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Chemical DNA damage activates p21$^{WAF1/CIP1}$-dependent intra-S checkpoint

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Abstract When cells progressing in G_1 phase are irradiated with UV light, two damage checkpoint pathways are activated: CHK1–Cdc25A and p53–p21$^{WAF1/CIP1}$, both targeting Cdk2 but the latter inducing long lasting inactivation. In similarly irradiated S phase cells, however, p21$^{WAF1/CIP1}$-dependent checkpoint is largely inactive. We report here that p21-dependent checkpoint can effectively be activated and induce a prolonged S phase arrest with similarly extended inactivation of Cdk2 by association of p21 if mid-S phase cells are damaged with a base-modifying agent instead of UV light, indicating that the poor utilization of p21-dependent checkpoint is not an innate property of S phase cells.

Keywords: p21$^{WAF1/CIP1}$; Cdk2; CHK1; Intra-S checkpoint; Methyl methanesulfonate

1. Introduction

When cells traversing G_1 phase are irradiated with UV light, the senor ATR detects damaged DNA and activates CHK1 kinase by phosphorylating at Ser345. Activated CHK1 inactivates Cdc25A phosphatase. This rapid reaction causes initial inactivation of Cdk2 resulting from accumulation of the Tyr15 phosphorylated form. In parallel, both ATR and activated CHK1 activate and stabilize p53, which in turn induces the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ that is responsible for long lasting inactivation of Cdk2 and consequently prolonged G_1 arrest [1–3].

When S phase-progressing cells are similarly irradiated, the same sensor detects damage and activates CHK1, but only the CHK1–Cdc25A pathway is efficiently utilized for inactivation of Cdk2 [2,3]. Consequently, inactivation of Cdk2 is transient and S phase arrest is short although late origin firing could also be blocked via an additional mechanism involving activated CHK1 [4]. The reason for the lack of efficient utilization of the p21-dependent system is not well understood, but it has been attributed at least in part to poor induction of p21 resulting from S phase-specific impairments of its transcription and facilitation of its proteasome-mediated degradation [5–11].

We have recently found that treatment of mid-S progressing cells with a base-modifying chemical, such as methyl methanesulfonate and cisplatin, effectively activates p21-dependent checkpoint and induces long lasting inactivation of Cdk2 and consequently a prolonged S phase arrest like in G_1 phase, indicating that the poor utilization of the p53–p21 pathway is not an intrinsic property of S phase cells.

2. Materials and methods

2.1. Antibodies

Methyl methanesulfonate (MMS) was purchased from Sigma; *cis*-diamminedichloroplatinum (cisplatin) from WAKO. The antibodies αCdk2 (M2), αCdk2 (M2)-G, αCyclin E (C-19), αCyclin A (C-19), αp21 (C-19), and αp53 (Pab240) were purchased from Santa Cruz Biotechnology; αp-Tyr15Cdc2, αp-Thr160Cdk2 and αp-CHK1 (Ser345) from Cell Signaling; αHistone H1 from Upstate Biotechnology; αGAPDH from Chemicon International.

2.2. Cells culture and preparation of mid-S progressing cells

Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5–10% FCS. Wild-type mouse embryonic fibroblasts were isolated from C-57 mouse embryos. The normal rat kidney fibroblast line NRK-49F and its Cdk2^{Y15} or Cdk2^{F15} overexpressers were synchronized to mid-S phase by 16 h incubation following G_0 arrest and release as described [12]. Wild-type and $p21^{-/-}$ mouse embryonic fibroblasts [13] were synchronized to early S by a double thymidine block [14] and cultured in fresh medium for 1–2 h to progress into mid-S phase.

2.3. DNA damage with chemicals and UV irradiation

Cells traversing mid-S phase ($2–5 \times 10^5$ in a 10 cm dish for each time point) were treated for 2–3 h with 120–150 μg/ml methyl methanesulfonate (MMS), 4 h with 20 μg/ml cisplatin contained in growth medium or irradiated with UV light at 7.5 J/m^2, washed twice with phosphate-buffered saline, and cultured in growth medium containing 25 ng/ml colcemid with cell sampling at the specified times.

2.4. Cytometric analysis

Flow cytometric analysis was performed with the FACScan flow cytometer (BECKMAN COULTER ECPIS XL) equipped with computer-assisted analysis of cell population in each cell cycle phase according to the manufacturer's instruction.

2.5. Preparation of whole cell lysates, Cdk2 kinase assay and immunoblot

Harvested cells were lysed with ice-cold immunoprecipitation buffer containing 0.35 M NaCl. One half of the lysates was used for detection of the specified proteins by immunoblot and the other half for assay of Cdk2 activity and associated proteins as described [15].

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Abbreviations: MMS, methyl methanesulfonate; Cisplatin, *cis*-diamminedichloroplatinum; DMEM, Dulbecco's modified Eagle medium; MEF, mouse embryonic fibroblast

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3. Results

3.1. Treatment of mid-S phase-progressing cells with methyl methanesulfonate induces a long-lasting S phase arrest with inactivation of Cdk2

During studies on DNA damage-induced arrest and recovery of S phase-progressing cells, we observed that treatment of mid-S progressing rat fibroblast line NRK-49F [12] with a comparable dose of methyl methanesulfonate (MMS) but not of UV irradiation led to a prolonged S phase arrest accompanied by similarly extended inactivation of Cdk2 with binding of p21 despite that the same sensor ATR was known to detect both damages [2]. In this experiment, mid-S progressing NRK cells were treated for 3 h with MMS at 120 μg/ml or irradiated with UV light at 7.5 J/m^2 and cultured further in growth medium containing colcemid, a metaphase blocker, to prevent their return to G_1 that could obscure the population of S phase-arrested cells. During MMS treatment and subsequent culturing, cells were collected every 4 h and analyzed for cell viability, cell cycle patterns and the levels of CHK1 and its Ser345 phosphorylated active form, Cdc25A, p21, p53, Cdk2 activity, Cdk2, its Tyr15 phosphorylated form and associated p21 by immunoblot, immunoprecipitation and kinase assay. The viability of these cells was 70–80% as assayed by colony forming ability, indicating that the extent of the damage induced by both treatments was comparable. In this series of experiment, mock-treated cells rapidly traversed S phase and reached G_2/M phase within 2–4 h (data not shown).

