p53 Ser\textsuperscript{15} phosphorylation disrupts the p53–RPA70 complex and induces RPA70-mediated DNA repair in hypoxia

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INTRODUCTION

RPA (replication protein A), the eukaryotic ssDNA (single-stranded DNA)-binding protein, is a heterotrimeric protein composed of three subunits, RPA70, RPA32 and RPA14 [1]. Along with ssDNA formation, RPA is required for cellular DNA metabolism processes, such as replication, recombination, checkpoints and repair. RPA70 interacts with genomic DNA and numerous other proteins involved in these processes [2,3], and is responsible for NER (nucleotide excision repair) and NHEJ (non-homologous end-joining) pathways of DNA repair [4,5]. It also interacts with BRCA1 (breast cancer early-onset 1) and BRCA2, two probable recombination mediators, as well as with p53 [6,7].

The p53 tumour suppressor co-ordinates a cellular response by transcriptional regulation of genes involved in cell-cycle arrest and apoptosis upon sensing DNA damage [8]. p53 is central to an extensive network of DNA-damage sensing, protein–protein–protein–nucleic acid interactions; RPA70 has been shown to interact with p53 under \textit{in vitro} and \textit{in vivo} conditions [9]. The interaction of p53 with RPA70 mediates suppression of homologous recombination [10], and in co-ordinating DNA repair through p53-dependent checkpoint control, by sensing UV damage [11]. The interaction between p53 and RPA70 inhibits p53’s ability to bind sequence-specific DNA [12,13] and UV exposure greatly reduces the ability of RPA70 to bind to p53. An NMR study confirmed that p53-NTD (amino acids 37–57; NTD is N-terminal domain) binds to residues 1–120 of RPA70 [6].

Hypoxia-mediated dysregulation of critical DNA repair pathways contributes to genetic instability and tumour progression in cancer cells [14] and is a critical factor limiting the efficacy of anticancer strategies. Within solid tumours, hypoxia-induced chemo-resistance is originally attributed to poor drug distributions and to the contention that hypoxic tumour cells are predominantly quiescent [15]; however, the underlying molecular mechanism of hypoxia-induced drug resistance remains unclear. Recent studies have shown the association of hypoxic tumours with increased forms of DNA damage, including DNA strand breaks and oxidative base damage, such as 8-oxoguanine and thymine glycols [16,17]. Hypoxia has been recognized to induce several DNA-damage-response factors, such as DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated)/ATR (ATM- and Rad3-related), CHK1 (checkpoint kinase 1)/CHK2 and BRCA1, and regulates the DNA repair [18]. NER and NHEJ are important DNA repair pathways that are responsible for the removal of helix-distorting DNA adducts, including UV-induced cyclobutane pyrimidine dimers and DNA DSBs (double-strand breaks). The hypoxia effector HIF (hypoxia-inducible factor)-\textalpha transcrionally regulates the expression of two NER proteins, XPC (xeroderma pigmentosum complementation group C) and XPD, after binding to HREs (hypoxia-responsive elements) [19], and the DNA repair pathway is also involved in DNA DSB repair through the targeting of HIF-\textalpha [20]. Although hypoxia-induced DNA repair is linked to chemo-resistance, little is known about the role of hypoxia on NER
and NHEJ; however, both of these pathways have been implicated in hypoxia-mediated chemoresistance [21].

In the present study we have demonstrated that hypoxic cells exhibit high RPA70-mediated DNA repair through NER and NHEJ pathways. Hypoxia increased RPA70 expression and induced dissociation of the p53–RPA70 complex, which is due to DNA-PK-mediated phosphorylation of p53Ser15. Furthermore, RPA70 silencing as well as inhibition of p53Ser15 phosphorylation induced apoptosis in the resistant hypoxic cancer cells.

