Lack of Effect of RuvB-Like Proteins on DNA Damage Signaling Activation

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Ataxia telangiectasia mutated (ATM) kinase is a central player in cellular response to DNA damage. Phosphorylation of the histone H2AX by ATM is required for the accumulation of repair proteins at the sites of double-strand breaks. Recently, it was reported that the histone acetyltransferase Tat interactive protein-60 (TIP60) is required to acetylate ATM prior to its activation. The RuvB-like proteins TIP48 and TIP49 are known to be necessary for the assembly and functional activity of the TIP60 acetyltransferase complex. In the present communication, we investigated the requirements of TIP48 and TIP49 for ATM activation by monitoring the cell cycle distribution and H2AX phosphorylation after irradiation of TIP48- and TIP49-depleted cells. We found that neither the cell cycle nor γ-H2AX were affected in TIP48- and TIP49-silenced cells, suggesting that the TIP60 chromatin modification complex is not engaged in DNA damage signaling upstream of ATM.

Key words: RuvB-Like Proteins, ATM, DNA Damage Signaling, Ionizing Radiation

Introduction

Ataxia telangiectasia mutated (ATM) kinase is a central player in cellular response to DNA damage. ATM is recruited to the sites of double-strand breaks (DSBs) (Falck *et al*., 2005; Lee and Paull, 2005) by the Mre11-Rad50-Nbs1 complex, and ATM activation blocks cell cycle progression and promotes DNA repair via phosphorylation of its downstream targets. Phosphorylation of the histone variant H2AX on serine 139 by the activated ATM serves to organize the repair process at the break sites, as it marks chromatin for repair, maintains retention of repair proteins there (Lee and Paull, 2005; Stucki and Jackson, 2006; Toh *et al*., 2006), and recruits chromatin modification and remodeling complexes (Downs *et al*., 2004; Morrison *et al*., 2004; van Attikum *et al*., 2004). The latter chromatin-modifying activities are indispensable for repair as they provide accessibility and interaction interfaces for the recruitment of DNA repair proteins (van Attikum and Gasser, 2005; Loizou *et al*., 2006; Downs *et al.,* 2007). Therefore, current models place ATM at the beginning of series of events that, via H2AX phosphorylation and downstream recruitment of chromatin modifiers, provide the necessary chromatin environment for DNA repair to occur. However, evidence has been provided for a reverse link between ATM and a chromatin modifier, showing that TIP60 is necessary for ATM activation (Sun *et al*., 2005, 2006, 2007; Jiang *et al.,* 2006). Other data contradict this finding. It is also difficult to reconcile the requirement of TIP60 for ATM activation with the finding that the TIP60 chromatin modification complex is recruited to break sites via interaction with H2AX (Downs *et al.*, 2004; Kusch *et al*., 2004). Knockdown of TIP60 in *Drosophila* cells did not impair phosphorylation of H2Av, which is the equivalent of mammalian H2AX and a direct substrate of ATM. In mouse cells, knockout of TRRAP, a subunit of the TIP60 acetyltransferase complex, did not impair activation of ATM (Murr *et al*., 2006). It is interesting whether other subunits of the TIP60 complex are required for the interaction between TIP60 and ATM.

The RuvB-like proteins TIP48 and TIP49 are conserved ATPases present in the mammalian TIP60 complex (Ikura *et al.*, 2000) as well as other chromatin modification/remodeling complexes. They are required for the correct assembly of the TIP60 complex and its histone acetylase activity (Jha *et al*., 2008). Recently we have shown that TIP49 and TIP48 play an important role in homologous recombination because they modulate

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the recruitment of the homologous recombination key protein Rad51 to the damaged chromatin most probably by affecting histone acetylation via their participation in the TIP60 chromatin modification complex (Gospodinov *et al.*, 2009). To further investigate the role of these proteins in DNA repair we studied whether TIP48 and TIP49 participate in the ATM-activating TIP60 complex by following cell cycle progression and phosphorylation of H2AX after induction of DNA damage under conditions of TIP48 and TIP49 depletion by RNA interference. The data we present here suggest that these proteins are not required for cell cycle block and H2AX phosphorylation after introduction of DSBs and are not present in the TIP60-containing complex that activates ATM.

