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MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells

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Abstract Mouse embryonic stem (ES) cells homozygous for disruption of the MSK1 gene had no detectable MSK1 activity. However, their activators (extracellular signal related kinase (ERK)1/ERK2) were stimulated normally in mitogen- and stress-activated protein kinase (MSK)1−/− and wild type cells in response to tetradecanoylphorbol acetate (TPA) and epidermal growth factor (EGF). TPA and EGF induced the phosphorylation of cyclic AMP-responsive element binding protein (CREB) at Ser-133 and ATF1 at Ser-63 in wild type cells and this was abolished by inhibition of the mitogen-activated protein kinase cascade. In contrast, the TPA- and EGF-induced phosphorylation of CREB/ATF1 was barely detectable in MSK1−/− cells. However, basal and forskolin-induced phosphorylation was similar, indicating that the MSK1 'knockout' did not prevent CREB phosphorylation by cyclic AMP-dependent protein kinase. Thus MSK1 is required for CREB and ATF1 phosphorylation after mitogenic stimulation of ES cells.

Key words: Cyclic AMP-responsive element binding protein; Phosphorylation; Mitogen- and stress-activated protein kinase 1; MAP kinase-activated protein kinase 1

1. Introduction

Cyclic AMP-responsive element binding protein (CREB) is the prototypic member of a family of transcription factors that regulate transcription by binding to cyclic AMP-responsive elements (CREs) in gene promoters. CREB family members have been implicated in the regulation of many processes, including cell proliferation, differentiation, metabolism and neuronal and immune responses [1,2]. Partial mouse 'knockouts' for CREB have suggested a role for this protein in memory [3], while the complete CREB 'knockout' leads to perinatal lethality as well as defects in T cell development [4].

The transactivation potential of CREB depends on phosphorylation of a serine residue in the kinase inducible domain (Ser-133 for CREB, Ser-63 for the closely related ATF1). These residues are phosphorylated by cyclic AMP (cAMP)-dependent protein kinase (PKA) in response to agonists that elevate intracellular cAMP. The same sites on CREB and ATF1 also become phosphorylated in response to growth factors and cellular stresses, but via the classical mitogen-activated protein kinase (MAPK) cascade [5,6] and the stress-activated protein kinase 2 (SAPK2, also called p38) pathway [7], respectively. However, while the phosphorylation of CREB is required for the full induction of many CRE-dependent genes, it is often insufficient to induce transcription per se, the activation of additional transcription factors being required.

The phosphorylation of Ser-133 (CREB) or Ser-63 (ATF1) is not catalysed by MAPK family members directly but by other protein kinases that they activate. Two protein kinases that are activated by extracellular signal related kinase (ERK)1 and ERK2, the MAPK family members of the classical pathway, are MAPK-activated protein kinase-1 (MAPKAP-K1, also called RSK) and mitogen- and stress-activated protein kinase (MSK). Both protein kinases phosphorylate CREB at Ser-133 in vitro, and mitogen-induced phosphorylation of this site is prevented by Ro 318220, a potent inhibitor of MAPKAP-K1 and MSK isoforms, consistent with the involvement of one or both enzymes [8]. However, there is controversy as to whether MAPKAP-K1 or MSK1 mediate the phosphorylation of CREB at Ser-133 in vivo. Several lines of evidence initially suggested that the phosphorylation of CREB at Ser-133 was catalysed by the MAPKAP-K1b (RSK2) isoform. For example, MAPKAP-K1b was reported to be the major CREB phosphorylating activity in extracts prepared from NGF-stimulated rat phaeochromocytoma 12 cells and to phosphorylate CREB more efficiently in vitro than the MAPKAP-K1a (RSK1) isoform [7,9]. In addition, fibroblast cell lines derived from human patients with Coffin–Lowry syndrome, which carry inactivating point mutations in the MAPKAP-K1b gene, were reported to be unable to phosphorylate CREB after stimulation with EGF [10]. More recently, however, IGF1 was found to induce a normal phosphorylation of CREB at Ser-133 in immortalised fibroblasts isolated from mouse 'knockouts' of the MAPKAP-K1b gene [11] and others found that MAPKAP-K1b did not phosphorylate CREB any more efficiently than MAPKAP-K1a in vitro [8].

