FEBS Letters 583 (2009) 680–684

FEBS Letters

journal homepage: www.FEBSLetters.org

Regulation of the activity and expression of ERK8 by DNA damage

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ARTICLE INFO

Article history:
Received 1 October 2008
Revised 12 January 2009
Accepted 12 January 2009
Available online 21 January 2009

Edited by Angel Nebreda

Keywords:
ERK8
MAPK
DNA damage
MMS

ABSTRACT

We have investigated the agonists that activate transfected extracellular signal-regulated kinase 8 (ERK8) in cells, and have found that the most potent activators are hydrogen peroxide, DNA alkylating and cross-linking agents and the poly (ADP-ribose) polymerase inhibitor KU-0058948. The feature shared by all these agents is that they lead to the accumulation of single strand breaks in DNA, suggesting a role for ERK8 in the response to, or repair of, DNA single strand breaks. The DNA alkylating agent MMS also induced the disappearance of endogenous ERK8 by a proteasome-dependent mechanism.

1. Introduction

The most recently described member of the mitogen-activated protein (MAP) kinase family is extracellular signal-regulated kinase 8 (ERK8) [1], which is most closely related to ERK1, ERK2 and ERK5. Like these protein kinases, ERK8 possesses a signature Thr-Glu-Tyr motif in its activation loop but, in contrast to ERK1, 2 and 5, ERK8 does not appear to require an "upstream" activating kinase, and instead phosphorylates and activates itself [1,2]. Moreover, in contrast to ERK1 and ERK2, whose activation requires the dual phosphorylation of the Thr as well as the Tyr of the Thr-Glu-Tyr motif, the activity of ERK8 is determined by level of phosphorylation of the Thr residue alone. The activity of transfected ERK8 is therefore a balance between the rate at which it autophosphorylates this Thr and the rate at which this residue is dephosphorylated by one or more members of the PPP family of protein serine/threonine phosphatases [2].

ERK8 has a low basal activity, but we found that its activity is increased 5-fold or more after exposure to hydrogen peroxide [2]. Hydrogen peroxide is a source of reactive oxygen species that attack cellular DNA and cause single strand breaks, which prompted us to investigate the effects of other DNA damaging agents on the activation of ERK8. Here, we report that DNA alkylating and cross-linking agents, as well as the poly (ADP-ribose) polymerase (PARP) inhibitor KU-0058948, which all lead to the accumulation of single strand breaks (SSBs) in DNA, are potent activators of ERK8. In contrast other agents that cause different types of DNA damage failed to activate ERK8 significantly. These observations suggest an important role for ERK8 in the regulation of DNA damage.

2. Materials and methods

2.1. Materials

Methyl methane sulphonate (MMS), hydroxyurea (HU) and cisplatin were purchased from Sigma Chemical Co (Poole, UK), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) from TCI Europe (Boerenveldseweg, Belgium) and MG132 and Proteasome Inhibitor I from Calbiochem (Nottingham, UK). KU-55933 [3] and KU-0058948 [4,5] were synthesised by established methods. The sources of other reagents and DNA constructs have been described [2].

2.2. Antibodies

Two different antibodies were raised in sheep that both recognise the phosphorylated and unphosphorylated forms of ERK8 equally well. One was raised against a glutathione S-transferase (GST)-ERK8 fusion protein (Sheep 324B, bleed 1) and the other against His_6-ERK8 (Sheep S78B, bleeds 1, 2 and 4). The antisera were affinity purified by Kerry Burness, Division of Signal Transduction Therapy, MRC Protein Phosphorylation Unit, University of Dundee [2]. A polyclonal antibody that recognises the phosphorylated Thr-Glu-Tyr motifs of ERK1 and ERK2, an antibody that

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0014-5793/$34.00
doi:10.1016/j.febslet.2009.01.011

recognises the phosphorylated Thr-Gly-Tyr motif of p38 MAP kinases, and antibodies that recognise Chkl phosphorylated at Ser345 and Chk2 phosphorylated at Thr68, were purchased from Cell Signaling Technologies (Hitchin, UK). The antibody that recognises the phosphorylated Thr-Pro-Tyr motif of c-Jun N-terminal kinases (JNKs) was from Biosource (Invitrogen, Paisley, UK). An anti-ubiquitin antibody was purchased from DakoCytomation (Cambridgeshire, UK), an anti-ATR antibody was from Santa Cruz, and an anti-HA antibody (clone 12CA5) from Roche (Lewes, UK).

