which kinase was activated in human MCF-7 cells exposed to ROSC. To identify the kinase responsible for Ser-46-p53 phosphorylation, kinase assays (KAs) were performed using cellular homeodomain-intereacting protein kinase-2 (HIPK2) and ATM kinase that were precipitated by specific polyclonal antibodies from cell lysates prepared from control cells and from cells treated with ROSC. We detected a strong activation of HIPK2 but not ATM kinase in ROSC-treated MCF-7 cells. The overexpression of wt but not mutant HIPK2 increased the basal and ROSC-induced level of p53 phosphorylation at Ser-46 and strongly enhanced the rate of apoptosis in cells exposed to ROSC.

MATERIALS AND METHODS

Cells

Human MCF-7 breast carcinoma cells were grown up to 60% confluence in Dulbecco's medium without phenol red supplemented with 10% FCS at 37°C in an atmosphere of 8% CO_2 and then treated with 20 μM ROSC for indicated periods of time. ROSC was dissolved as a stock solution in DMSO and stored at −20°C until used.

Expression Vectors

Established expression vectors for FLAG-HIPK2 and FLAG-HIPK2 K221A were previously described in detail [Hofmann et al., 2002].

Antibodies

We used the following antibodies: monoclonal anti-p53 antibody DO-1, a kind gift from Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno), and monoclonal anti-Hdm-2 (2A10), a kind gift from Dr. A. Levine (Institute for Advanced Study, Princeton, NJ). The monoclonal anti-AIF antibody (E-1), anti-PARP-1 (C-2-10), and polyclonal anti-ATM antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-FLAG (clone M2), and anti-cyclin D3 (clone DCS-22) were from Sigma Aldrich (St. Louis, MO). Monoclonal anti-$p21^{waf1}$ (clone DCS60), polyclonal anti-phospho-Thr14/Tyr15 CDK1, anti-phospho-Thr160 CDK2, anti-phospho-Ser15-p53, anti-phospho-Ser46-p53 antibodies, and corresponding antibodies against the total antigen were from New England Biolabs (Beverly, MA). The polyclonal anti-HIPK2 antibody was described [Hofmann et al., 2002]. Monoclonal anti-actin (Clone C4) antibodies were from ICN Biochemicals (Aurora, OH). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from Amersham International (Little Chalfont, Buckinghamshire, UK).

Measurement of DNA of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described previously by Vindelov et al. [1983]. Cells were stained with propidium iodide and then the fluorescence was measured using the Becton Dickinson FACScan after at least 2 h incubation at +4°C in the dark.

Quantitative Analysis of the Mitochondrial Membrane Potential by Flow Cytometry

Mitochondrial depolarization was monitored using the cationic carbocyanine dye JC-1 (Molecular Probes, Inc., Eugene, OR) as previously described [Kovar et al., 2000; Wesierska-Gadek et al., 2005]. Control and ROSC-treated cells were trypsinized, washed and incubated with the dye at a final concentration of 10 μM for 30 min followed by extensive washings in PBS and immediate two-color analysis under fluorescence microscopy and by flow cytometry. Alternatively, cells in Petri dishes were PBS washed and directly incubated with JC-1. After washing, samples were inspected under fluorescence microscopy using a band-pass filter (detects fluoresceine and rhodamine). JC-1 exists in form of red-fluorescent aggregates (excitation/emission at 488 nm/570 nm) in intact cells due to potential-driven mitochondrial accumulation and aggregation. Upon mitochondrial depolarization it occurs as green-fluorescent monomer (excitation/emission at 488 nm/530 nm).

Subcellular Fractionation of Cells

In experiments designed to examine the release of distinct proteins from mitochondria during the execution of apoptosis, a cell fractionation procedure described by Fiskum et al. [2000] was used. Cells were collected, PBS washed and suspended in a buffer containing 250 mM sucrose and low concentration of digitonin. After vigorous vortexing and homogenization using a glass potter, the homogenate was spun down yielding a supernatant and crude nuclei. Supernatant was centrifuged at higher speed to separate soluble cytosolic proteins from mitochondria (pellet).