There are two isoforms of MSK, termed MSK1 and MSK2, which share 75% amino acid sequence identity [8,12,13]. MSK1 was found to phosphorylate CREB in vitro with a far lower K_m value than MAPKAP-K1a or MAPKAP-K1b and to be localised to nuclei in both unstimulated and stimulated cells. This raises the possibility that MSK1 rather than MAPKAP-K1 mediates the mitogen-induced phosphorylation of CREB at Ser-133. In order to determine if MSK1 is a mitogen-induced CREB kinase in vivo, we have developed embryonic stem (ES) cell lines with targeted disruption of the MSK1 gene locus. These cells do not express MSK1 activity and, in this paper, we use them to study the effect of mitogens on the phosphorylation of CREB and ATF1.

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2. Materials and methods

2.1. Materials

Peptides were synthesised by Dr G. Blomberg (University of Bristol, UK). Peptide antibodies raised in sheep against residues 26–44 (LTVKHELRTANLTGHAEKV), 384–402 (FKRNAAVIDPLQFHMGVER) of human MSK1 (which do not recognise MSK2) and 712–734 (RNQSPVLEPVGRSTLAQRRGIKK) of human MAPKAP-K1b have been described previously [8,19]. The MAPKAP-K1 antibody recognises both MAPKAP-K1a (RSK1) and MAPKAP-K1b (RSK2). Isoform-specific antibodies for MAPKAP K1a, MAPKAP-K1b and MAPKAP-K1c (RSK3) were Santa Cruz products obtained from Autogen Bioclear (Calne, UK). A phospho-specific antibody, raised against the peptide KRREILSRRP*SYRK (where * denotes a phosphorylated residue), which recognises CREB phosphorylated at Ser-133 and ATF1 phosphorylated at Ser-63, was purchased from UBI (Lake Placid, USA), and an antibody which recognises an epitope near the carboxy terminus of CREB and ATF1 was from Santa Cruz (sc186). A phospho-specific antibody that recognises ERK1 and ERK2 phosphorylated at their Thr–Glu–Tyr motifs and another antibody that recognises phosphorylated and dephosphorylated ERK1/ERK2 equally well were purchased from New England Biolabs (Hitchin, UK). Antibodies that recognise GSK3α phosphorylated on Ser-21 were raised in sheep against RARTSS*FAEPG [17]. Mouse EGF and microcystin-LR were purchased from Life Technologies (Paisley, UK) and tetradecanoylphorbol acetate (TPA) and forskolin from Sigma (Poole, UK).

2.2. Cloning of the MSK1 genomic locus

A 129Sv mouse BAC library (Incyte Genomics, MO, USA) was screened using the open reading frame from a human MSK1 cDNA [8], to obtain a clone containing the mouse MSK1 gene. *Eco*RI and *Hin*dIII restriction fragments that hybridised to the human probe, were subcloned into pBluescript and sequenced. This allowed the identification of several MSK1 exon sequences, including the exon encoding the start of the open reading frame. The area around this region was mapped further and sequenced by subcloning overlapping fragments from the BAC clone.

A targeting vector was constructed to delete from the MSK1 start codon to the end of the first coding exon and insert transcription termination sequences. The first arm of homology was generated by PCR, during which *Not*I and *Bam*HI sites were introduced at the 5′ and 3′ ends, respectively. The arm was sequenced to ensure that there were no PCR-generated mutations. The second arm of homology was generated by subcloning the 4.8 kb *Xba*I fragment 3′ to the deletion. A thymidine kinase (TK) promoter-neo-pA cassette was inserted for positive selection, and a TK cassette included for negative selection. The vector was linearised at the *Not*I site before use.