RESULTS AND DISCUSSION

Hypoxic cancer cells show increased DNA repair and inhibition of apoptosis

DNA repair protects cancer cells from apoptosis by reducing the accumulation of genomic insult [24]. Hypoxic cancer cells are resistant to drug-mediated apoptosis [25] and NER/NHEJ are known to play important roles in the repair of hypoxia-induced DNA anomalies. DNA repair pathways are shown to regulate hypoxia-induced genetic instability within the tumour [26,27], thus making tumours resistant, aggressive and metastatic [28]. We have recently shown through EPR spectroscopy that the oxygen concentration is 1.8% in the hypoxic core of the MCF-7 tumour [29]. To establish the relationship between hypoxia-mediated DNA repair and inhibition of apoptosis, we analysed the status of cellular NER and NHEJ in MCF-7 cells that were maintained at 1.8% oxygen. Cisplatin and UV, which are known to induce NER [30,31] and NHEJ [32,33] were used as the positive controls. NER was analysed by evaluation of the removal of UV-induced lesions from UV-irradiated plasmid (substrate) as described previously [23]. The relative percentage of NER repair was calculated for normoxic and hypoxic MCF-7 cells that were treated with UV (25 J/cm²) and cisplatin (20 μM) (see the Supplementary Materials and methods section at http://www.BiochemJ.org/bj/443/bj4430811add.htm) (Figure 1A). In normoxic MCF-7 cells, NER was 20% (baseline); UV and cisplatin increased NER to 49% and 41%, respectively. Hypoxia exposure alone increased NER to 48%, whereas UV and cisplatin significantly increased NER to 58% and 54%, respectively in hypoxic cells. Antibodies against XPA were used as a negative control to block NER [34]. NHEJ was measured using an assay based on fluorescent detection of repaired products [22] (Figure 1B). In comparison with normoxic MCF-7 cells, UV- and cisplatin-treated cells showed 2.89- and 2.36-fold increases in NHEJ, respectively. Interestingly, hypoxia induced 2.1-fold higher NHEJ than in normoxic MCF-7 cells. Furthermore, both UV and cisplatin treatment induced >3-fold increase in NHEJ in hypoxic cells in comparison with the UV- and cisplatin-treated normoxic cells. The anti-Ku70 antibody was used as a negative control to block NHEJ [35]. These results established high levels of NER and NHEJ in hypoxic cancer cells. Since hypoxia up-regulated NER and NHEJ, the cellular level of proteins involved in regulation of these DNA repair pathways were further analysed. Western blots were conducted to study the expression of the proteins involved in NER (XPC, RAD23B, CETN2, XPA, XRC5, XBP, GF2H1 and CDK7) and NHEJ [ERCC4 (excision repair cross-complementing rodent repair deficiency complementation group 4), Ku70, Ku80, PRKDC and NHEJ1] in normoxic and hypoxic MCF-7 cells (Figure 1C). The results showed a significant increase in expression of these proteins under hypoxic conditions. The cDNA expression vectors of these proteins were used as negative and positive controls respectively. Although the expression of key proteins such as Ku70, Ku80 and TFIIH were shown to be increased in hypoxic cells previously
Figure 1  Hypoxic cancer cells show high NER/NHEJ repair and chemoresistance

(A) Nucleotide excision repair (NER) was analysed in UV (25 J/cm²)- and cisplatin (20 μM)-treated normoxic and hypoxic (1.8% O₂) MCF-7 cells. Results show that NER-mediated DNA repair is 2.4-fold higher in hypoxic MCF-7 cells (dark grey bar) in comparison with normoxic cells (light grey bar). UV- and cisplatin-treated hypoxic MCF-7 cells also show a significant increase in NER. The antibody against XPA was used as a negative control to block NER [31]. (B) DNA repair through NHEJ was observed in UV (25 J/cm²)- and cisplatin (20 μM)-treated normoxic and hypoxic (1.8% O₂) MCF-7 cells, as described previously [22]. NHEJ was 2.1-fold higher in hypoxic (dark grey bar) than in normoxic (light grey bar) MCF-7 cells. The anti-Ku70 antibody was used as a negative control to block NHEJ. For (A) and (B), n = 10, error bars represent S.D. and significance was measured using ANOVA (A, * P < 0.038; B, * P < 0.036). (C) The effect of hypoxia on the cellular expression of key proteins involved in NER and NHEJ machinery was observed. Western blots show a significant increase in the expression of NER/NHEJ proteins in hypoxic MCF-7 cells (cDNA of these proteins was used as a positive control) (n = 5, significance was measured using ANOVA). (D) Hypoxia-mediated chemoresistance was observed in UV- and cisplatin-treated hypoxic MCF-7 cells using annexin V staining and flow cytometry. UV and cisplatin induced 36% and 60% apoptosis in MCF-7 cells, whereas in hypoxic cells it was 12% and 16% respectively, suggesting that hypoxic MCF-7 cells are resistant to UV- and cisplatin-induced apoptosis. In order to analyse the role of DNA repair in hypoxia-induced resistance to apoptosis, cellular NER and NHEJ were inhibited by incubating hypoxic cells with antibodies

[36,37], the expression of other DNA repair proteins was reported to be unaltered in hypoxic cancer cells [38].

We then determined the role of NER and NHEJ in hypoxia-mediated chemoresistance in UV- and cisplatin-treated hypoxic MCF-7 cells using annexin V staining and flow cytometry (Figure 1D). In normoxic MCF-7 cells, UV and cisplatin induced 36% and 60% apoptosis in MCF-7 cells, whereas in hypoxic cells it was 12% and 16% respectively, suggesting that hypoxic MCF-7 cells are resistant to UV- and cisplatin-induced apoptosis. In order to analyse the role of DNA repair in hypoxia-induced resistance to apoptosis, cellular NER and NHEJ were inhibited by incubating hypoxic cells with antibodies...
Figure 2  Hypoxia-mediated increase in NER/NHEJ is RPA70-dependent