2.3. Cell culture, transfection, stimulation and lysis

The cell lines used were maintained in a 95% air/5% CO_2 atmosphere at 37 °C. HEK293 cells were cultured as described [2], whilst HCT116 and ATRflox/- cells were cultured in McCoy's 5A medium according to instructions provided by the American Tissue Culture Collection. HEK293 cells were transiently transfected with a pCMV5 ERK8 vector that expresses haemaglutinnin (HA)-tagged ERK8 using polyethylenimine (PEI) [2]. ATRflox/- cells were infected with adenovirus-expressing CRE recombinase, the medium changed after 24 h, and the cells transfected with a vector expressing HA-ERK8 using lipofectamine 2000. Forty-six and forty-eight hours later the medium was changed and the cells were exposed for 2 h to 2 mM MMS or for 10 min to hydrogen peroxide, and then lysed. For all other experiments the cells were stimulated with 2 mM MMS, 10 μM cisplatin, 2 μM MNNG or 2 mM HU, or exposed to 1 μM KU-0058948 for the times indicated in the figure legends. For ionising (γ)-radiation (IR), cells were exposed to a ^{137}Cs source at 10 Gy, whilst ultraviolet radiation was administered at 254 nm (UV-C) using a Spectrolinker (Spectronics Corporation) at 50 J/m^2. The cells used in the present study were lysed [4], centrifuged at 13 000×*g* for 10 min at 4 °C, and the supernatants (termed cell extracts) removed, frozen and stored at −20 °C until use.

2.4. Immunoprecipitation of endogenous ERK8 from cell extracts

Extracts prepared from HCT116 or HeLa cells (10 mg extract protein) were immunoprecipitated with an anti-ERK8 antibody coupled covalently to Protein G-Sepharose. Routinely, 3 μg of antibody was used per mg of extract protein. Immunoprecipitation was carried out for 6 h at 4 °C with continuous agitation. Following centrifugation for 2 min at 5000×*g*, the supernatants were discarded and the pellet washed once in lysis buffer containing 0.15 M NaCl, then twice in lysis buffer alone. Samples were denatured in SDS, subjected to SDS–PAGE, transferred to PVDF membranes and immunoblotted using the ECL detection system.

3. Results and discussion

3.1. Phosphorylation and activation of ERK8 in response to DNA damaging agents

We reported previously that hydrogen peroxide induced the phosphorylation and activation of transfected ERK8 in HEK293 cells (see 1). Hydrogen peroxide is a source of reactive oxygen species that could exert this effect in several ways, for example by attacking cellular DNA and cause single strand breaks (SSBs) in DNA (see 1). We therefore examined whether the DNA-akylating agent MMS, which also leads to the accumulation of SSBs, was able to activate ERK8. We found that MMS did indeed activate transfected ERK8 (Fig. 1A) and induced the phosphorylation of its Thr-Glu-Tyr motif (Fig. 1A) to a similar extent to hydrogen peroxide, although activation by MMS was slower. Activation by hydrogen peroxide was maximal after 10 min and sustained for at least