Immunoprecipitation and *In Vitro* Kinase Assays

For immunoprecipitation (IP) cell lysates were obtained from parental MCF-7 cells and from cells transfected with plasmids encoding WT-HIPK2 and MT-HIPK2. Whole cell lysates (WCLs) (150 μg protein) prepared in a modified RIPA buffer from control, and ROSC-treated cells were precleared and immunoprecipitated using 2 μg of anti-HIPK2 or of anti-FLAG antibody and 25 μl prewashed Protein A/G-Sepharose beads (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). The immunoprecipitates were washed as previously described [Hofmann et al., 2002]. The KA was performed in a final volume of 40 μl kinase buffer containing 4 μM ATP and ^{32}P-γATP and 1 μg template protein (histone H1, His-p53 or GST-p53 (1–393)). After incubation for 30 min the reaction was stopped. Samples were separated on 10 or 12% SDS gels, blotted onto a polyvinylidene difluoride (PVDF) membrane and exposed against an X-ray film.

Electrophoretic Separation of Proteins and Immunblotting

Total cellular proteins or proteins of the distinct subcellular fractions dissolved in SDS sample buffer were separated on 10 or 15% SDS slab gels, transferred electrophoretically onto PVDF membrane (PVDF) (Amersham Biosciences) and immunoblotted as previously described [Wojciechowski et al., 2003]. Equal protein loading was confirmed by Ponceau S staining. To determine the phosphorylation status of selected proteins, antibodies recognizing site specifically phosphorylated proteins were diluted to a final concentration of 1:1,000 in 1% BSA in Tris-saline-Tween-20 (TST) buffer. In some cases, blots were used for sequential incubations.

RESULTS

ROSC, a Strong CDK2 Inhibitor Activates wt p53 Protein Via Site-Specific Phosphorylation at Serine 46

According to the predictions, ROSC but not olomoucine negatively affected the functional status of CDK2. Exposure of MCF-7 cells to ROSC resulted in the loss of the activating phosphorylation of CDK2 at Thr160 (Fig. 1A), an increase of inhibiting phosphorylation of CDK1 (Fig. 1B) and the inhibition of CDK2 and CDK1 activity (not shown). This was preceded by blocking of the cell cycle in the G_2 phase (Fig. 1C). Analysis of the WCLs by immunoblotting revealed a strong increase of p53 protein