(A) RPA70 mRNA level was analysed in UV- and cisplatin-treated normoxic/hypoxic MCF-7 cells, using RT (reverse transcription)–PCR (see the Supplementary Materials and methods section and Supplementary Table S1 at http://www.BiochemJ.org/bj/443/bj4430811add.htm for more details). Hypoxia induced a 6-fold increase in RPA-70 mRNA level; UV and cisplatin treatment also significantly increased RPA70 mRNA expression. RPA70 siRNA was used as a negative control. n = 5. (B) RPA70 protein level was analysed using Western blotting. The results show an increase in RPA70 protein in hypoxic MCF-7 cells, and UV and cisplatin increase the expression further. n = 5. (C) NER (i) and NHEJ (ii) were observed in hypoxic MCF-7 cells when the RPA70 gene was silenced using RPA70 siRNA. RPA70 silencing reduced NER to 16 % from 48 % (compare with Figure 1A); NHEJ was reduced 2.85-fold (grey bars). n = 10, error bars indicate S.D., significance was measured using ANOVA (*P < 0.039), suggesting that hypoxia-mediated NER/NHEJ are RPA-70 dependent. (D) The effect of RPA-70 on hypoxia-mediated chemoresistance was observed by silencing RPA-70 in UV/cisplatin-treated hypoxic MCF-7 cells. Annexin V staining shows a significant increase in the apoptotic fraction from 12 % and 16 % (in hypoxic cells) to 52 % and 84 % (in hypoxic cells with RPA silencing) respectively in RPA70 silenced MCF-7 cells (n = 7). *P < 0.042. (E) The effect of RPA70 on cellular expression of key NER and NHEJ protein was analysed in hypoxic MCF-7 cells. RPA70 siRNA reduced the expression of NER/NHEJ proteins. UV-induced overexpression of NER/NHEJ proteins was reversed upon RPA70 silencing; cDNA of the NER/NHEJ proteins was used as positive controls (n = 4). *P < 0.046.

against the proteins that are involved in DNA repair pathways. NER was inhibited using antibodies against XPC, XPA, ERCC5, XPB and XPD; NHEJ was inhibited using antibodies against Ku70 and Ku80. Annexin V staining showed that inhibition of NER and NHEJ led to a significant increase in UV- and cisplatin-induced apoptosis in hypoxic MCF-7 cells (Figure 1E). Besides, simultaneous inhibition of NER and NHEJ showed a more than 5-fold increase in UV- and cisplatin-induced apoptosis in hypoxic MCF-7 cells (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430811add.htm). These results established that NER and NHEJ are increased during hypoxia and their inhibition induces apoptosis in hypoxic/resistant cells.

RPA70 mediates DNA repair in hypoxic cancer cells

Hypoxia induces a variety of genetic alterations, including activation of oncogenes and inactivation of tumour-suppressor
genes. RPA70 plays a major role in the NER and NHEJ [39] pathways of DNA repair, but the cellular mechanism of these pathways in hypoxic cancer cells is unknown. Since levels of NER and NHEJ were increased in hypoxic MCF-7 cells, RPA70 mRNA and protein levels were analysed. A 6-fold increase in RPA70 mRNA (Figure 2A) and a 4.8-fold increase in RPA70 protein level were observed in hypoxic MCF-7 cells (Figure 2B); RPA70 siRNA and RPA70 cDNA were used as negative and positive controls. Furthermore, the role of RPA70 in NER/NHEJ was established by analysis of NER/NHEJ-mediated DNA repair in hypoxic MCF-7 cells after RPA70 gene silencing (Figure 2C, panels i and ii, grey bar). RPA70 silencing reduced NER by 69% (compare with Figure 1A) and NHEJ by 73% (compare with Figure 1B). To establish whether hypoxia- and RPA70-induced DNA repair is linked to chemoresistance, RPA70 gene silencing was performed in UV- and cisplatin-treated hypoxic MCF-7 cells (Figure 2D). Annexin V staining showed 52% and 84% apoptosis in UV- and cisplatin-treated hypoxic MCF-7 cells. The apoptosis induced by RPA70 silencing and by inhibition of NER/NHEJ (compare with Figure 1F) was similar, suggesting that a hypoxia-mediated increase in DNA repair may be responsible for chemoresistance in hypoxic cancer cells. To study the role of RPA70, we studied the expression of proteins involved in NER/NHEJ by Western blotting. RPA70 siRNA significantly decreased the expression of proteins involved in NER/NHEJ (Figure 2E). Irradiation with UV led to a substantial increase in the expression of NER/NHEJ member proteins, but RPA70 siRNA abolished the UV-induced increase in protein expression. The results confirmed the role of RPA70 in hypoxia-mediated DNA repair.

**Hypoxia dissociates the p53–RPA70 complex**

DNA-damage-induced activation of p53 is always accompanied by the release of the heterotrimetric RPA protein [9]. However, the mechanism of hypoxia-mediated RPA70 activation is unknown. As the p53–RPA70 complex is shown to exist in cancer cells [6], and this complex dissociates upon UV exposure [9], we asked if such a complex is present in hypoxic cancer cells. IP (immunoprecipitation) using anti-p53 and anti-RPA70 antibodies showed that the p53–RPA70 complex is intact in normoxic MCF-7 cells (Figure 3A); p53 and RPA70 show binding in normoxic MCF-7 cells, however, in hypoxic MCF-7 cells, IP with anti-p53 and anti-RPA70 antibodies showed no binding between p53 and RPA70. For all experiments, n = 5. C-ter, C-terminal; N-ter, N-terminal; Nor, normoxia.
addition of the NTD led to disruption of the p53–RPA70 complex and formation of the NTD–RPA70 complex, suggesting selective binding of NTD towards RPA70 (Figure 3B). IP with an anti-HA (haemagglutinin) antibody showed that HA-tagged NTD binds to both p53 and RPA70 (Figure 3B). Interaction of the NTD with RPA70 was also confirmed in H1299 cells that were transfected with NTD cDNA (Figure 3). These results confirmed that p53 interacts with RPA70 through its N-terminus in cancer cells.