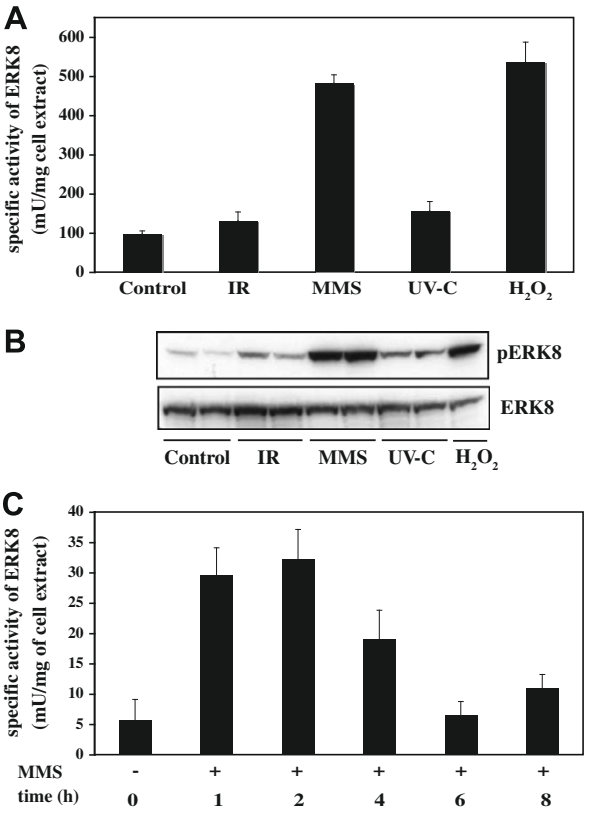

Fig. 1. The effect of DNA damaging agents on the activity and phosphorylation of ERK8. (A) HEK293 cells were transfected with a vector encoding wild type HA-ERK8. Thirty-six hours post-transfection, the cells were treated for 10 min with 1 mM H_2O_2 or for 2 h with 2 mM MMS, or exposed to 50 J/m^2 UV-C or 10 Gy γ-radiation (ionising radiation, IR) followed by incubation for 2 h at 37 °C. The cells were lysed, ERK8 was immunoprecipitated from 0.5 mg of cell extract protein and its activity measured using myelin basic protein (MBP) as a substrate [2]. (B) The cell extracts from (A) were immunoblotted with an antibody that recognises the phosphorylated Thr-Glu-Tyr motifs of ERK1, ERK2 and ERK8 (pERK8), and an antibody that recognises all forms of ERK8 equally well (ERK8). (C) The cells were transfected and 36 h later exposed to 2 mM MMS and lysed at the times indicated. ERK8 was immunoprecipitated from the cell extracts and its activity measured as in (A).

30–60 min [2]. In contrast, little activation by MMS was observed after 30 min (results not shown) and activation only became maximal after 1–2 h, declining thereafter (Fig. 1C). Several other DNA-damaging agents only activated ERK8 slightly up to 8 h of treatment. These included ionising radiation that causes double-strand breaks and UV-C that causes DNA photoproducts (Fig. 1A) and HU (an inhibitor of ribonucleotide reductase) (data not shown).

These results described above suggested that ERK8 might be activated specifically when SSBs in DNA accumulate. To investigate this further, we therefore exposed the cells to MNNG, another DNA alkylating agent, and to cisplatin, a DNA cross-linking agent, both of which also cause SSBs, possibly in the form of single strand gaps. These experiments showed that MNNG (Fig. 2A) and cisplatin (Fig. 2B) were potent, but slower activators of ERK8 than MMS. However, the activation of ERK8 attained after a 24 h exposure to MNNG or cisplatin was much higher than the maximal activation by MMS, which occurred after 2 h (Fig. 1C).

The compound KU-0058948 inhibits members of the poly (ADP-ribose) polymerase (PARP) family, which includes PARPl, an enzyme required for the repair of single strand breaks [4]. The prolonged incubation of cells with KU-0058948 therefore also leads to the accumulation of SSBs, and we found that this compound also activated ERK8 with a time course similar to MNNG and cisplatin (Fig. 2C).