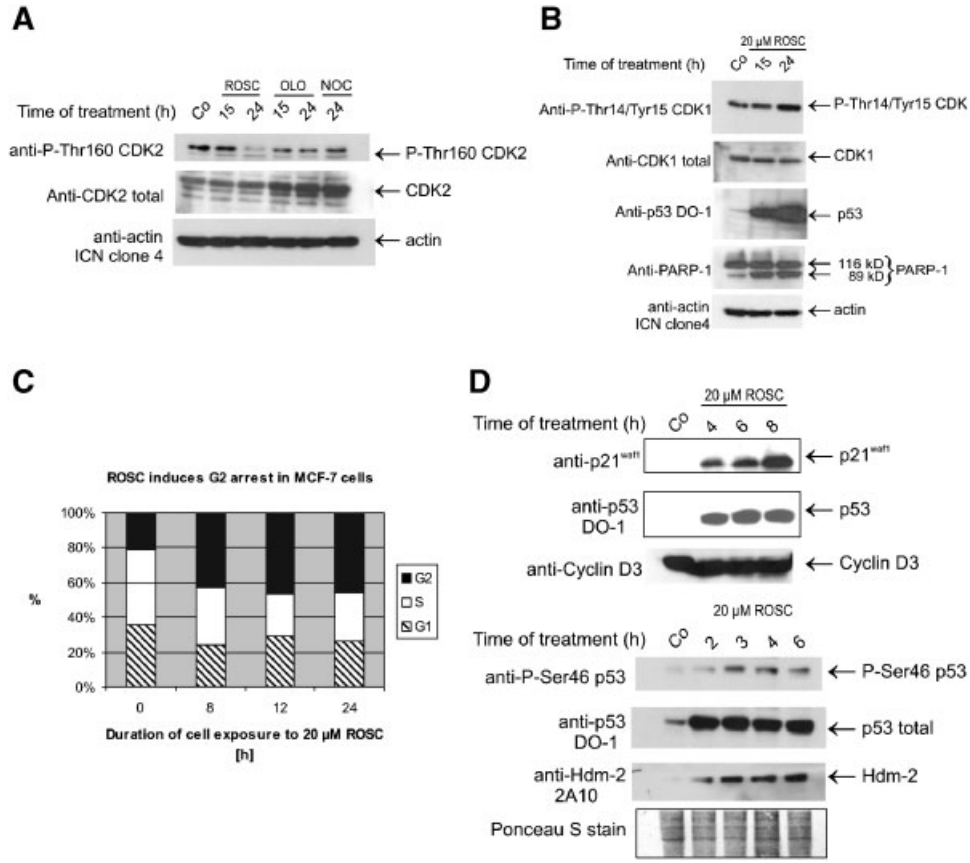

Fig. 1. ROSC induces cell cycle arrest by modulation of the activity of CDKs and the p53 tumor suppressor protein. MCF-7 cells were exposed to 20 μM ROSC, 20 μM olomoucine (OLO) or 0.05 μg/ml nocodazole (NOC) for indicated periods of time. Thereafter, control and drugs treated cells were collected and stained with propidium iodide (PJ) or lysed in RIPA puffer. PJ stained cells were analyzed by flow cytometry and WCLs were electrophoretically separated on 10 or 12% SDS slab gels. Proteins immobilized on PVDF membranes were subjected to immunoblotting using different primary antibodies and appropriate secondary antibodies coupled to HRP. The immune complexes were detected by chemiluminescence using luminol (ECL+) as a substrate. For control of the equal protein loading, membranes were stained with Ponceau S and incubated with anti-cyclin D and anti-actin antibodies. **A**: ROSC abolished the site-specific phosphorylation of CDK2 in human MCF-7 cells. MCF-7 cells were exposed to 20 μM ROSC, 20 μM olomoucine (OLO) or 0.05 μg/ml NOC for indicated periods of time. Cellular proteins (WCLs) were separated on 12% SDS gel. The status of site-specific phosphorylation of CDK2 was determined using anti-P-Thr160-CDK2 antibodies. **B**: A strong up-regulation of p53 protein and cleavage of PARP-1 in ROSC treated cells. Cellular proteins (WCLs) were separated on 12% (for detection of CDK1) and 10% (for detection of p53 and PARP-1) SDS gels. Immunoblotting was sequentially performed with distinct antibodies as indicated. **C**: The accumulation of G_2 arrested MCF-7 cells after treatment with 20 μM ROSC. Control and ROSC treated cells were collected and stained with propidium iodide (PJ). DNA concentration in single cells was measured by flow cytometry. Diagram depicts the changes of distribution of cells in distinct cell cycle phases after treatment with ROSC. **D**: Transcriptional competence of p53 protein up-regulated after exposure of MCF-7 cells to ROSC. Cellular proteins (WCLs) were separated on 12% (for detection of $p21^{waf1}$ and cyclin D_3) and 10% (for detection of Hdm-2) SDS gels. The increase of $p21^{waf1}$ and Hdm-2, two p53-dependent proteins, correlated with appearance of p53 protein upon ROSC treatment.

(Fig. 1B) beginning very early after onset of ROSC treatment (Fig. 1D). The accumulated p53 protein was phosphorylated at serine 46 (Figs. 1D and 2A) but not at serine 15 (Fig. 2A). The results of Western blotting (WB) were additionally confirmed by ELISA (data not shown) [Wesierska-Gadek et al., 2005]. The upregulation of p53 in MCF-7 cells was attributable to its post-translational modification induced by ROSC. The exposure of MCF-7 cells to ROSC resulted in the site-specific phosphorylation and stabilization of p53 protein. The half-life of p53 was approximately 40-fold extended after 15 h ROSC treatment [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005]. Moreover, the up-regulated p53 protein was active as a transcription factor. ROSC-induced p53 protein transactivated its specific downstream targets such as $p21^{waf1}$ and Hdm-2 (Fig. 1D). Moreover, P-Ser-46-p53 activated the