The influence of genotoxic/cellular stresses such as cisplatin or hypoxia on the stability of the p53–RPA70 complex was then analysed. IP showed that, in cisplatin-treated MCF-7 cells, the p53–RPA70 complex is also disrupted (Figure 3C). Cisplatin also disrupted the NTD–RPA70 complex both in MCF-7 and H1299 cells (Figure 3D). Interestingly, IP using anti-p53 and anti-RPA70 antibodies showed that the p53–RPA70 complex is disrupted in hypoxic MCF-7 cells (Figure 3E). In a similar manner, hypoxia disrupted the NTD–RPA70 complex in MCF-7 and H1299 cells (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/443/bj4430811add.htm), which might be due to p53-NTD post-translational modifications.

\[ \text{p53}^{\text{Ser15}} \] phosphorylation is responsible for disruption of the p53–RPA70 complex

The dissociation of the p53–RPA70 complex in hypoxia led us to hypothesize that p53 post-translational modifications might be responsible for this. The p53 NTD (amino acids 1–126) [40] has no known site for acetylation; however, it contains nine serine and threonine residues which undergo phosphorylation [41]. The post-translational phosphorylation of p53 and NTD were analysed in UV-, cisplatin- and hypoxia-treated MCF-7 and H1299 cells. IP using anti-phospho-p53 antibodies showed that both p53 and NTD are phosphorylated upon UV, cisplatin and hypoxia treatment (Figure 4A). To establish the role of p53 phosphorylation, the p53–RPA70 and NTD–RPA70 complexes were analysed in the presence of serine/threonine kinase inhibitors. Serine/threonine kinase inhibitors abolished the disruption of both the p53–RPA70 and NTD–RPA70 complexes in UV-, cisplatin- and hypoxia-treated MCF-7 (Figure 4B, panel i) and H1299 (Figure 4B, panel ii) cells. The efficiency of serine/threonine kinase inhibitors in inhibition of p53 phosphorylation was tested as a control (Supplementary Figure S3 at http://www.BiochemJ.org/bj/443/bj4430811add.htm). These results suggest that p53-NTD phosphorylation might regulate the dissociation of the p53–RPA70 complex. We then sought to find the residues in p53-NTD whose phosphorylation was crucial for the disruption of the p53–RPA70 complex. UV- and hypoxia-treated H1299 cells that were transfected with p53 cDNA carrying mutations at Ser6, Ser9, Ser20, Ser33 and Ser37 and Thr18, Thr55 and Thr81 residues [42] showed that these mutations were unable to dissociate p53 from RPA70 (Figure 4C). This data suggests that phosphorylation of either one or a group of these residues were responsible for the regulation of the p53–RPA70 complex. In order to identify those key amino acid residues, p53 cDNA constructs coding for the point mutations at Ser6, Ser9, Ser15, Ser20, Ser33 and Ser37 and Thr18, Thr55 and Thr81 residues were used [43], and the p53–RPA70 complex was analysed by IP (Figure 4D). H1299 cells transfected exclusively with the 

\[ \text{p53}^{\text{Ser15}} \] mutation showed stabilization of the p53–RPA70 complex in the presence of UV and hypoxia (Figure 4D, top panel). p53 cDNA constructs carrying point mutations at Ser6, Ser9, Ser20, Ser33, Ser37, Thr18, Thr55 and Thr81 were unable to inhibit the disruption of the p53–RPA70 complex. These results established that p53-NTD was responsible for the regulation of the p53–RPA70 protein complex. Inhibition of Ser15 phosphorylation using serine/threonine kinase inhibitors or by mutating the p53Ser15 residue prevented the UV-, cisplatin- and hypoxia-mediated disruption of the p53–RPA70 complex. The UV- and hypoxia-treated H1299 cells transfected with wild-type p53 cDNA were used as a control (Figure 4D, top panel). These results convincingly established that the phosphorylation of p53 at Ser15 disrupted p53–RPA interaction.

\[ \text{DNA-PK} \] phosphorylates p53Ser15 in hypoxia

DNA-PK is known to induce p53Ser15 phosphorylation under \textit{in vitro} conditions [44,45], and ATR kinase is also activated in an RPA-dependent manner [46]. We thus asked whether DNA-PK could phosphorylate p53 under hypoxia. The DNA-PK activity was increased in hypoxic MCF-7 cells (Figure 5A), and IP (using anti-DNA-PK antibodies) showed that there was cellular interaction between p53 and DNA-PK (Figure 5B). The observed interaction was further disrupted by DNA-PK siRNA with a decrease in total p53 phosphorylation as well as p53Ser15 phosphorylation in UV-treated normoxic MCF-7 cells (Figure 5C). The fraction of total phosphorylated p53 as well as p53 phosphorylated at the Ser15 residue was low in hypoxic MCF-7 cells (Figure 5C), whereas DNA-PK silencing abolished the total p53 phosphorylation and p53Ser15 phosphorylation in hypoxic MCF-7 cells (Figure 5C). In addition, co-IP showed that silencing of DNA-PK abolished the disruption of the p53–RPA70 complex in hypoxic MCF-7 cells (Figure 5D) and caused an increase in the apoptotic fraction of cisplatin-treated hypoxic cells (69 %) (Figure 5E). An interaction between BRCA2 and RAD51 was shown to be disrupted due to CDK-mediated phosphorylation of BRCA2 at its Ser2051 residue [47].