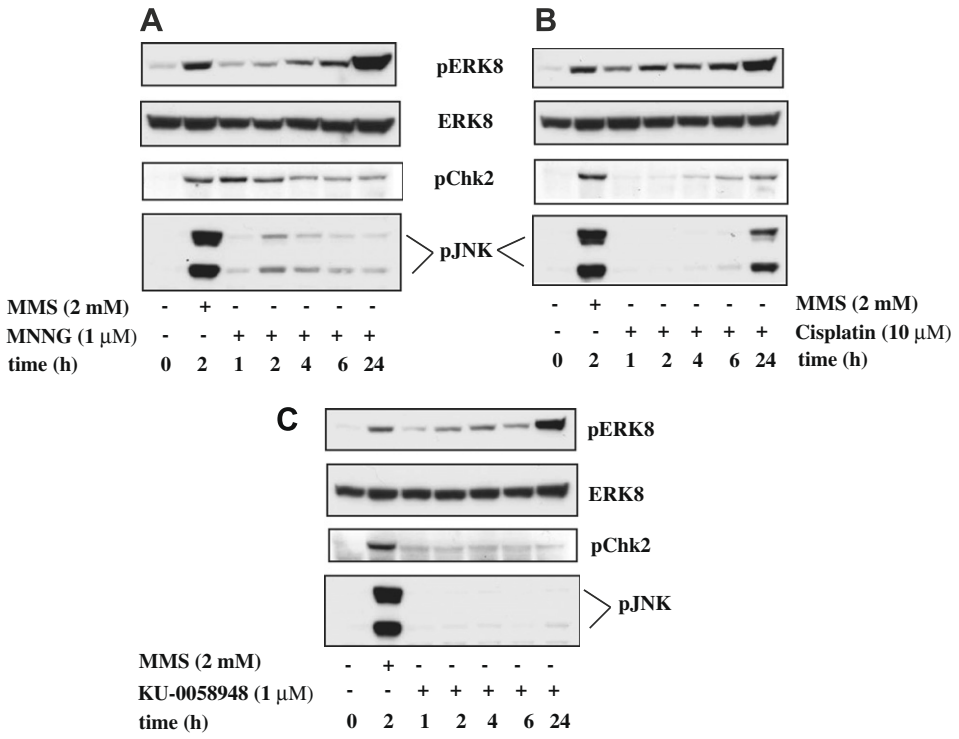

Fig. 2. The effect of MNNG, cisplatin and KU-0058948 on the phosphorylation of ERK8. HEK293 cells transfected as in Fig. 1A were treated with 2 mM MMS or 2 μM MNNG (A), 2 mM MMS or 10 μM cisplatin (B) or 2 mM MMS or 1 μM KU-0058948 (C) and lysed at the times indicated. The cell extracts (60 μg protein) were immunoblotted with the antibodies used in Fig. IB, an antibody that recognises Chk2 phosphorylated at Thr 68 (pChk2), and an antibody that recognises the phosphorylated Thr-Pro-Tyr motif of JNK isoforms (pJNK).

MMS not only activates ERK8 but also the MAP kinases JNK (Fig. 2) and p38 MAPK (not shown). However, although MNNG, cisplatin and the PARP inhibitor KU-0058948 all activated ERK8 more potently than MMS, they were much weaker activators of JNK (Fig. 2) and p38 MAPK (results not shown). Indeed, KU-0058948 was unable to activate these MAP kinase at all under the conditions tested (Fig. 2).