Material and Methods

Cells and treatment

The human prostate cancer cell line PC3 was grown in DMEM supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2 atmosphere. The TIP49 sequence targeted for RNAi was 5'-GAGUCUUCUAUCGCUCCCAUCGU-3' (1034 – 1056 bp) and the targeted sequence for TIP48 was 5-GAGAGUGACAUGGCGCCUGUCCU-3 (962 – 984 bp) according to Watkins *et al.* (2004). TIP49 and TIP48 small interfering RNA (siRNA) and siRNA against luciferase (GL2, Eurogentec) were introduced in exponentially growing cells by transfection with Lipofectamine 2000 (Invitrogen, Paisley, UK) following the manufacturer's recommendations, and protein abundance was assessed 72 h after transfection using Western blot analysis. Irradiation was done using exponentially growing cells with a ^{137}Cs γ-ray source at a rate of 1.57 Gy min^{-1}. Fluorescence-activated cell sorting (FACS) analysis was carried out as previously described (Mladenov *et al.*, 2007).

Western blotting and immunofluorescence

To prepare a total cell extract, cells were collected using a cell scraper, washed twice in phosphate buffered saline (PBS), and boiled in Laemmli loading buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 1% SDS, 15% glycerol, 0.0025% Bromphenol blue. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% polyacrylamide gels according to Laemmli (1970). For Western blot analysis the proteins were transferred on 0.45 μm nitrocellulose membranes using a semidry Western blot device (Bio-Rad, Hercules, CA, USA). Equal loading and transfer were monitored by Ponceau S staining of the membranes and by β-actin.

The following primary antibodies were used: rabbit TIP49 antibody prepared as previously described (Sigala *et al.*, 2005), rabbit TIP48 antibody (Sigala *et al.*, 2005), mouse monoclonal β-actin antibody (Sigma), rabbit polyclonal γ-H2AX antibody (Abcam, Cambridge, UK). The secondary antibodies used were: goat anti-rabbit IgG-horseradish peroxidase-conjugated (HRP) (Amersham Biosciences, Uppsala, Sweden), sheep anti-mouse IgG HRP (Abcam), goat anti-mouse IgG-AlexaFluor700-conjugated (Molecular Probes, Paisley, UK), and goat anti-rabbit IgG-AlexaFluor800-conjugated (Molecular Probes). For the last two antibodies, membranes were scanned using the Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA). The rest of the antibody reactions were developed by the ECL plus Western blotting detection system (Amersham Biosciences) as recommended by the manufacturer.

To stain for γ-H2AX, cells were grown on cover slips, washed in PBS, fixed with 2% formaldehyde in PBS for 10 min at room temperature, washed in 150 mM glycine in PBS to quench crosslinking, permeabilized with 0.2% Triton X-100 in PBS on ice for 5 min, blocked in 3% goat serum/0.3% BSA in PBS for 1 h and stained with rabbit polyclonal γ-H2AX antibody (Abcam). The secondary antibody was Texas Red-conjugated goat anti-rabbit IgG (Abcam). Cells were examined with an Axiovert 200 fluorescent microscope (Carl Zeiss) using 63x and 100x objectives.

Results and Discussion

To study whether TIP48 and TIP49 participate in ATM activation we monitored the cell cycle distribution after introduction of DSBs by treatment with ionizing radiation (IR) under conditions of TIP49 and TIP48 depletion. The human prostate cancer cell line PC3 was transfected with siRNA against either TIP48 or TIP49. As a control siRNA against luciferase was used. 72 h after transfection the abundance of proteins was assayed by Western blot analysis (Fig. 1A). The results showed that the levels of both proteins were reduced to about 30% of the initial. If TIP48 and TIP49 participate

