Cellular Signalling 22 (2010) 1783–1789

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Both ERK1 and ERK2 kinases promote G2/M arrest in etoposide-treated MCF7 cells by facilitating ATM activation

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ARTICLE INFO

Article history:
Received 24 June 2010
Accepted 8 July 2010
Available online 15 July 2010

Keywords:
ERK1/2 kinases
DNA damage response (DDR)
ATM
CHK2
CDC25C
G2/M arrest

ABSTRACT

The MEK–ERK pathway plays a role in DNA damage response (DDR). This has been thoroughly studied by modulating MEK activation. However, much less has been done to directly examine the contributions of ERK1 and ERK2 kinases to DDR. Etoposide induces G2/M arrest in a variety of cell lines, including MCF7 cells. DNA damage-induced G2/M arrest depends on the activation of the protein kinase ataxia-telangiectasia mutated (ATM). ATM subsequently activates CHK2 by phosphorylating CHK2 threonine 68 (T68) and CHK2 inactivates CDC25C via phosphorylation of its serine 216 (S216), resulting in G2/M arrest. To determine the contribution of ERK1 and ERK2 to etoposide-induced G2/M arrest, we individually knocked-down ERK1 and ERK2 in MCF7 cells using specific small interfering RNA (siRNA). Knockdown of either kinases significantly reduced ATM activation in response to etoposide treatment, and thereby attenuated phosphorylation of the ATM substrates, including the S139 of H2AX (γH2AX), p53 S15, and CHK2 T68. Consistent with these observations, knockdown of either ERK1 or ERK2 reduced etoposide-induced CDC25C S216 phosphorylation and significantly compromised etoposide-induced G2/M arrest in MCF7 cells. Taken together, we demonstrated that both ERK1 and ERK2 kinases play a role in etoposide-induced G2/M arrest by facilitating activation of the ATM pathway. These observations suggest that a cellular threshold level of ERK kinase activity is required for the proper checkpoint activation in MCF7 cells.

1. Introduction

Eukaryotic cells employ multiple mechanisms to ensure accurate transmission of genetic information between generations. Critical surveillance of this transmission is provided by the DNA damage response (DDR) [1]. Disruption or attenuation of DDR plays an essential role in promoting tumorigenesis [2–5]. DDR is coordinated by activation of ATM and ATR (ATM- and Rad3-related) kinases, leading to the activation of DNA damage checkpoints to arrest cell cycle progression for repairing DNA damage [1,6,7].

One of the early events initiated by double-stranded DNA breaks (DSBs) is the phosphorylation of serine 139 in the SQE motif located on the tail of histone H2AX (γH2AX) by ATM/ATR kinases and the subsequent rapid formation of γH2AX foci at the DSB sites [8]. These foci are essential in facilitating the assembly of repair factors on damaged DNA sites [9,10] and also aid in the activation of DNA damage checkpoints [11–13]. γH2AX foci also form at sites of physiological DSBs in lymphocytes and germ cells [11,14–16]. Knockout of H2AX produces mice with immune deficiency and male infertility [10], while loss or reduction of H2AX compromises genome stability and facilitates tumorigenesis [10,17,18]. Taken together, a large body of evidence demonstrates that γH2AX foci play an essential role in the cellular DNA damage response.

In addition to facilitate the formation of γH2AX nuclear foci, ATM also induces cell cycle arrest through phosphorylation of its targets, p53 and CHK2 [1,19]. CHK2 then phosphorylates CDC25C on serine 216 (S216), which prevents CDC25C from dephosphorylating tyrosine 15 and threonine 14 of CDC2 (or CDK1), resulting in cell cycle arrest at the G2/M phase [19–22].