3.2. Mechanism of action of ERK8

Upon DNA damage, two signaling pathways are activated that are important in mediating DNA damage responses and DNA repair. One of these pathways leads to the activation of the Ataxia Telangactasia Mutated (ATM) protein kinase, which then phosphorylates and activates the protein kinase Chk2, as well as many other substrates. The second pathway leads to the activation of the ATM and Rad3-related kinase (ATR) which phosphorylates and activates the protein kinase Chkl and other substrates. To determine whether ERK8 is activated downstream of ATM, we incubated cells with KU-55933, a specific ATM inhibitor [3]. These experiments showed that KU-55933 prevented the MMS-stimulated phosphorylaton of Chk2 as expected, without affecting the MMS-induced activation of ERK8 (Fig. 3A). Thus the MMS-induced activation of ERK8 does not require the ATM-Chk2 signaling pathway. To determine whether ERK8 was activated downstream of ATR we depleted this protein kinase from ATRflox/- colon carcinoma cells [6]. The complete depletion of ATR had no effect on the MMS or hydrogen peroxide induced activation of ERK8 (Fig. 3B), demonstrating that the activation of ERK8 does not require the ATR-CHK1 pathway. Taken together, these results demonstrate that the MMS-stimulated activation of ERK8 is not mediated by ATM-Chk2 or ATR-Chkl.

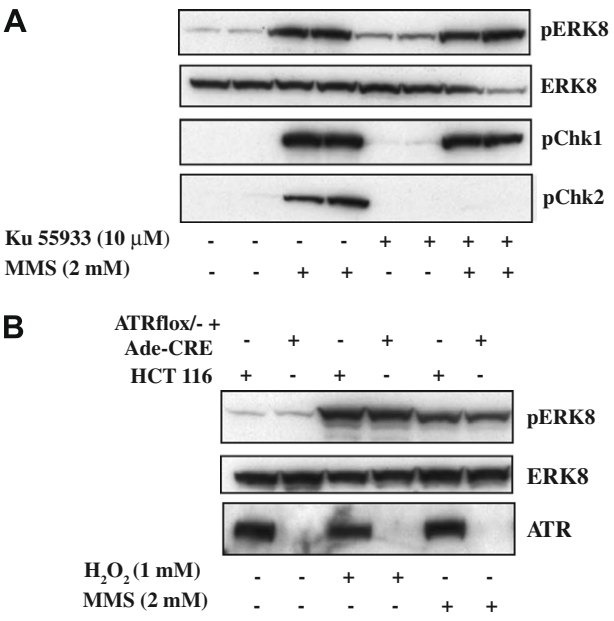

Fig. 3. The effect of the ATM inhibitor KU-55933 or ATR depletion on the phosphorylation of ERK8. (A) HEK293 cells were transfected as in Fig. 1A and 36 h post-transfection incubated with (+) or without (−) 10 μM KU-55933, then exposed for 2 h to 2 mM MMS and lysed. The cell extracts (60 μg) were immunoblotted with the antibodies used in Fig. 2 and an antibody that recognises Chkl phosphorylated at Ser 345 (pChkl). (B) HCT116 and ATRflox/- cells were infected in the presence (+) or absence (−) of adenovirus-expressing CRE recombinase (Ade-CRE). Forty-eight hours post-infection the cells were transfected with a vector expressing HA-ERK8, and after a further 24 h the cells were exposed for 10 min to 1 mM hydrogen peroxide or for 1 h to 2 mM MMS and lysed. The cell extracts (60 μg protein) were immunoblotted as in A and with an anti-ATR antibody (ATR).

3.3. Prolonged treatment with MMS decreases the level of ERK8

Although the MMS-induced activation of transfected ERK8 peaked after 2 h and declined thereafter, we noticed that the decline in activity did not result from dephosphorylation, but correlated with the loss of the ERK8 protein, which had almost disappeared after a 6–8 h exposure to MMS (Fig. 4A). In contrast, the levels of ERK1 and ERK2 did not decline. The MMS-stimulated disappearance of ERK8 did not require ERK8 activity, since the expression of the transfected catalytically inactive ERK8[D154A] declined in parallel (Fig. 4B). As reported previously for hydrogen peroxide [2], MMS failed to induce the phosphorylation of ERK8[D154A] (Fig. 4B), indicating that activation of the wild-type ERK8 had resulted from autophosphorylation.