2.3. ES cell targeting

Mouse ES cells were grown in DMEM (high glucose) supplemented with 15% foetal bovine serum, 0.1 mM non-essential amino acids, antibiotics (50 units/ml penicillin G, 50 μg/ml streptomycin), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and 1000 U/ml leukaemia inhibitory factor (LIF). ES cells were maintained on a feeder layer of primary embryonic fibroblasts before and during the initial selections, which had been derived from transgenic mice homozygous for an inserted neo gene. Targetting of the MSK1 gene to obtain ES cells heterozygous for the disruption was performed using standard procedures [20]. Targeted cell lines were identified by PCR using an antisense primer in the neo gene (P2, ACTGCTCGACATTGGGTGGAAACATTCCAG) and a primer outside the targeting vector (P1, CTTCCCGTCAGCCTCATGGGATTCGACAAG). A wild type (WT) control was also used that included a primer in the region to be deleted (P3, CTTCCCGTCAGCCTCATGGGATTCGACAAG). Targeted cell lines were confirmed by Southern analysis using a probe outside the 3′ arm of the vector (Fig. 1A).

Cell lines homozygous for the disruption were obtained by culturing the cells in high concentrations of G418, which selected for gene conversion. For this and subsequent assays, cells were grown on gelatinised tissue culture plastic in knockout DMEM supplemented with 10% ES replacement serum (Life Technologies) with the non-essential amino acids, antibiotics, L-glutamine, 2-mercaptoethanol and LIF as described above. Cells were selected in 4 mg/ml G418 for 15 days,

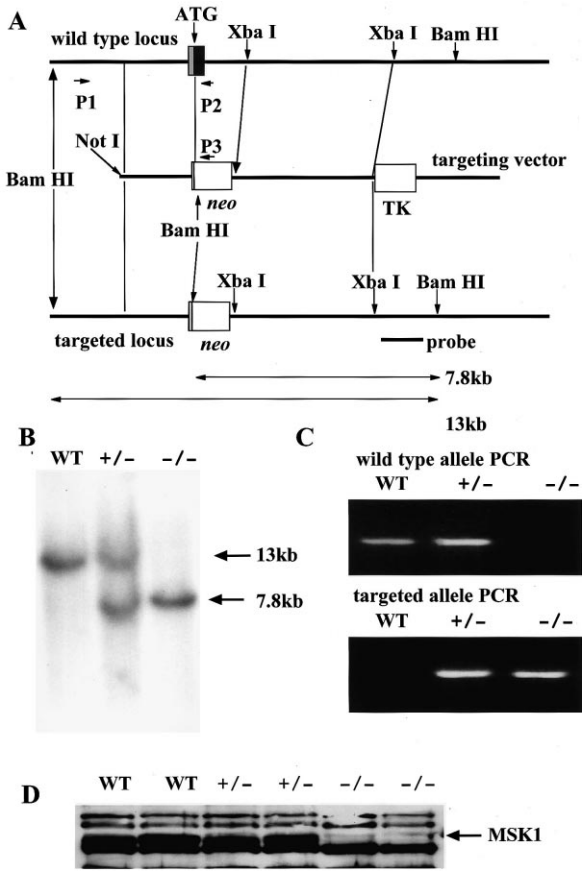

Fig. 1. Generation of MSK1−/− ES cells. A: A targeting vector was made to delete part of the first coding exon of the MSK1 gene. The 5′ untranslated region (grey box) was unchanged while the start of the coding region from the ATG (black box) was deleted and replaced with a neomycin selection cassette and polyadenylation sequences. Also shown are the positions of the probe to screen for the correct incorporation of the 3′ end targeting vector by *Bam*HI digestion (horizontal bar), and PCR primers (P1, P2 and P3) to screen for the 5′ end. B: Genomic DNA from WT, MSK1+/− and MSK1−/− cells was digested with *Bam*HI, run on an 0.8% agarose gel and Southern-blotted using the 3′ probe. 13 kb bands indicate a WT locus and 7.8 kb bands a targeted locus. C: PCR analysis in WT, MSK1+/− and MSK1−/− cells using P1 and P2 (to demonstrate the presence of a WT allele) or P1 and P3 (to show a targeted allele). D: 20 μg of lysate protein from WT, MSK1+/− and MSK1−/− cells was electrophoresed on a 10% SDS/polyacrylamide gel and immuno-blotted using an antibody raised against the whole MSK1 protein. The position of MSK1 is indicated by an arrow.

after which surviving colonies were isolated and expanded in the absence of G418. Cell lines were analysed by PCR and Southern blotting to identify homozygous cell lines. When cells were to be serum-starved they were incubated for 4–5 h in knockout DMEM (Gibco) supplemented with non-essential amino acids, antibiotics, L-glutamine and 0.1 mM 2-mercaptoethanol.