Cell cycle progression is regulated by the ERK (extracellular-signal-regulated) kinases, members of the mitogen-activated protein kinase (MAPK) family [23]. ERK kinases are activated by their upstream kinases, MEK1 and MEK2 [24]. Activation of ERK is sufficient to transform NIH3T3 cells or mouse embryonic fibroblasts lacking either p53 or p16 [25,26]. Additionally, ERK also plays a role in the cellular DNA damage response, a major process of tumour

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doi:10.1016/j.cellsig.2010.07.007

suppression. ERK activation was observed in response to multiple DNA damage stimuli [27–31]. The impact of the MEK–ERK pathway on DDR is cell-type-dependent [32]. While ERK activity facilitates DNA damage-induced cell cycle arrest or apoptosis in several mammalian cell lines and Drosophila [29,30,33–36], ERK activation also prevents DNA damage-induced apoptosis [31,37,38].

We report here that both ERK1 and ERK2 kinases are required for the proper checkpoint activation in etoposide-treated MCF7 cells. While etoposide treatment induces G2/M arrest in MCF7 cells by activating the ATM–CHK2–CDC25C pathway, knockdown of either ERK1 or ERK2 compromises this process, thereby reducing etoposide-induced G2/M arrest in MCF7 cells.

2. Materials and methods

2.1. Materials, cell lines, plasmids, and cell cycle determination

Propidium iodide (PI) and etoposide were purchased from Sigma. MEK1 inhibitor U0126 was obtained from Promega. Etoposide and U0126 were prepared in DMSO. Cell cycle distribution of MCF7 cells was determined by individualizing cells using PBS containing 0.02% EDTA, followed by examination of cell cycle profile according to our published procedure [30].

2.2. Cell lysis and western blot

Cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. 50 μg of total cell lysate was separated on SDS-PAGE gel and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% skim milk and then incubated with the indicated antibodies at room temperature for 1 h. Signals were detected using an ECL Western Blotting Kit (Amersham). Primary antibodies and concentrations used were: anti-p53 (FL-353 at 1 μg/ml, Santa Cruz); anti-phospho-p53(S15) (Cell Signaling, 1:1000); anti-ERK (1:500, New England Biolabs); anti-H2AX (Santa Cruz, 1:1000); anti-γH2AX (1:1000, Upstate), anti-phospho-ATM (S1981) (Rockland, 1:500), anti-ATM (Rockland, 1:500), anti-phosph-CHK2 (T68) (Cell Signalling, 1:500), anti-CHK2 (Cell Signalling, 1:1000), anti-phospho-CDC25C (S216) (Cell Signalling, 1:500), anti-CDC25C (Santa Cruz, 1:1000), and anti-actin (Santa Cruz, 1:1000).

2.3. Immunofluorescence staining

Cells were treated as defined in the figure legends. Immunofluorescent staining was carried out by fixing cells with prechilled (−20 °C) acetone–methanol for 15 min. The primary antibodies, anti-γH2AX (Upstate, 0.5 μg/ml) and anti-phospho-ATM (S1981) (Cell Signalling, 1:100), were then added to the slides at 4 °C overnight. After washing, secondary antibodies, FITC-Donkey anti-rabbit IgG (1:200, Jackson Immuno Research Lab) and Rhodamine-Donkey anti-mouse IgG (1:200, Jackson Immuno Research Lab), were then applied for 1 h at room temperature. The slide was then covered with VECTASHIELD mounting medium with DAPI (VECTOR Lab Inc., Burlingame, CA94010). Images were taken with a fluorescent microscope (Carl Zeiss, Axiovert 200).

2.4. Knockdown of ERK1 and ERK2 in MCF7 cells

Hairpin-based ERK1 and ERK2 shRNA plasmids (pSUPER-Erk1shRNA, pSUPER-Erk2shRNA) have been used to reduce ERK1 and ERK2 gene expression in multiple myeloma cells [39]. Dr. Chatterjee kindly provided us with these constructs. Since the pSUPER-based vector contains no antibiotic selection marker, we have co-transfected MCF7 cells with pcDNA3 (containing a Geneticin or G418 selection marker)