3.4. Studies on the expression of endogenous ERK8

The endogenous ERK8 protein has never been studied previously due to its low abundance in cells and lack of sufficiently sensitive antibodies for its detection. To investigate whether the disappearance of transfected ERK8 after prolonged MMS-treatment was also observed with the endogenous protein, we raised two different antibodies that were capable of immunoprecipitating endogenous ERK8 from cell extracts. Following immunoprecipitation with either antibody we immunoblotted with the S324B antibody (Fig. SIA) or with the S78B antibody (results not shown). In each case an identical doublet was observed (Fig. SIA), the upper band of the doublet co-migrating with transfected ERK8 (Fig. SIB). These results suggested that this doublet did indeed correspond to the endogenous ERK8 and that the lower band, which

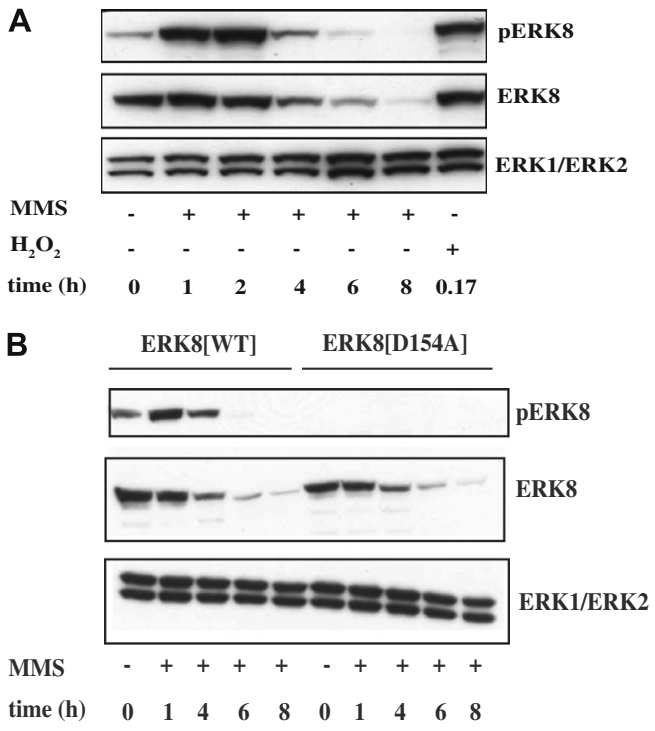

Fig. 4. Effect of MMS on the phosphorylation and expression of ERK8. (A) HEK293 cells were transfected with a vector expressing wild type HA-ERK8. Thirty-six hours post-transfection, the cells were either exposed for 10 min (0.17 h) to 1 mM hydrogen peroxide or to 2 mM MMS for the times indicated. Cell extracts (60 μg protein) were immunoblotted with the antibodies used in Fig. 1B and an antibody that recognises all forms of ERK1 and ERK2. (B) The cells were transfected with vectors encoding either wild type (WT) HA-ERK8 or a catalytically inactive mutant HA-ERK8[D154A]. Thirty-six hours post-transfection, the cells were exposed to MMS and lysed at the times indicated. Cell extract protein (60 μg) was denatured in SDS, resolved by SDS–PAGE and immunoblotted with the antibodies used in A.