2.4. Cell stimulations

Cells were cultured to confluence on gelatinised tissue culture plastic, then incubated for 5 h in serum free medium. The cells were then incubated with either no agonist (unstimulated), TPA (400 ng/ml), EGF (100 ng/ml) or forskolin (20 mM)+isobutylmethyl xanthine (IBMX, 10 mM). The cells were lysed in ice cold 50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and complete proteinase inhibitor cocktail (Roche, East Sussex, UK, one tablet per 50 ml). For immunoblotting of CREB,

cells were grown to confluence on 6 cm gelatinised plates, serum-starved, then stimulated and lysed in 0.5 ml boiling 50% (v/v) lysis buffer, containing 1% (w/v) SDS and 5% (v/v) glycerol and heated for 10 min at 95°C.

2.5. Other procedures

Immunoprecipitation and assay of MSK1 and MAPKAP-K1 was carried out as described previously [16]. MAPKAP-K1 activity was assayed with CROSSTIDE (GRPRTSSFAEG) as substrate and MSK1 using glutathione-*S*-transferase–CREB fusion. One unit of protein kinase activity was defined as that amount of enzyme, which phosphorylates 1 nmol of substrate in 1 min. Immunoblotting of cell lysates was performed as described [16].

3. Results

3.1. Generation of MSK1 knockout cells

The disruption of the MSK1 gene was performed as described under Section 2. Following transfection of the targeting vector, 386 ES cell colonies were screened for homologous recombination. Of these, 12 were found to be heterozygous for the disruption of the MSK1 gene. One of these was further selected using high concentrations of G418 to obtain cell lines homozygous for the disruption by gene conversion; from 24 colonies screened, three homozygous cell lines were obtained. The genotype of these cell lines was confirmed by PCR and Southern analysis (Fig. 1A–C). Due to the low level of MSK1 in WT ES cells compared to many transformed cell lines, antibodies against the MSK1 protein do not detect a single band in immunoblotting experiments. However, a band was detected at the correct molecular mass for MSK1 in MSK1+/− and WT cells which was absent in the MSK1−/− cells (Fig. 1D). MSK1 was also immunoprecipitated using two different anti-peptide antibodies (see Section 2), and the immunoprecipitates examined by immunoblotting using the MSK1 antibody raised against the whole protein. In each case a single band was obtained in lysates from WT cells, which was absent in the lysates from the MSK1−/− cells (data not shown).

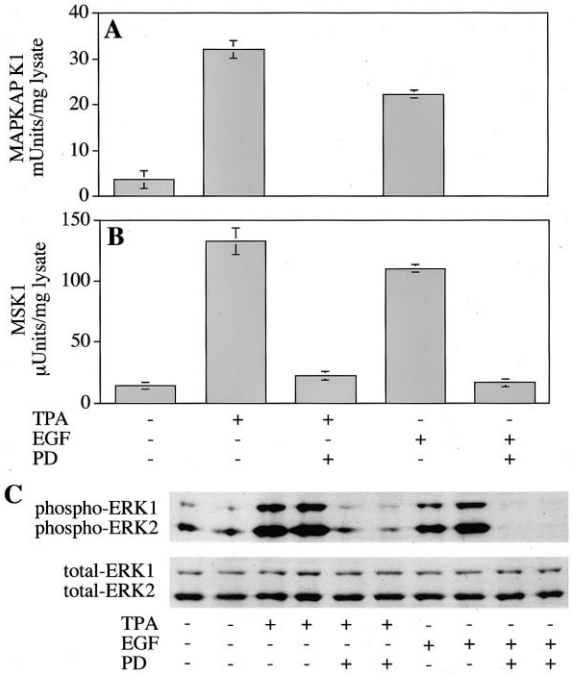

Fig. 2. Effect of PD 184352 on TPA- and EGF-induced activation of MAPKAP-K1, MSK1 and ERK1/ERK2 in WT ES cells. Cells were serum-starved for 4 h, PD 184352 (PD, 10 μM) being added where indicated 1 h prior to stimulation. The cells were then either left unstimulated or stimulated with TPA (400 ng/ml, 10 min) or EGF (100 ng/ml, 5 min). A: MAPKAP-K1a and MAPKAP-K1b were coimmunoprecipitated from the lysates and assayed as described in Section 2. Error bars represent S.E.M. of six points. B: MSK1 was immunoprecipitated from the lysates and assayed as described in Section 2. Error bars represent S.E.M. of six points. C: An aliquot of each lysate (20μg protein) was electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose and immunoblotted using antibodies that recognise phospho-ERK1 and ERK2 or dephospho plus phospho-ERK1 and ERK2 (total-ERK).