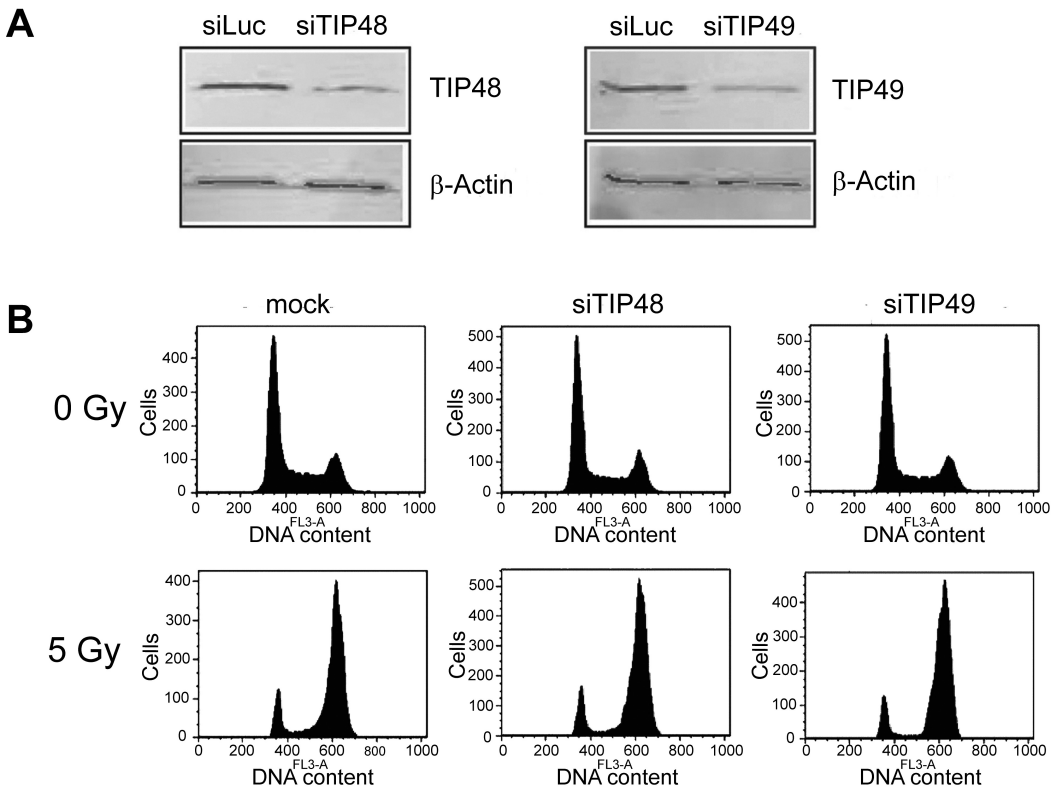

Fig. 1. Silencing of TIP48 and TIP49 and cell cycle distribution of knockdown cells. (A) PC3 cells were transfected with siRNA against TIP48 (siTIP48), TIP49 (siTIP49), or luciferase (siLuc) as a control. 72 h after transfection the abundance of TIP48 and TIP49 was assessed by Western blotting; β-actin was used as loading control. (B) TIP48-silenced, TIP49-silenced, and mock-silenced PC3 cells were irradiated with 5 Gy. FACS analysis of irradiated and non-irradiated control cells was carried out 24 h after irradiation.

in ATM activation as part of TIP60, it would be expected that such a significant reduction of protein amount would affect DNA damage signaling. ATM plays a critical role in activation of cell cycle checkpoints that lead to IR-induced arrest of cell cycle progression. If TIP48 and TIP49 participate in the activation of ATM under these conditions, their reduction would affect the cell cycle distribution. Cells were transfected with siRNA against TIP48 and TIP49 or transfected with siRNA against luciferase (mock-silenced). 72 h later, cells were treated with 5 Gy of IR and FACS analysis was performed 24 h after irradiation. Irradiated cells were blocked in the G2 phase of the cell cycle, but there were no differences in the cell cycle distribution between the mock-silenced and TIP48- or TIP49-silenced cells (Fig. 1B). This is an indication that TIP48 and TIP49 are not components of the TIP60 (sub-)complex that activates ATM. To further analyze this problem we studied the phosphorylation of H2AX, which is a direct substrate of the activated ATM. TIP48-, TIP49- or mock-silenced cells were treated with 5 Gy of IR and left to recover for 1 h. Total extracts of the irradiated and non-irradiated cells were analyzed by Western blotting with an antibody against γ-H2AX. Cells that were not irradiated displayed no detectable amounts of γ-H2AX. Extracts from TIP48-silenced, TIP49-silenced and control cells that were subjected to DNA damaging treatment showed substantial levels of H2AX phosphorylation, but there were no differences between the silenced and control cells (Fig. 2A). These results suggest that H2AX phosphorylation does not depend on TIP48 or TIP49. To verify this conclusion by using an independent approach we examined the formation of γ-H2AX foci. Irradiated and non-irradiated cells were fixed and stained

with antibody against γ-H2AX. As expected, IR induced extensive γ-H2AX foci formation, but again changes in the number or the pattern of foci between the TIP48- and TIP49-silenced and the mock-silenced cells were not observed (Fig. 2B).