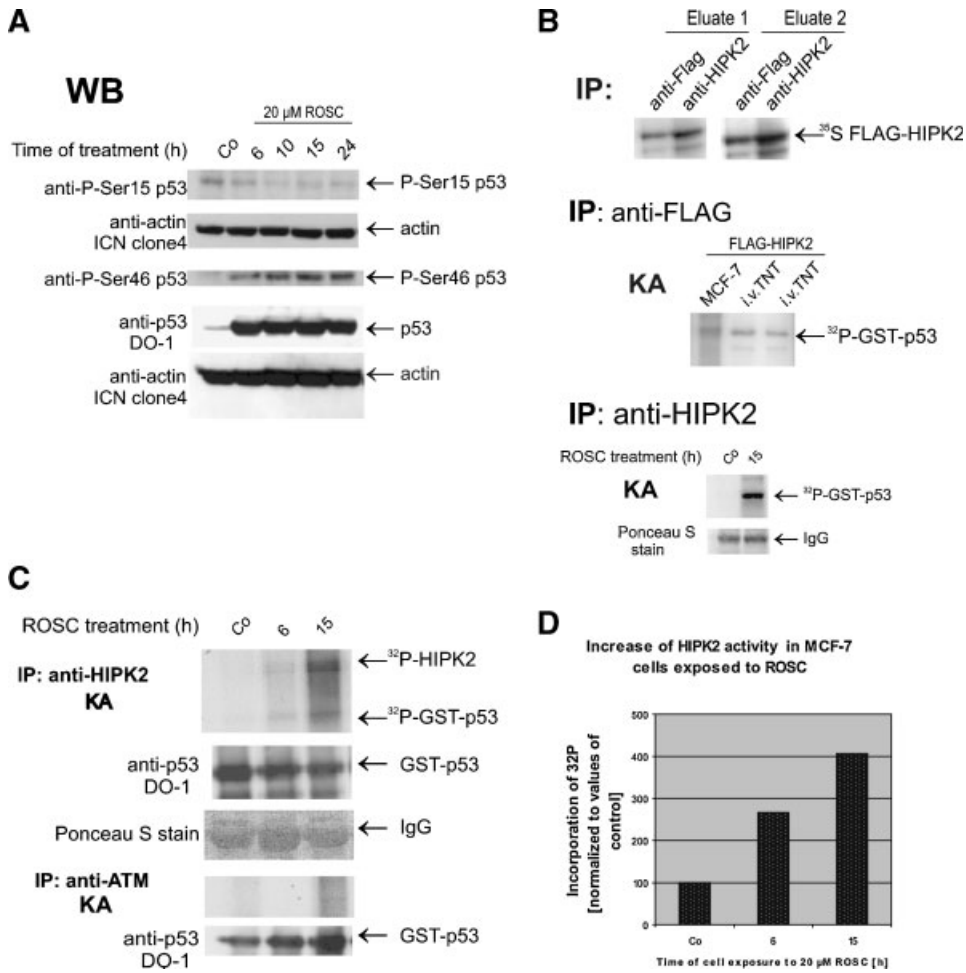

Fig. 2. HIPK2 is activated in MCF-7 cells exposed to ROSC. **A**: ROSC induces site-specific phosphorylation of p53 at Ser 46. WCLs were analyzed by immunoblotting after separation on 10 % slab gels. Details of WB as indicated in Figure 1. **B**: Kinase assay (KA) using human recombinant HIPK2 expressed in MCF-7 cells and by in vitro translation. Human ^{35}S-labelled recombinant HIPK2 was precipitated by different antibodies (**upper panel**) and taken for the kinase assay using human recombinant GST-p53 protein (**medium panel**). The activity of endogenous HIPK2 was determined in control MCF-7 cells and cells exposed to ROSC for 15 h (**lower panel**). Precipitated ^{35}S-labelled HIPK2 as well as p53 template ^{32}P-labelled during kinase reaction were detected by autoradiography. **C**: ROSC induces cellular HIPK2. Endogenous HIPK2 and ATM kinase precipitated by polyclonal anti-HIPK2 and anti-ATM antibodies from cell lysates prepared from untreated MCF-7 control cells and cells exposed to 20 μM ROSC for 6 and 15 h were used for the kinase assay. ^{32}P-ATP was used as a substrate and GST-p53 protein was used as a template. The GST-p53 protein was finally detected by immunoblotting using anti-p53 antibodies DO-1. **D**: Quantification of the HIPK2 activity. ^{32}P-labelled proteins eluted from the beads after kinase assay were measured by liquid scintillation counting.

transcription of p53AIP1 protein [Wesierska-Gadek et al., 2005].

The strong cell cycle arrest at the G_2 phase was occurring after 8 h ROSC treatment and preceded the inhibition of activating phosphorylation of CDK2.