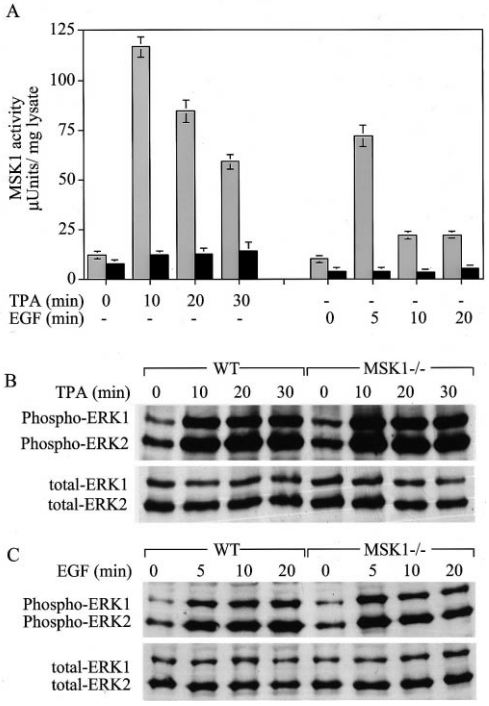

Fig. 3. Activation of MSK1 and ERK in MSK1−/− cells. MSK−/− (grey bars) and WT (black bars). ES cells were serum-starved for 4 h and then stimulated with TPA (400 ng/ml) or EGF (100 ng/ml) for the times indicated. A: MSK1 was immunoprecipitated from the lysates with an antibody raised against amino acids 712–734 of human MSK1 from WT (grey bars) and MSK1−/− (black bars) cell lysates and assayed. Details are given under Section 2. Error bars represent S.E.M. of four points. B and C: Aliquots of the cell lysates (20 μg of protein) from TPA- (B) and EGF- (C) stimulated cells were electrophoresed on 10% SDS–polyacrylamide gels and immunoblotted using the antibodies described in Fig. 2.

The MSK1−/− and MSK1+/− cell lines had similar growth rates and morphology to the WT cells, and were also able to undergo in vitro differentiation via embryoid bodies into erythrocytes and macrophages. Immunoblotting for ERK1/ERK2 (see later) and MAPKAP-K1 isoforms (data not shown) revealed that these other components of the MAPK cascade were present at similar levels in WT and MSK1−/− cells.

3.2. TPA and EGF activate MSK1 and MAPKAP-K1 in ES cells via the classical MAPK cascade

Stimulation of WT ES cells with either TPA or EGF resulted in the activation of MSK1 and MAPKAP-K1a/b (Fig. 2A). The activation of both enzymes was blocked by PD 184352, a specific inhibitor of the classical MAPK cascade [14]. Consistent with these findings, TPA and EGF also acti-

vated the MAPK family members ERK1 and ERK2 measured in the same lysates, and the phosphorylation of these enzymes was also blocked by preincubation with PD 184352 (Fig. 2B). Together, these results demonstrate that TPA and EGF activate MAPKAP-K1 and MSK1 via the classical MAPK cascade.