Taken together, these results show that knockdown of TIP48 or TIP49 does not cause defects in DNA damage detection and signaling cascade, suggesting that these proteins are not components of the TIP60 complex that acetylates and activates ATM upon DNA damage. It is still possible that a pool of TIP60 molecules different from the TIP60 chromatin modification complex may be engaged in DNA damage sensing and signaling upstream of ATM (Squatrito *et al*., 2006). Our data are in agreement with the results of Murr *et al.* (2006) showing that cells lacking the TRRAP subunit of the TIP60 chromatin modification complex have normal activation of ATM and downstream IR-induced DNA damage signaling, including normal phosphorylation of H2AX. The fact, that a second module of the TIP60 chromatin modification complex, that of TIP48/TIP49, is not engaged in DNA damage cell signaling, strongly suggests that the role of this complex is limited to the modulation of loading of repair proteins to the damaged sites (Murr *et al.*, 2006; Gospodinov *et al.*, 2009) and the TIP60 chromatin modification complex is not engaged in the activation of the ATM-dependent cell cycle checkpoint signaling.

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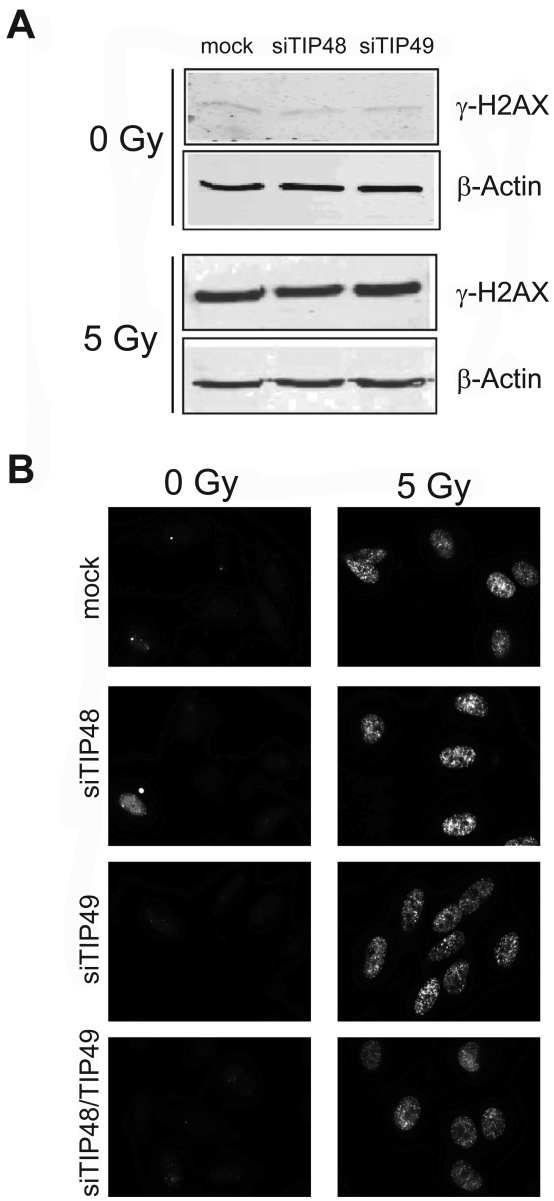

Fig. 2. TIP48 and TIP49 silencing do not affect H2AX phosphorylation. (A) PC3 cells were silenced as in Fig. 1, irradiated with 5 Gy of ionizing radiation or left unirradiated. After 1 h recovery, total extracts were prepared and the amount of γ-H2AX was analyzed by Western blotting; β-actin was used as loading control. (B) PC3 cells were transfected with siRNA against TIP48, TIP49, TIP48 and TIP49 simultaneously, or luciferase as a control. 72 h after transfection cells were irradiated with 5 Gy. After 1 h, cells were fixed and immunofluorescently stained with an antibody γ-H2AX.

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