ROSC Induces the Activity of the Endogenous HIPK2

To assess which protein kinase catalyzes the site-specific phosphorylation of p53 at serine 46, KAs were performed. In the first step the KA was established using recombinant human FLAG-tagged HIPK2 enzyme and GST-p53, and histone H1 as a substrate. As shown in Figure 2B, ^{35}S-labelled HIPK2 was precipitated by anti-FLAG as well as by specific polyclonal anti-HIPK2 antibodies. According to the predictions, wt but not mutant HIPK2 phosphorylated recombinant human p53 protein and histone H1 (Fig. 2B).

In the next step the activity of two potential kinases, HIPK2 and ATM, was determined in cellular lysates. WCLs obtained from untreated

controls and cells treated for 6 and 15 h with 20 μM ROSC were precipitated using specific anti-HIPK2 and anti-ATM kinase polyclonal antibodies, and the immobilized enzymes were used for the assay. As shown in Figure 2C, HIPK2 and ATM kinase were almost inactive in untreated MCF-7 cells. However, the exposure of MCF-7 cells to ROSC strongly activated HIPK2 activity. The quantification revealed roughly a stepwise increase of the HIPK2 activity upon ROSC treatment (Fig. 2D). No substantial increase of the activity of ATM kinase after exposure of human MCF-7 cells to ROSC was detected (Fig. 2C). The concomitant determination of the activity of two other kinases, namely CDK2 and CDK1, revealed the opposite effect of ROSC treatment. ROSC inhibited the activity of CDK2 and CDK1 (not shown).

Overexpressed HIPK2 Increases the Level of P-Ser-46-p53 Protein in Human MCF-7 Cells

To prove the consequences of the elevation of the HIPK2 expression, we transfected MCF-7 cells with wt HIPK2 and then exposed the cells transiently expressing HIPK2 to ROSC. The ectopical (over)expression of HIPK2 resulted in an elevation of the basal level of p53 phosphorylation at serine 46 (Fig. 3A) and additionally increased the extent of site-specific phosphorylation of p53 protein in ROSC treated cells. This coincided with the increased HIPK2 activity (Fig. 3B). Mutant HIPK2 K221A affected neither the basal nor ROSC-induced phosphorylation level of p53 protein (Fig. 3A) and did not phosphorylate recombinant p53 protein (Fig. 3B).

Enhancement of the Apoptosis Rate in MCF-7 Cells Overexpressing HIPK2

Finally, we examined the effect of an increased expression of HIPK2 on the rate of apoptosis. The potential of the mitochondrial membrane was determined by flow cytometric measurement of the formation of J-aggregates. Moreover, the release of mitochondrial proteins into the cytosol was assessed by WB. In untransfected control MCF-7 cells an increased apoptosis rate was induced after exposure to ROSC for 15 and 24 h as evidenced by detection of caspase-cleaved poly(ADP-ribose) polymerase-1 (PARP-1) at 89 kDa (Fig. 1B). As shown in Figure 3C, elevated expression of HIPK2 accelerated the execution of apoptosis in MCF-7 cells exposed to ROSC and enhanced the rate of apoptosis. At 24 h the population of apoptotic cells increased by 35% in the presence of increased HIPK2 levels and reached 85%. Moreover, 6 h after administration of ROSC a substantial amount of apoptosis inducing factor (AIF) was released from mitochondria into the cytosol (data not shown). The comparable effect in non-transfected MCF-7 was observed at 15 h after onset of ROSC treatment indicating that HIPK2 accelerates the execution of apoptosis.

DISCUSSION

Wt p53 is present in unstressed cells at low levels but after exposure to a variety of stress stimuli it is stabilized and activated. The stabilization of p53 is a very complex process involving different post-translational modifications, a few ubiquitin ligases and a number of other interacting proteins that influence the interaction between p53 protein and its negative regulators. It is widely accepted that the increase of cellular levels of p53 and its activity is regulated by a number of different post-translational modifications (phosphorylation, acetylation, poly(ADP-ribosyl)ation, sumoylation, NEDDylation, and others) that change the susceptibility of p53 protein for the E3 ligase-mediated ubiquitylation [Lavin and Gueven, 2006]. Furthermore, more recent data evidence that the modulation of the stability of p53 protein is more complex. Stability of p53 protein is regulated by a number of newly detected cellular E3 ligases: COP1, Prh2, promyelocytic leukemia (PML), by isomerization of prolyl binding induced by prolyl isomerase Pin1, by pVHL protein, and by deubiquitination (HAUSP) [Lavin and Gueven, 2006].