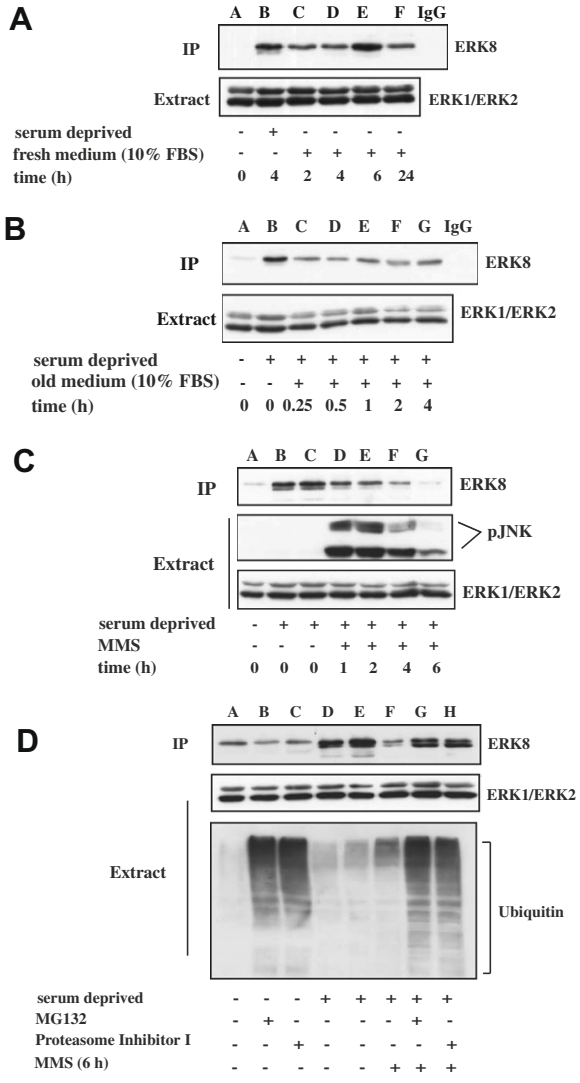

Fig. 5. Regulation of the expression of endogenous ERK8 in HCT116 cells. (A) HCT116 cells were seeded and kept for 48 h in culture medium containing 10% FBS. The cells were lysed (Lane A) or the medium removed and either replaced with serum-free medium and kept for 4 h before lysis (Lane B) or replaced with fresh medium containing 10% FBS and kept for the times indicated before lysis (Lanes C–F). ERK8 was immunoprecipitated (IP) using anti-ERK8 (Lanes A–F) or control sheep IgG (IgG) and immunoblotted with anti-ERK8 (upper panel). Cell extracts (60 μg protein) were immunoblotted with anti-ERKl/ERK2 (lower panel). (B) The cells were seeded and maintained for 48 h as in (A). The culture medium (termed "old medium") was removed and the cells either lysed (Lane A) or incubated for 4 h with fresh serum-free medium (Lanes B–G). They were then either lysed (Lane B) or the serum-free medium replaced with the original "old medium" (Lanes C–G). After incubation for the times indicated, the cells were lysed, ERK8 immunoprecipitated (IP) and immunoblotted with anti-ERK8 (upper panel labelled IP). Non-immune IgG replaced anti-ERK8 in control experiments (IgG). The cell extracts were also immunoblotted with antibodies recognising all forms of ERK1 and ERK2 (lower panel). (C) The cells were seeded and maintained for 48 h in culture medium containing 10% FBS. The medium was removed and the cells either lysed (Lane A) or the "old medium" replaced with fresh medium without serum (Lanes B–G). After 4 h, the cells were either lysed (Lanes B and C) or exposed to 2 mM MMS and lysed at the times specified (Lanes D–G). ERK8 was immunoprecipitated from the cell extracts and the immunoblotted with anti-ERK8 (top panel), while 60 μg of cell extract protein was immunoblotted to detect phospho-JNK and total ERKl/2. (D) The cells were seeded, maintained in culture for 48 h, then incubated for 2.5 h with (+) or without (−) 25 μM MG132 or 10 μM Proteasome Inhibitor I and lysed (Lanes A–C). Alternatively, the "old medium" was removed after 48 h and the cells incubated for 4 h with fresh serum-free medium to elevate ERK8 expression before lysis (Lanes D and E). In lanes F–H the cells were treated as for Lanes D and E except that, after 3 h in serum-free medium, they were incubated for 1 h without (−) or with (+) MG132 or Proteasome Inhibitor I. The cells were then exposed to 2 mM MMS for 6 h followed by lysis. Immunoprecipitation and immunoblotting of ERK8 was then carried out as in A and 60 μg of cell extract protein was immunoblotted with anti-ERKl/ERK2 and anti-ubiquitin.

