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BTG3, a candidate tumor suppressor, promotes methylation of checkpoint kinase CHK1

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ABSTRACT

B-cell translocation gene 3 (BTG3), recognized as a member of an antiproliferative B-cell translocation gene/Transducer of ErbB2 (BTG/Tob) gene family, is a downstream target of p53 and is induced upon genotoxic stress in a p53 and Checkpoint kinase 1 (CHK1), a vital checkpoint kinase which contributes significantly in cell survival and cell cycle checkpoints, dependent manner. Post-translational modifications of CHK1 (phosphorylation and ubiquitination) facilitated by interaction with BTG3 have been observed suggesting their possible role in tumorigenesis, although the underlying mechanisms are unclear. Methylation, as one of the types of post-translational modifications, is a critical event during cell cycle checkpoint controls and DNA damage repair. Here, for the first time, it is reported that overexpression of BTG3 vividly enhances the methylation of CHK1. Expression of CHK1 was detected in a cancer cell line in this study. This work also reveals the significant role of the kinase domain of CHK1 for BTG3 facilitated methylation as BTG3 overexpression only promoted the methylation of wild type CHK1 but failed to promote the methylation of CHK1 mutants which were impaired for their kinase domain. This novel finding would therefore greatly enhance our understanding of the mechanisms underlying interactions between important cancer biomarkers.

1. Introduction

CHK1 is a ubiquitous serine/threonine kinase, essential to normal cellular functions. It phosphorylates numerous targets, including Cdc25, to allow the cell to progress through cellular division. It is activated predominantly via ATR-mediated phosphorylation but also by ATM in response to DNA damage or inhibition of DNA replication (Abraham, 2001; Shiloh, 2003). Its prevalence in the cell division cycle has led to it being the target for numerous anticancer therapies. Many drugs are being developed that are CHK1 inhibitors that bind to the ATP binding site. CHK1 inhibition is meant to stop the cell in S phase and G2/M to allow DNA damaging chemotherapeutic treatments to work on tumor cells.

CHK1 is an evolutionarily conserved protein kinase. In addition to ATR-mediated CHK1 phosphorylation, previous studies have shown that CHK1 is also subjected to other post-translational modification including mono- and poly-ubiquitination (Puc et al., 2005; Puc & Parsons, 2005; Zhang et al., 2005; Zhang et al., 2009; Cheng et al., 2013). Proteins, which generally are post-translationally methylated at arginine and/or lysine, are involved in a number of different cellular processes, including transcriptional regulation, RNA metabolism and DNA damage repair (Bedford & Clarke, 2009). Several proteins involved in DNA repair (MRE11, p53, DNA polymerase b) have been shown to be regulated by arginine/lysine methylation (Bedford & Clarke, 2009).

BTG3 (B-cell translocation gene 3), a candidate tumor suppressor gene, is a member of the antiproliferative BTG/Transducer of ErbB2 gene family and is induced by genotoxic stress in a p53- and CHK1-dependent manner (Cheng et al., 2013). BTG4 overexpression suppresses colony formation in colorectal cancer cells and its expression is frequently down-regulated in primary gastric cancers (Dong et al., 2009). BTG3 expression also has a suppressive role in cancer progression as evidenced from previous studies (Yoneda et al., 2009; Lin et al., 2012). Cheng et al. (2013) reported that BTG3 interacts with CHK1 and regulates its phosphorylation and activation. However, BTG3 mediated methylation of CHK1 have not been previously examined. In this study, a possible role in the interaction between BTG3 and CHK1, important cancer biomarkers, was identified in a cancer cell line. CHK1 was found to interact with and be methylated by BTG3. In addition to other types of post-translational modifications, methylation has an important role to play in maintaining the integrity of the genome. This

Abbreviations: BTG3, B-cell translocation gene 3; CHK-1, Checkpoint kinase 1; SDS, sodium dodecyl sulfate; PAGE, PA-gel electrophoresis; DTT, dithiothreitol.

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study also demonstrates that in the event of BTG3 over-expression, methylation was only observed in the wild type CHK1 (CHK1-WT), but not in the kinase-dead CHK1 mutant (CHK1-KD/D130A) nor in the N-terminal deleted (residues 1 to 276) CHK1 mutant (CHK1-N), truncated form of CHK1 which lacks the kinase domain. This signifies the important role of kinase domain of CHK1 for such modifications.

2. Materials and methods

2.1. Cell culture

293T cells were cultured in DMEM supplemented with 10% FBS, and 100 U/mL penicillin and 100 μg/mL streptomycin.

2.2. Cell lysis and immunoblotting

For immunoblot analysis of proteins, cell lysates were prepared in TEGN buffer (10 mM Tris at pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing 10 mM NaF, 10 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitor mixture (Roche). The antibodies used for immunoblotting were as follows: rabbit anti-actin (Sigma), mouse anti-MeK (Santa Cruz), mouse anti-Chk1 (Sigma), mouse anti IgG (Sigma), mouse anti-myc (Santa Cruz) and mouse anti-HA (Medical & Biological Laboratories). Anti-mouse HRP (Roche) and anti-rabbit HRP (Roche) were used as secondary antibody.

2.3. 293T cells transfection

Transfection of plasmids (mycBTG3, HA-CHK1-WT/wild type, HA-CHK1-N/N-terminal deleted mutant, HA-CHK1-KD/kinase-dead mutant-D130A) was performed by calcium phosphate precipitation method. The constructs were kindly provided by Dr. Sheau-Yann Shieh, IBMS, Academia Sinica.