Since ATR was previously shown to regulate p53 Ser15 phosphorylation during anoxia [48], we asked whether ATR kinase has any role in controlling p53 Ser15 phosphorylation during hypoxia (1.8 % O2). ATR kinase is not linked to p53 Ser15 phosphorylation at 1.8 % O2 concentration (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/443/bj4430811add.htm), although it is responsible for inducing the phosphorylation of p53 at the Ser15 residue in anoxic (0 % O2) cancer cells. ATR gene silencing did not affect the p53–RPA70 interaction in hypoxic MCF-7 cells [see Supplementary Figure S5 at http://www.BiochemJ.org/bj/443/bj4430811add.htm], although it was found to increase RPA70 protein expression in hypoxic MCF-7 cells (see Supplementary Figure S6 at http://www.BiochemJ.org/bj/443/bj4430811add.htm) and \textit{HIF-1} gene silencing reduced RPA70 protein expression (Supplementary Figure S6).

Hypoxia itself is insufficient to induce DNA damage; however, it can induce genetic instability through resistance to apoptosis and decreased DNA repair in tumour tissue [16]. Once DNA damage signalling pathways are initiated in response to hypoxia, a number of kinases, including ATR and DNA-PK, are activated, leading to phosphorylation of p53 [48]. ATM, ATR and DNA-PK belong to the phosphoinositide 3-kinase-related kinase family and phosphorylate substrates, which are essential to transduce checkpoint signals to downstream effectors, including the p53 and the BRCA1 breast cancer tumour-susceptible protein [49].

Previously, the role of ATR in inducing the phosphorylation of H2AX in hypoxic (2 % O2) and anoxic (0.02 % O2) RKO cells was observed by Hammond et al. [48]. It was reported that histone H2AX was clearly phosphorylated by ATR in response to extreme hypoxia/anoxia, but remained unaffected at 2 % oxygen. These finding suggest that, similar to p53, the histone H2AX might also be phosphorylated by another stress-activated kinase pathway in...
p53 Ser15 phosphorylation disrupts the p53–RPA complex

**Figure 4** p53Ser15 phosphorylation controls dissociation of the p53–RPA70 complex

(A) IP with anti-p53 and anti-phospho-p53 (phos-p53) antibodies shows the absence of p53 phosphorylation in normoxic MCF-7 cells. UV and cisplatin significantly induced p53 phosphorylation. Hypoxia exposure induces minimal phosphorylation of p53 protein; UV and cisplatin treatment induce p53 phosphorylation in hypoxic cells. Hypoxic (1.8 % O2) H1299 cells were transfected with NTD cDNA; IP confirms that hypoxia induces minimal phosphorylation of NTD in the presence of UV and cisplatin (n = 7). (B) The effect of serine/threonine kinase inhibitors on the stability of p53–RPA70 and NTD–RPA70 complexes was analysed in (i) MCF-7 and (ii) H1299 cells (transfected with NTD cDNA). p53 and RPA-70 were present in a complex in untreated cells. UV and hypoxia disrupted the p53–RPA70 complex. Addition of serine/threonine kinase inhibitors to UV, cisplatin and hypoxia-treated cells stabilized the p53–RPA70 complex. Similar results were observed for NTD–RPA70 binding in NTD-transfected H1299 cells (ii) (n = 6). (C) H1299 cells were transfected with p53 cDNA construct carrying mutations at serine and threonine residues of p53 NTD; p53 mutated at Ser6, Ser9, Ser15, Ser20, Ser33, Ser37 and Thr18, Thr55 and Thr81 residues are unable to disrupt the p53–RPA70 complex (n = 8). (D) To identify the phosphorylated residues, H1299 cells were transfected with a series of mutant p53 cDNA constructs which code for S6A, S9A, S15A, S20A, S33A, S37A, T18A, T55A and T81A. p53–RPA70 complex formation was analysed in each case. UV and hypoxia were used as signals to induce dissociation of the p53–RPA70 complex. The results show that transfection of the p53-Ser15 mutant abolished the hypoxia- and UV-induced dissociation of the p53–RPA70 complex. No other p53 mutant was able to show any effect on hypoxia- or UV-induced disruption of the p53–RPA70 complex, suggesting that p53Ser15 phosphorylation was mandatory for hypoxia- or UV-induced dissociation of the p53–RPA70 complex (n = 5). *P < 0.02. WT, wild-type; MT, mutant.

hypoxic cancer cells (2 % O2). ATM and DNA-PK matched cell lines showed that both p53Ser15 and H2AX were phosphorylated in response to hypoxia; however, a deficiency in ATR had no effect on H2AX phosphorylation. These results support our observation in the present study that both DNA-PK and ATR are active and induce p53Ser15 phosphorylation in anoxic cancer cells; however, only DNA-PK is responsible for p53Ser15 phosphorylation in cells exposed to physiological levels of hypoxia (1.8 %).