was not always present (for example see Fig. 5), may arise from the proteolytic removal of a small peptide from one end of the protein. The identification of this band as ERK8 was confirmed by an siRNA "knock-down" experiments in HCT116 cells (Fig. SIC) and HeLa cells (results not shown). The amount of ERK8 immunoprecipitated from 10 mg cell extract protein was less than the amount of ERK8 present in 60 μg of cell extract prepared from transfected cells (Fig. SIB), explaining why the endogenous ERK8 cannot be detected by immunoblotting of cell extracts.

The level of endogenous ERK8 in HCT116 cells was extremely low after incubation for 48 h in normal growth medium. Interestingly, however, we found that the level of endogenous ERK8 rose sharply if the "old medium" was replaced by fresh medium, reaching a maximum after 4–6 h and then declining by about 50% over the next 24 h (Fig. 5A) and to almost nothing after 48 h (results not shown). A similar increase in ERK8 was observed when "old medium" was replaced by fresh, serum-free medium, demonstrating that the decreased expression of ERK8 after 48 h was not caused by the loss of a component present in foetal bovine serum (FBS). Instead, the results suggested that a factor accumulating in the culture medium after 48 h might be responsible for the decreased expression of ERK8. To test this hypothesis, we incubated HCT116 cells for 48 h in normal medium, then removed this "old" medium and set it aside. The cells were then cultured for 4 h in serum-free medium to elevate ERK8 expression, followed by incubation once again with "old medium". The "old medium" induced a rapid decline in the expression of ERK8, which was essentially complete after 15 min (Fig. 5B). These results again indicated that a factor(s) secreted from HCT116 cells was responsible for decreasing the level of expression of ERK8 after prolonged incubation in cell culture.

3.5. MMS decreases the expression of endogenous ERK8 by a proteasome-dependent event

To study the effect of MMS, HCT116 cells were cultured for 48 h in normal medium, then transferred to fresh serum-free medium for 4 h to increase the level of endogenous ERK8, before exposure to MMS. As observed for the transfected protein (Fig. 4A), MMS induced a gradual decline in the level of endogenous ERK8, hardly any protein remaining after 6 h (Fig. 5C). Two structurally distinct proteasome inhibitors, MG132 and Proteasome Inhibitor I, suppressed the MMS-induced decline in the level of the endogenous ERK8 protein, as well as increasing the overall extent of protein polyubiquitination in cells as expected (Fig. 5D, lanes D–H). However, incubation with these proteasome inhibitors did not prevent the decline in the expression of ERK8 that occurred after incubation in culture medium for 48 h (Fig. 5D, lanes A–C), and nor did it prevent the MMS-induced decrease in the expression of transfected ERK8 (results not shown)

3.6. Concluding remarks

The results presented in this paper demonstrate that the most potent activators of ERK8 all lead to the accumulation of SSBs in DNA by a mechanism that is independent of the activation of ATM or ATR. These findings suggest that ERK8 may play a role in the response to, or repair of, SSBs. It will clearly now be of great interest to investigate whether the depletion of ERK8 from cells affects cellular sensitivity to agents that cause SSBs.

Acknowledgements

We thank Dr. Steven Elledge for the ATRflox/- cells, the Antibody Production Team of the Division of Signal Transduction Therapy in the MRC Protein Phosphorylation Unit at Dundee (coordinated by Dr. James Hastie) for generating and purifying the anti-ERK8 antibodies, and Dr. John Rouse for valuable suggestions and critical reading of the manucscipt. DNA sequencing was performed by the Sequencing Service (MRC Protein Phosphorylation Unit, Dundee). This study was supported by the UK Medical Research Council, The Royal Society, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck KGaA and Pfizer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.01.011.

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