2.4. CHK1 and MeK immunoprecipitation

CHK1 and MeK were immunoprecipitated from 293T cells using mouse anti-Chk1 antibody or mouse anti-MeK antibody, respectively. For immunoprecipitation cells were harvested, washed in ice-cold PBS, and extracted using TEGN lysis buffer (10 mM Tris at pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing 10 mM NaF, 10 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitor mixture. Extracts were clarified by centrifugation and the cleared lysates were incubated with antibody and protein G beads and then rocked for 2–3 h at 4 °C. The beads were washed three times using TEG/TEGN wash buffer, boiled in the protein sample buffer and resolved by 8–10% SDS/PAGE followed by western blot.

3. Results

3.1. BTG3 promotes methylation of CHK1

In order to understand the link between BTG3 overexpression and CHK1 methylation, 293T cells, grown in DMEM supplemented with 10% FBS, were transfected with full length BTG3 and CHK1-WT. The 293T cells were also transfected with full length BTG3 and the constructs of CHK1 (CHK1-N and CHK1-KD) to elucidate the importance of the kinase domain of CHK1 for BTG3 facilitated methylation.

Result showed that in absence of BTG3, CHK1-WT methylation level was nil. However, when co-expressed, the overexpression of BTG3 leads to the dramatically increased methylation of CHK1-WT (Fig. 1a). On the other hand, it was noted that in comparison to the CHK1-N expression without BTG3, co-expression of CHK1-N with BTG3 was not different (Fig. 1a) indicating that BTG3 was unable to promote the methylation of CHK1-N which lacks the N terminal. Similarly, CHK1-KD expression, which is a kinase-dead mutant, with and without BTG3 was not different again suggesting the role of kinase domain of CHK1 for BTG3 promoted methylation (Fig. 1a).

From this experiment it was concluded that BTG3 overexpression facilitates and promotes the methylation of CHK1. To check the efficiency of immunoprecipitation, membrane was stripped and re-probed with Met antibody (Fig. 1b). Fig. 1c is the input control result for this experiment.

3.2. Kinase domain of CHK1 is essential for BTG3 facilitated methylation

Fig. 1a clearly shows that BTG3 was unable to promote the methylation of CHK1 mutants (CHK1-N and CHK1-KD). BTG3 only promoted the methylation of CHK1-WT which indicates that the kinase domain of CHK1 is essential for such modification. However, for some unknown reasons, mutant CHK1-KD showed a basal level of methylation with and without BTG3 which was not increased when co-expressed with BTG3. This could be due to the stress generated upon CHK1-KD because of the single point mutation (D130A) in this mutant which also prevented CHK1-KD from further being methylated by BTG3.

4. Discussion

In this study, a novel role in the interaction between BTG3 and CHK1 was identified. This work presented evidence that BTG3 overexpression is associated with CHK1 methylation. Most importantly, CHK1 is one of the key strand break repair proteins which functions portentously in cell cycle checkpoints.

BTG3 expression have been shown to be negatively correlated with lymph node metastasis of lung cancer (Li et al., 2006), distant metastasis of gastric (Lu et al., 2005) and hepatocellular (Kumagai & Dunphy, 2000) cancers. Here, it was found that BTG3 over expression was positively linked to CHK1 methylation. .Gou et al. (2015) reported that BTG3 overexpression in vitro inhibited proliferation, induced apoptosis and senescence, and S/G2 arrest of gastric cancer cells, and in vivo suppressed the growth of gastric cancer cells by inhibiting proliferation, inducing apoptosis and autophagy, and indicated that BTG3 might be employed as a molecular target of gene therapy to reverse the aggressive phenotypes of gastric cancer.

Cheng et al. (2013) demonstrated the functional interplay among BTG3, CHK1, and $CRL4^{Cdt2}$ and suggested that genotoxic stress promotes the interaction and K63-linked ubiquitination of CHK1. Involvement of BTG3 in CHK1 methylation is thought-provoking and for the first time this work demonstrates that BTG3 overexpression and CHK1 methylation are linked. The observation that only wild type CHK1 (CHK1-WT), but not the CHK1 kinase domain impaired mutants (CHK1 N and CHK1-KD), was modified for methylation when co-expressed with BTG3 suggests the important role of kinase domain as well for such alteration.

The other observation that CHK1-KD had basal level of methylation which was not promoted further with BTG3 co-expression is interesting. This could possibly be due to the single point kinase dead mutation (CHK1-KD) in CHK1 which generated stress upon the mutant and also prevented the further methylation of CHK1-KD when co-expressed with BTG3. This could support the impression that methylation might be the key for CHK1 kinase activity. However, single point kinase dead mutation (CHK1-KD) of CHK1 does not interfere its interaction with BTG3 as interaction of BTG3 with CHK1 does not require the kinase activity of CHK1 (Cheng et al., 2013). This suggests that intact kinase domain of CHK1 is prerequisite for BTG3 facilitated methylation of CHK1.

The exploration of BTG3 interaction with CHK1, as described in this study, may lead to improved understanding of CHK1-regulated signal transduction. CHK1 modification/inhibition enhances cellular sensitivity to anti-cancer drugs (Okita et al., 2012). There are still a large number of issues to be addressed before the current knowledge about BTG3