The increase in the DNA-PK kinase activity was shown further to be HIF-1-dependent, and HIF-1 siRNA significantly (50 %)
Figure 5  DNA-PK phosphorylates p53 at Ser15 in hypoxic cancer cells

(A) The kinase activity of DNA-PK was analysed in hypoxic MCF-7 cells. Results showed a 3-fold increase in the DNA-PK activity in hypoxic MCF-7 cells (grey bar). DNA-PK siRNA was used as a control. Error bars indicate S.D., significance was calculated using ANOVA (*P < 0.018), n = 10. (B) The binding between DNA-PK and p53 was observed in UV-, cisplatin- and hypoxia-treated MCF-7 cells. IP using anti-DNA-PK antibody shows the p53–DNA-PK interaction in all three cases. Hypoxia induced a marked increase in this interaction (n = 4). (C) The role of the p53–DNA-PK interaction upon total p53 as well as p53Ser15 phosphorylation was determined in UV- and hypoxia-treated MCF-7 cells; IP performed using anti-phospho-p53 and anti-phospho-p53Ser15 antibodies show that the level of the total phosphorylated p53 and p53 phosphorylated at the Ser15 residue was low in hypoxic MCF-7 cells. DNA-PK siRNA abolished total p53 phosphorylation in hypoxic MCF-7 cells, suggesting the role of DNA-PK in hypoxia-mediated p53 phosphorylation (n = 4). (D) Effect of DNA-PK silencing on dissociation of the p53–RPA70 complex was analysed. IP shows inhibition of hypoxia-mediated dissociation of p53 and RPA70 binding (n = 3). (E) The effect of DNA-PK gene silencing on the cell viability and apoptosis in the hypoxic MCF-7 cells was observed. Annexin V staining showed 12 % and 69 % apoptosis upon treatment with cisplatin (n = 10). IB, immunoblot; phospho-p53, phosphorylated p53.

Reduced the hypoxia-induced increase in DNK-PK kinase activity (see Supplementary Figure S7 at http://www.BiochemJ.org/bj/443/bj4430811add.htm). HIF-1 also regulated the cellular expression of the DNA-PK protein level (see Supplementary Figure S8 at http://www.BiochemJ.org/bj/443/bj4430811add.htm). The DNA-PK protein level increased in hypoxic cancer cells (Supplementary Figure S8), and this increase was HIF-1-dependent; HIF-1 gene silencing reduced the hypoxia-induced increase in the DNA-PK protein level (Supplementary Figure S8). HIF-1 gene silencing also reduced p53 Ser15 phosphorylation in hypoxic MCF-7 cells (see Supplementary Figure S9 at http://www.BiochemJ.org/bj/443/bj4430811add.htm). These results collectively suggest that the pathway of p53- and RPA70-mediated DNA repair in hypoxic cancer cells is HIF-dependent.

HIF-1 is the key regulator of the cellular response to oxygen deprivation. Under hypoxia, HIF-1 is stabilized, enters the nucleus and binds to HREs to transactivate a variety of hypoxia-responsive genes [50], therefore contributing to the adaptive response to hypoxic conditions. Um et al. [51] previously showed that hypoxia-induced accumulation of the transcription factor HIF-1 is reduced in DNA-PK-deficient cells. However, the authors did not show whether DNA-PK is activated under this stress condition. The results of the present study show that hypoxia induces DNA-PK kinase activity and HIF-1 regulates the cellular level of DNA-PK protein.
In hypoxic cancer cells, this study may be of importance in developing new chemoresistance in hypoxic cancer cells. Since the expression of DNA repair proteins remains unaltered in hypoxic cells [38]. Thus the present study shows that hypoxia-induced chemoresistance might be due to high RPA70-mediated NER/NHEJ DNA repair. We propose a model to explain the mechanism of hypoxia-induced dissociation of the p53–RPA70 protein complex and its probable effect upon hypoxia-induced RPA70-mediated NER/NHEJ and chemoresistance (Figure 6). In conclusion, we have demonstrated a novel mechanism that shows how DNA-PK phosphorylates p53–NTD, thus disrupting the p53–RPA70 complex. This finding might explain the reason for induction of NER/NHEJ repair and chemoresistance in hypoxic cancer cells. Since RPA70 gene silencing induces significant apoptosis in the resistant hypoxic cancer cells, this study may be of importance in developing new cancer therapeutics based upon regulation of RPA70-mediated NER and NHEJ repair pathways.

REFERENCES


AUTHOR CONTRIBUTION

Esha Madan designed the study, performed the experiments and wrote the paper. Rajan Gogna designed the study and performed the experiments. Uttam Pati designed the study, analysed the results, provided research material and wrote the paper.

ACKNOWLEDGEMENTS

We thank Professor Abbas Ali Mahdi (Chhatrapati Shahaji Maharaj Medical University, Lucknow, India) for the cDNA clones and the antibodies used in Figure 1(C).