ROSC, a very potent inhibitor of CDKs [De Azevedo et al., 1997; Meijer et al., 1997] strongly upregulates and activates p53 [Ljungman and Paulsen, 2001; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005]. It has been shown that the stability of p53 protein increased approximately 40-fold in human MCF-7 cells exposed to ROSC [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005] and that upregulated p53 was competent as a transcription factor and induced p53-responsive genes such as $p21^{waf1}$ and mouse double minute-2 (Mdm-2) [Wojciechowski et al., 2003; Wesierska-Gadek

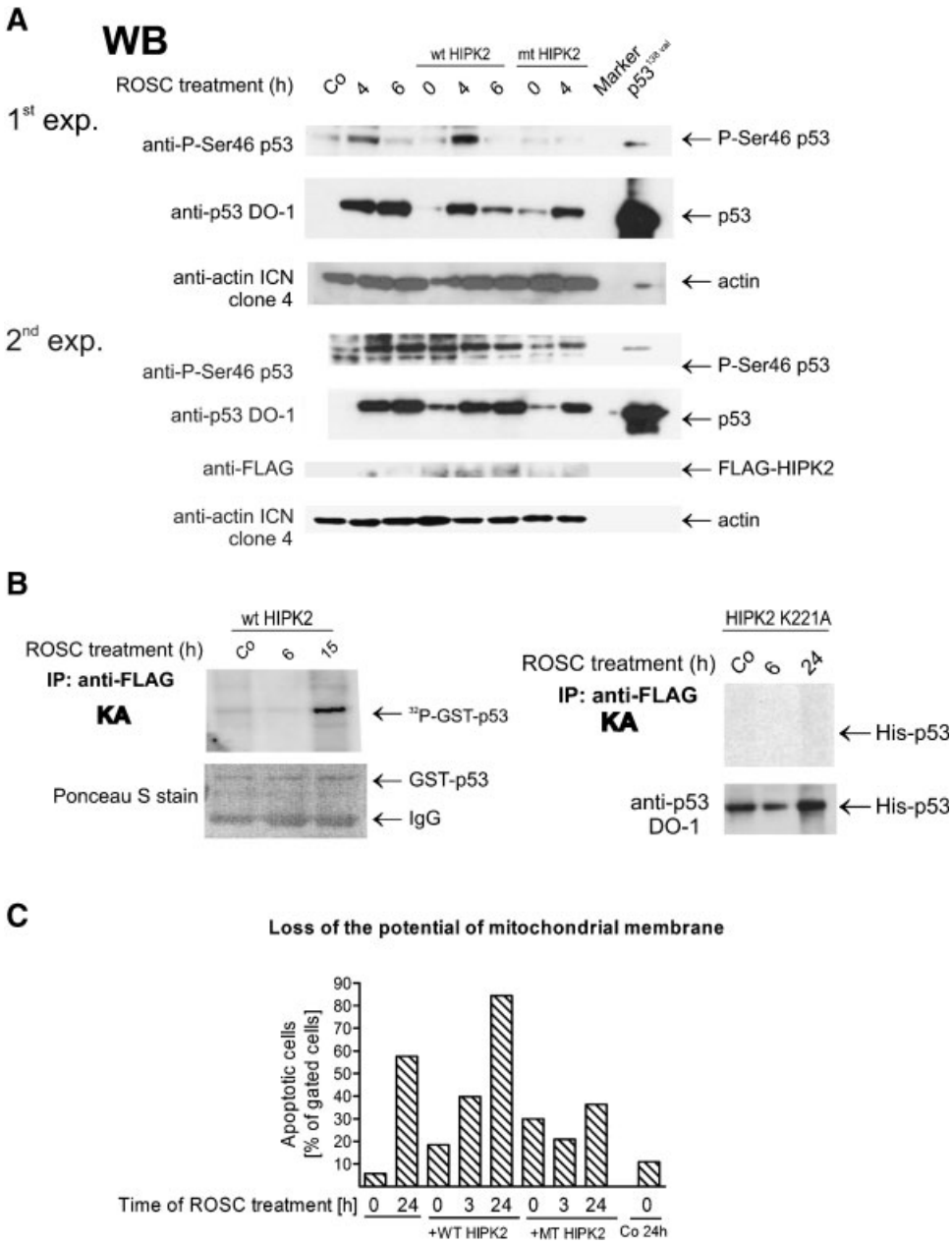

Fig. 3. Overexpressed HIPK2 increases site specific phosphorylation of p53 and enhances the apoptosis rate in ROSC-treated MCF-7 cells. MCF-7 cells transfected with wt HIPK2 or with mutant HIPK2 K221A were treated with 20 μM ROSC for indicated periods of time and lysed or incubated with JC-1. WCLs were used for immunoblotting (**A**) and for kinase assay (**B**) and JC-1 stained cells were analyzed by flow cytometry (**C**).

et al., 2005]. The ROSC-mediated stabilization of p53 seems to be attributable to its phosphorylation on serine 46. It has been observed previously that the extent of p53 phosphorylation on serine 46 closely correlates with the extent of its elevation (MCF-7 parental cells versus MCF-7 cells reconstituted with human caspase-3) and with the extension of the p53 half-life [Wesierska-Gadek et al., 2005]. The observation that the selective inhibitor of CDKs induces site-specific phosphorylation of p53 is not only a surprising finding, but it is also biologically highly important. It indicates that ROSC is able to simultaneously induce several pathways, thereby increasing its efficacy in anti-cancer therapy.

The ROSC-mediated phosphorylation of serine 46 of p53 protein is of great functional