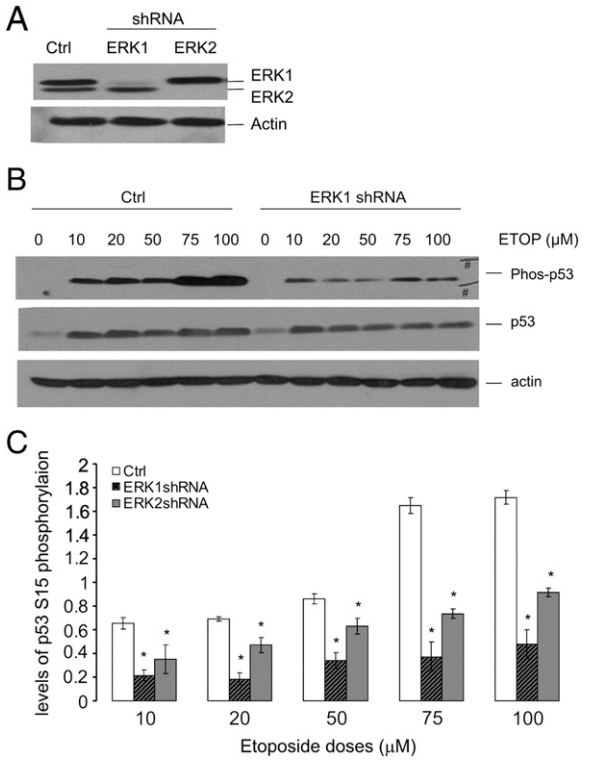

Fig. 1. Knockdown of either ERK1 or ERK2 reduces etoposide-induced p53 S15 phosphorylation. A) MCF7 cells were stably infected with empty vector (Ctrl), ERK1 shRNA, and ERK2 shRNA, followed by examination of the expression of ERK1, ERK2, and actin by western blot using the specific antibodies. B) Ctrl and ERK1 shRNA MCF7 cells were treated with the indicated doses of etoposide (ETOP) for 8 h. The expressions of p53 S15 phosphorylation (phos-p53), total p53, and actin were determined by western blot. #s indicate protein molecular markers. Experiments were repeated three times and a typical experiment was presented. C) p53 S15 phosphorylation in three independent experiments in Ctrl, ERK1 shRNA and ERK2 shRNA cells was quantified against the respective actin and presented as means ± standard errors. *: $p<0.05$ in comparison to the respective p53 S15 phosphorylation in Ctrl cells.

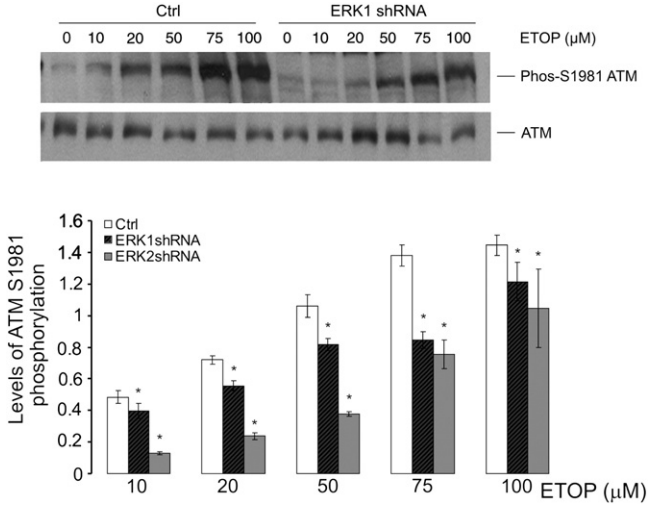

Fig. 2. ERK1 and ERK2 kinases facilitate etoposide-induced ATM activation. Ctrl, ERK1 shRNA, and ERK2 shRNA MCF7 cells were treated with the indicated doses of ETOP for 8 h and then examined for ATM activation (ATM S1981 phosphorylation: phos-S1981 ATM) and total ATM by western blot. Experiments were repeated three times. Typical western blot images derived from Ctrl and ERK2 shRNA cells were shown (up panel). ATM S1981 phosphorylation in three independent experiments was quantified against total ATM and presented as means ± standard errors. *: $p<0.05$ in comparison to the respective ATM S1981 phosphorylation in Ctrl cells.