FUNDING

We thank Jawaharlal Nehru University GRE and UGC (University Grants Commission (India)) for funding support to U.P.
SUPPLEMENTARY ONLINE DATA

p53 Ser15 phosphorylation disrupts the p53–RPA70 complex and induces RPA70-mediated DNA repair in hypoxia

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MATERIALS AND METHODS

UV exposure and drug treatment

Exponentially growing cells were exposed to UV at a dose of 25 J/cm2 with a Stratalinker 2400 UV cross-linker for 4 h (Stratagene). To equalize the irradiation conditions, medium was removed from the culture dishes during UV irradiation. Immediately after irradiation, the cells were incubated under standard culture conditions (normoxia) or exposed to hypoxia for 24 h. The optimal concentration of cisplatin was established using a standard curve (5–50 μM) and set as the maximal nontoxic effect at 20 μM for a period of 24 h. In each protocol, experiments were repeated at least three times and representative data are shown.

Hypoxia exposure

For hypoxic exposure, cells were placed in a humidified chamber maintained at 1.8% O2 and 5% CO2 and balance in N2 for 24 h. The oxygen level in the chamber was monitored with an oxygen analyser (Vascular Technology). As a control, cells were cultured in a standard incubator (normoxia; air with 5% CO2) for 24 h.

Plasmids

siRNA for p53, RPA70, XPC, RAD23B, XPA, XRCC5 (Ku86), XPB (TFIIH), CDK7, Ku80, PRKDC (DNA-PKcs), NHEJ1 (XLF), CETN2, TFIIH, XRCC4 and Ku70 were obtained from Santa Cruz Biotechnology. The reporter cassette construct for XLF, CETN2, TFIIH, XRCC4 and Ku70 were obtained from Santa Cruz Biotechnology. The reporter cassette construct for detection of NHEJ repair was constructed as described previously [1].

Flow cytometry

To quantify NHEJ events, the cells were examined by flow cytometry on day 4 after transfection to allow for maximum GFP (green fluorescent protein) expression. To normalize for the efficiency of transfection, the ratio of GFP+ to DsRed+ cells was used as a measure of NHEJ efficiency.

Annexin V staining

A Beckton Dickinson flow cytometer was used to detect the apoptotic cell surface shift of phosphatidylserine using the binding of FITC-conjugated annexin V to the outer membrane of intact cells. Floating cells were collected by centrifugation and these, as well as the attached cells, were washed in PBS (Ca2+- or Mg2+-free) with 0.1% EDTA. The attached cells were gently scraped off the dish without trypsin digestion and the floating

and attached cell populations were combined and centrifuged (5 min at 500 g at 25°C). After centrifugation, the cells were incubated with 500 μl binding buffer (Abcam) followed by 5 μl of annexin V–FITC and 5 μl of propidium iodide. Cells were incubated at room temperature (25°C) for 5 min. These cells were then filtered through 70 μm mesh to eliminate cell aggregates and were analysed by flow cytometry.

RT–PCR

Cells were lysed and pipetted in TRIzol® (1 ml of TRIzol® per well of a 6 well plate for cultured cells). Cells were then kept at room temperature for 5–10 min, 200 μl of chloroform per 1 ml of TRIzol® was added and mixed thoroughly. The cells were then left at room temperature for 10 min. Cells were then centrifuged at 12000 rev./min at 4°C for 15 min and the upper aqueous colourless layer was transferred to a fresh microfuge tube. To this microfuge tube, 75 μl of LiCl followed by 1 ml of ice-cold ethanol were added and kept at −20°C for 2–3 h. The microfuge tube was centrifuged at maximum speed (5000 g) for 15 min at 4°C. The supernatant was discarded and 250 μl of 70% (v/v) ethanol was added and the tube was kept at room temperature for 2 min. The tube was again centrifuged at 32000 g at 4°C for 5 min, and finally the supernatant was discarded and the pellet was resuspended in RNA-grade water until it was completely dissolved. Single-strand cDNA was synthesized on incubation with sense and anti-sense primers using a RevertAid™ H Minus first strand cDNA synthesis kit (Fermentas). The resulting cDNA was diluted 1:10 before proceeding with the PCR reaction. PCR was conducted in a master-cycler gradient (Brinkmann Instruments). Each PCR reaction used 50 μl of cDNA, 2.5 units of Taq polymerase (Eppendorf), 0.2 mM dNTPs and 0.5 μM primer. PCR products were resolved on a 2% agarose gel containing 0.01% ethidium bromide and visualized using an UV illuminator. Another set of primers was used in all reactions to amplify an endogenous control gene (β-actin). Primers for RT–PCR are given in Table S1.

### Table S1

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5′–3′)</th>
</tr>
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<tr>
<td>p53 forward</td>
<td>CTGCCCCCGTGTACCTTCGTGC</td>
</tr>
<tr>
<td>p53 reverse</td>
<td>CACGCAAAATCCCTCCACCTCG</td>
</tr>
<tr>
<td>RPA70 forward</td>
<td>GCGGGGCGCGCAACCTTCGCGCGC</td>
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<tr>
<td>RPA70 reverse</td>
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<tr>
<td>β–Actin forward</td>
<td>AGTAGAGTGGACCTGATCACCG</td>
</tr>
<tr>
<td>β–Actin reverse</td>
<td>GCTGCTGATCCACATCTGCTG</td>
</tr>
</tbody>
</table>

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2 To whom correspondence should be addressed (email uttam@mail.jnu.ac.in).
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Figure S1  The effect of simultaneous inhibition of NER and NHEJ in UV/cisplatin-treated hypoxic MCF-7 cells

Inhibition of NER and NHEJ was performed using anti-XPA, anti-XPB, anti-Ku70 and anti-Ku80 antibodies. Results show 88% apoptosis in cisplatin-treated hypoxic cells, and other treatments were also significantly more effective in inducing apoptosis. n = 10, error bars indicate S.D., significance was measured using ANOVA; *P < 0.045.