importance. P-Ser46-p53 protein induces expression of its target, p53AIP1 protein that after *de novo* synthesis translocates into the mitochondria and is then involved in the induction of apoptosis [Oda et al., 2000; Wesierska-Gadek et al., 2005]. Considering the functional consequence of site-specific phosphorylation of p53 after treatment with ROSC, the identification of the kinase modifying p53 protein on serine 46 was of great importance. Thus, our results show that ROSC activates HIPK2 kinase in human MCF-7 cells which subsequently modifies p53 protein at serine 46 and induces its downstream effects resulting in the initiation of apoptosis. Moreover, the above results identified the mechanism by which ROSC mediates stabilization of wt p53. It has been suggested previously that the increased half-life of p53 upon ROSC treatment is due to down-regulation of Hdm-2, a negative regulator of p53 protein, as a consequence of the inhibition of global transcription [Lu et al., 2001]. In contradiction to this report O'Hagan and Ljungman [2004] showed that after treatment with the CDK inhibitor DRB, that is known to inhibit global transcription, p53 accumulated in nuclei despite the fact that mdm-2 levels remained high. This observation is in clear concordance with the results that p53 protein was upregulated after ROSC treatment in human cells despite the persistence of high Mdm-2 levels [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005; Ribas et al., 2006]. Furthermore, p53 accumulation after DRB treatment and blockage of transcription was also detected in the colon cancer cell line SJSA that has increased endogenous Mdm-2 levels [O'Hagan and Ljungman, 2004] and after overexpression of Mdm-2 in normal human fibroblasts [O'Hagan and Ljungman, 2004].

Our results confirm previous observations that wt p53 protein is up-regulated in MCF-7 cells exposed to ROSC despite an increased Hdm-2 level, thereby indicating that ROSC-induced p53 protein became resistant to the negative action of Hdm-2.

The above data show a close correlation between the extent of ROSC-induced phosphorylation of p53 at serine 46 and apoptosis. They are in concordance with our previous results evidencing that the therapeutic effect of ROSC was markedly weaker in MCF-7 cells reconstituted with caspase-3 [Wesierska-Gadek et al., 2005]. In the latter, lack of phosphorylation of p53 at serine 46 after treatment with ROSC