3.3. MSK1 activity is abolished in MSK1−/− cells but ERKs, MAPKAP-K1 and GSK3 activities are unaffected

The activation of MSK1, ERK1/ERK2 and MAPKAP-K1 was compared in MSK1−/− and WT ES cells. The TPA-induced and EGF-induced activation of MSK1 in WT cells peaked after 10 and 5 min stimulation, respectively, but MSK1 activity was not significantly above background at any time point in the MSK1−/− cells (Fig. 3A). In contrast TPA (Fig. 3B) and EGF (Fig. 3C) both induced ERK1 and ERK2 phosphorylation, similarly in the MSK1−/− and WT cells. Consistent with this finding, all three isoforms of MAPKAP-K1 were activated in the same lysates (Fig. 4A–C). The

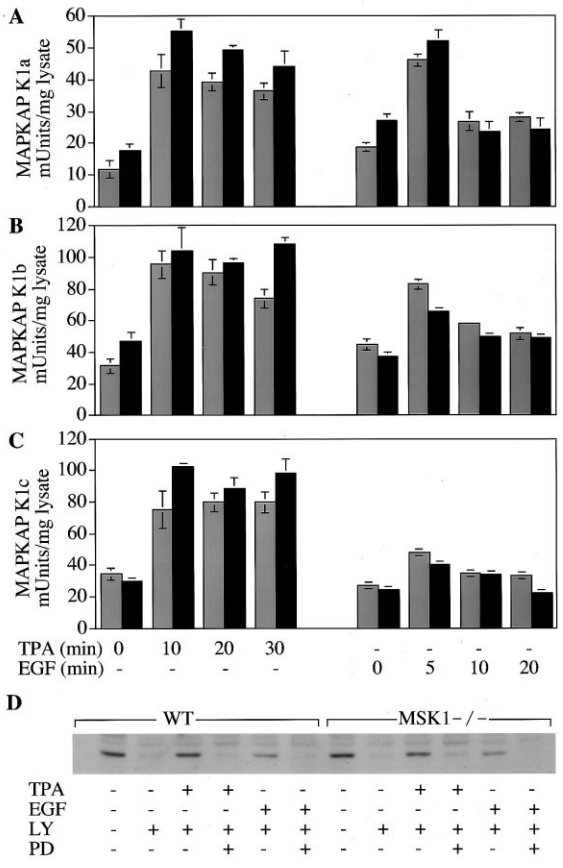

Fig. 4. Activation of MAPKAP K1 isoforms and inhibition of GSK3α by TPA and EGF in MSK1−/− and WT ES cells. MSK1−/− (grey bars) and WT (black bars) ES cells were serum-starved for 4 h and then left unstimulated or stimulated with TPA (400 ng/ml) or EGF (100 ng/ml) for the times indicated. MAPKAP-K1a (A), MAPKAP-K1b (B), and MAPKAP-K1c (C) were immunoprecipitated individually using isoform-specific antibodies and assayed as described in Section 2. Error bars represent the S.E.M. of four points. D: Cells were serum-starved in the presence of LY 294002 (LY, 100 μM) or PD 184352 (PD, 10 μM) where indicated and then left unstimulated, or stimulated with TPA (400 ng/ml, 10 min) or EGF (100 ng/ml, 5 min). 20 μg of protein from the cell lysate was run on a 4–12% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with a phospho-GSK3α antibody.