Western blotting

Whole-cell lysates were prepared using RIPA buffer [10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 0.1% SDS and 1 mM DTT (dithiothreitol)]. Proteins were resolved by SDS/PAGE (10% or 12% gels) and transferred on to PVDF membranes (Invitrogen). The membranes were stripped according to the molecular mass of the protein. Incubations with primary antibodies were followed by incubations with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibodies and detected using the DAB (diaminobenzidine) staining kit (Vector Lab). To quantify Western blotting signals, densitometry was performed using ImageJ software.

DNA-dependent protein kinase assay

The kinase activity of DNA-PK was determined using the Sigma TECTM DNA-dependent protein kinase assay system from Promega. Briefly, 10 μg of nuclear extract was incubated with activator DNA, a biotinylated p53-derived peptide substrate, and [α-32P]ATP at 30°C for 5 min. The sample was terminated by adding termination buffer. Each termination reaction sample was spotted onto SAM2TM Biotin Capture Membrane and washed with 2 M NaCl and 2 M NaCl in 1% (v/v) H3PO4.

Figure S2  NTD–RPA70 binding in hypoxic MCF-7 and H1299 cells transfected with NTD–HA

In MCF-7 cells, IP with anti-p53, anti-RPA70 and anti-HA antibodies showed no binding between p53 and RPA70 or NTD and RPA70. In H1299 cells, IP using anti-RPA70 and anti-HA antibodies showed no binding between NTD and RPA-70. These results establish that, in normal cells, RPA70 interacts with p53–NTD and hypoxia dissociates p53–RPA70 binding (n = 5). C-ter, C-terminal; N-ter, N-terminal.
Figure S3 Efficiency of serine/threonine kinase inhibitors to inhibit p53 phosphorylation in MCF-7 cells

IP shows that serine/threonine kinase inhibitors abolished p53 phosphorylation (phos-p53) in UV-, cisplatin- and hypoxia-treated MCF-7 cells.

Figure S4 Role of ATR kinase in hypoxia-mediated phosphorylation of p53 at Ser^{15}

The IP with anti-phospho-p53 antibody shows that silencing DNA-PK in MCF-7 cells exposed to 1.8 % O_2 concentration led to a significant decrease in total p53 phosphorylation. However, silencing of ATR did not result in such a drastic loss in total p53 phosphorylation at 1.8 % O_2 concentration. When MCF-7 cells were exposed to anoxia (0 % O_2 concentration), total p53 phosphorylation was similar to that observed during 1.8 % O_2 concentration. DNA-PK silencing was unable to influence the total p53 phosphorylation at 0 % O_2 concentration. On the other hand, ATR silencing induced a significant decrease in the total p53 phosphorylation at 0 % O_2 concentration, whereas ATR gene silencing did not affect p53^{Ser^{15}} phosphorylation at 1.8 % O_2 concentration. At 0 % O_2 concentration, the p53^{Ser^{15}} phosphorylation was comparable with its value at 1.8 % O_2 concentration. Both DNA-PK and ATR gene silencing experiments led to a significant decrease in p53^{Ser^{15}} phosphorylation.

Figure S5 Role of ATR gene silencing on the cellular interaction of p53 and RPA70 in hypoxic MCF-7 cells

Hypoxia exposure disrupted p53–RPA70 binding, and DNA-PK gene silencing restored their interaction. ATR silencing did not show a restoration of the p53–RPA70 interaction, suggesting that the interaction was independent of ATR. In MCF-7 cells exposed to 0 % O_2 concentration, anoxia induced disruption of the p53–RPA70 complex. Interestingly, both DNA-PK and ATR gene silencing did not restore the p53–RPA70 interaction. However, the combined gene silencing of ATR and DNA-PK led to restoration of the p53–RPA70 interaction in anoxic MCF-7 cells.
Western blotting analysis shows that RPA70 expression is increased during hypoxia (1.8% \( \text{O}_2 \) concentration) and that HIF-1 siRNA causes a significant decrease in RPA70 expression. The results suggest that HIF-1 plays a crucial role in increasing the cellular expression of RPA70 in hypoxic cancer cells.

The DNA-PK kinase activity increases by 65% during hypoxia (1.8% \( \text{O}_2 \) concentration) in comparison with normoxia. Upon silencing HIF-1, the DNA-PK kinase activity shows a significant 54% decrease (grey bar). These results suggest that DNA-PK kinase activity is HIF-1 dependent.

A significant increase is seen in the DNA-PK protein expression upon hypoxia exposure (1.8% \( \text{O}_2 \) concentration). Upon HIF-1 gene silencing, the hypoxia-induced increase in HIF-1 protein expression is significantly reduced.

IP shows a significant decrease in p53\(^{\text{Ser15}}\) phosphorylation in hypoxic MCF-7 cells upon HIF-1 gene silencing.

REFERENCE