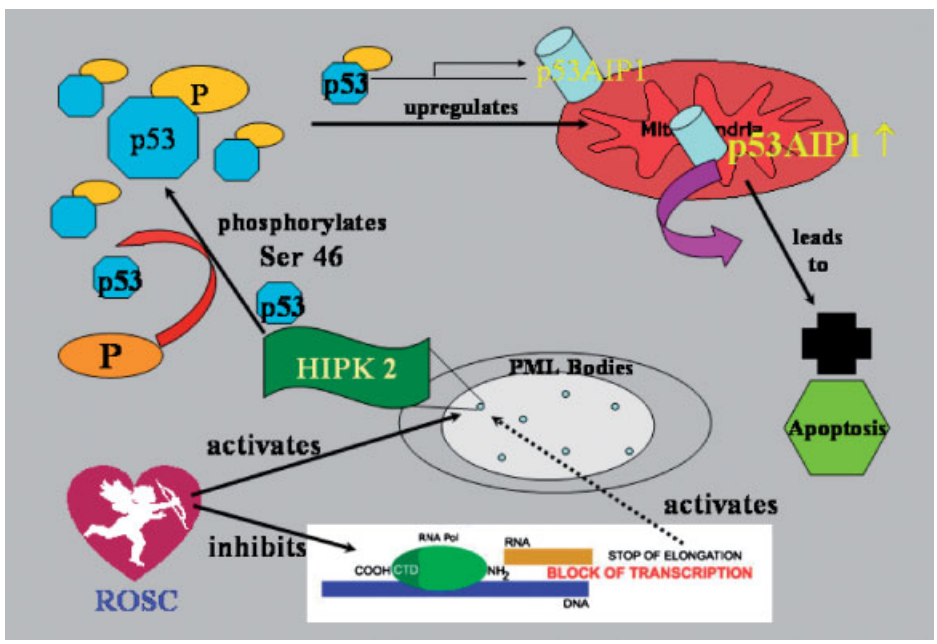

Fig. 4. A new proposed model explaining the mechanism of ROSC-mediated p53 stabilization. ROSC inhibits the global transcription and concomitantly activates HIPK2 localized in PML bodies. Activated HIPK2 phosphorylates p53 protein at serine 46 resulting in its strong stabilization. P-Ser46-p53 protein up-regulates transcription of mitochondrial p53AIP1 protein which after *de novo* synthesis translocates into mitochondria and induces dissipation of the mitochondrial membrane, and in consequence the onset of apoptosis associated with the release of mitochondrial proteins into the cytosol.

closely correlated with the low rate of apoptosis despite expression of caspase-3. More recent data substantiate these observations. MCF-7 cells cultivated in tissue culture medium containing phenol red responded much weaker to ROSC treatment than cells maintained in phenol red-free medium [Wesierska-Gadek et al., 2006]. In the latter the site-specific phosphorylation of p53 was diminished and the number of cells undergoing apoptosis after longer exposure to ROSC was significantly reduced [Wesierska-Gadek et al., 2006].

How can one interpret the above data? Wt p53 is a short living protein and its stability is tightly regulated by the combination of post-translational modifications and the interaction of p53 protein with specific regulators [Blagosklonny, 2002; Lavin and Gueven, 2006]. Mdm-2, a RING finger protein possessing E3 ubiquitin ligase activity, has been first recognized as a key factor mediating the stability of cellular wt p53 protein under physiological conditions. More recently, a number of additional regulators mediating the ubiquitylation of p53 such as COP1, Pirh2 or gankyrin have been found [Lavin and Gueven, 2006]. Interestingly, PML protein, a component of PML bodies, was also identified as RING finger protein mediating the stability of p53 [Fogal et al., 2000]. Since PML protein is aberrantly expressed in some cancer cells, it does not play a general role in the control of p53 stability. However, it has been shown that PML protein is essential in human cells [Moller et al., 2003; Bao-Lei et al., 2006]. The inactivation of PML by siRNA or gene disruption impaired the p53 signal transduction pathway and inhibited irradiation-induced apoptosis in MCF-7 cells [Bao-Lei et al., 2006]. Moreover, HIPK2 has been shown to phosphorylate p53 at serine 46 after UV irradiation [Moller et al., 2003]. Considering the fact that both, PML and HIPK2, are located in PML bodies and cooperate [Moller et al., 2003], the interaction between them seems to be essential for the regulation of the p53 steady state after ROSC treatment.

In the light of these facts we assume that exposure of MCF-7 cells to ROSC activated HIPK2 which in turn modified p53 at serine 46, thereby preventing its ubiquitylation by PML and its subsequent degradation in the proteasome. However, it remains unknown whether ROSC directly modulates the activity of HIPK2 or via an unknown factor (Fig. 4).

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