Cytometric analysis with computational estimation of cell population in each cell cycle phase revealed that the MMS-treated cells arrested in S phase for at least 20 h (Fig. 1A left upper panel). In response to MMS, Ser345 phosphorylation of CHK1 was detected at 4 h and disappeared by 8 h. The onset of CHK1 phosphorylation was followed by induction of p53 and p21 with concomitant inactivation of Cdk2, which persisted for at least 20 h. As indicated by a sudden increase of G2/M cells with a proportional decrease of S phase cells, recovery from S phase arrest began by 24 h. During arrest, Cdk2 underwent no apparent Tyr15 phosphorylation but association with significant amounts of p21, which diminished at the time of its re-activation.

By contrast, the UV-irradiated NRK cells arrested for less than 12 h accompanied by inactivation of Cdk2 with no apparent binding of p21 but an elevation of Tyr15 phosphorylation at 4–8 h with a slight decrease of Cdc25A protein although the extents of CHK1 phosphorylation and p21 induction were very similar between the two treatments (Fig. 1A right panels). Furthermore, re-activation of Cdk2 coincided roughly with the diminishment of Tyr15 phosphorylation to the original level that occurred at 12 h. These results confirm the well established finding that Cdc25A-dependent checkpoint is predominantly utilized in S phase upon UV damage and reveal that MMS treatment induces a prolonged S phase arrest accompanied by a similarly extended inactivation of Cdk2 with binding of p21 but no apparent elevation in Tyr15 phosphorylation.

MMS is not the only chemical that can induce a prolonged S phase arrest with inactive Cdk2 bound by p21. When treated with *cis*-diamminedichloroplatinum (cisplatin), another frequently used DNA base-modifying agent [16], mid-S progressing NRK cells similarly underwent an extended arrest for more than 16 h, again accompanied by inactivation of Cdk2 with p21 binding but no obvious elevation of Tyr15 phosphorylation, long after the Ser345 phosphorylation of CHK1 disappeared (Fig. 1B).

3.2. MMS treatment induces long lasting S phase arrest to primary mouse embryonic fibroblasts

Similarly, NRK is not the only cell that responds MMS and arrests long in S phase with extended inactivation of Cdk2 with binding of p21. When wild-type mouse embryonic fibroblasts (MEF) traversing mid-S phase were treated with MMS and cultured further, they arrested for more than 20 h with concomitant inactivation of Cdk2, just like NRK cells (Fig. 2 left panel). During the prolonged arrest, Tyr15 phosphorylation of Cdk2 did not elevate whereas induced p21 bound the inactive Cdk2. Furthermore, there were no significant changes in the amounts of Cdk2-associated cyclins A and E or in the level of Thr160 phosphorylation that was essential for activation of this kinase. Prolonged S phase arrest with Cdk2 inactivation was observed also with MMS-treated mouse fibroblast line C3H10T1/2 (data not shown).

3.3. $p21^{WAF1}$ but not tyrosine phosphorylation is responsible for the inactivation of Cdk2 during MMS-induced extended S phase arrest

The critical involvement of p21 in MMS-induced long lasting Cdk2 inactivation and S phase arrest was established by analysis of $p21^{-/-}$ mouse embryonic fibroblasts ($p21^{-/-}$ MEF). When mid-S progressing $p21^{-/-}$ MEF [15] was treated with MMS and analyzed just like for wild-type MEF, its S phase arrest was short (<12 h) and Cdk2 remained active despite that CHK1 was similarly activated (Fig. 2 right panel).

By contrast, there was no noticeable involvement of Cdc25A-dependent checkpoint in the extended inactivation of Cdk2 that was induced by MMS treatment. When two NRK cell clones both 4–5-folds overexpressing wild type Cdk2 ($Cdk2^{Y15}$) and unphosphorylatable $Cdk2^{F15}$, respectively, were treated with MMS during their mid-S progression and similarly analyzed, both of them arrested in S phase for roughly the same duration with inactive Cdk2 bound by p21 (Fig. 3). During the arrest, the level of Tyr15 phosphorylation of wild-type Cdk2 elevated slightly after 2 h whereas the onset of inactivation of $Cdk2^{F15}$ delayed about 2 h, suggesting that Cdc25A-dependent checkpoint was also activated albeit very weakly and participated in the initial phase of Cdk2 inactivation upon MMS treatment. Like in Fig. 1A, reactivation of Cdk2 roughly coincided with a reduction of bound p21.

3.4. Subcellular localization of induced p21 is similar between MMS treatment and UV irradiation

As shown above, unlike UV irradiation, MMS treatment let induced p21 to bind and inactivate Cdk2 despite the same level of induction. But this was not made by changes in subcellular localization of induced p21 or Cdk2. The same experiment as in Fig. 1A was carried out with additional comparison of the amounts of cytosolic and nuclear/chromatin-bound p21 as well as Cdk2 between MMS- and UV-treated cells. As shown in Fig. 4, despite the preferential association of induced p21 with the Cdk2 only in MMS-trea-