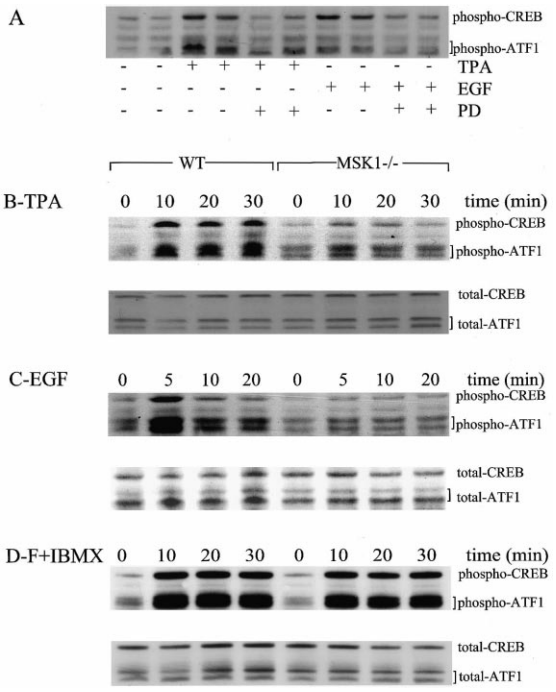

Fig. 5. CREB and ATF1 phosphorylation in MSK1−/− cells. CREB and ATF1 phosphorylation was examined in serum-starved MSK1−/− and WT ES cells after stimulation with TPA (400 ng/ml), EGF (100 ng/ml), or forskolin (20 μM) and IBMX (10 mM) for the times indicated. In A, the cells were preincubated with 10 μM PD 184352 where indicated. Lysates were run on a 4–12% gradient polyacrylamide gels, transferred to nitrocellulose and immunoblotted using antibodies that recognised Ser-133-phosphorylated CREB and Ser-63-phosphorylated ATF1 or dephospho plus phospho-CREB/ATF1 (total CREB). A: TPA- and EGF-induced phosphorylation of CREB and ATF1 in WT ES cells is mediated by the classical MAPK cascade. B: Phosphorylation of CREB and ATF1 after TPA stimulation in WT and MSK1−/− ES cells. C: Phosphorylation of CREB and ATF1 after EGF stimulation of WT and MSK1−/− ES cells. D: Phosphorylation of CREB and ATF1 after forskolin (F) plus IBMX stimulation in WT and MSK1−/− ES cells.

kinetics of activation and inactivation of MAPKAP-K1 in the MSK1−/− and WT cells were similar to those observed for MSK1 in WT cells.

The protein kinase GSK3 is inactivated by phosphorylation of a serine residue (Ser-21 in GSK3α and Ser-9 in GSK3β). This can be catalysed by PKB in response to agonists that activate PI 3-kinase [15]) or by MAPKAP-K1 in response to agonists that activate the classical MAPK cascade [16,17]. In WT ES cells, GSK3α was partially phosphorylated at Ser-21 in unstimulated cells and this was prevented by preincubation with LY 294002, an inhibitor of PI 3-kinase, indicating that phosphorylation was catalysed by PKB. TPA and EGF both stimulated the phosphorylation of Ser-21, even in the presence of LY 294002, and this was blocked by the MAPK cascade inhibitor PD 184352. Similar results were obtained in WT and MSK−/− cells (Fig. 4D).

3.4. Phosphorylation of CREB and ATF1 in WT and MSK1−/− cells

The phosphorylation of CREB and ATF1, at Ser-133 and Ser-63 respectively, was stimulated by TPA and EGF with similar kinetics to the activation of MSK1 and MAPKAP-

K1 in WT ES cells. The phosphorylation of both proteins was blocked by the MAPK cascade inhibitor PD 184352 (Fig. 5A). In contrast, CREB and ATF1 phosphorylation induced by either agonist was greatly reduced in MSK1−/− cells (Fig. 5B,C). This was not due to a decrease in the level of the CREB and ATF1 proteins, which were similar in the WT and MSK1−/− cells. Moreover, forskolin (an activator of adenylate cyclase) and IBMX (an inhibitor of cAMP phosphodiesterases) induced a similar phosphorylation of CREB and ATF1 in WT or MSK1−/− cells (Fig. 5D), indicating that these proteins were still available for phosphorylation by PKA.

4. Discussion

In this paper we show that, as in other cell types, mitogen-induced CREB and ATF1 phosphorylation at Ser-133 and Ser-63, respectively, is mediated via the classical MAPK pathway. Importantly, we also show that the mitogen-induced phosphorylation of these proteins is decreased in ES cells that lack MSK1 (Fig. 5). This indicates that MSK1 is the major protein kinase that mediates the TPA- and EGF-induced phosphorylation and activation of these transcription factors in ES cells. MSK1 is not acting indirectly by controlling the synthesis of MAPKAP-K1 isoforms, because the levels of these protein kinases and their activation in response to mitogens was similar in the MSK1−/− and WT ES cells (Fig. 4A–C). MAPKAP-K1 was also able to phosphorylate its substrate GSK3α normally in MSK1−/− cells in response to TPA or EGF (Fig. 4D). These observations, together with the normal activation of ERK1/ERK2 in MSK1−/− cells (Fig. 3B), indicates that the MSK1 'knockout' does not adversely affect upstream MAPK signalling. We are currently addressing whether the closely related MSK2 [8] or one or more MAPKAP-K1 isoforms are responsible for the trace residual TPA- and EGF-induced phosphorylation of CREB in the MSK1−/− cells by the generation of an MSK1−/−/MSK2−/− double 'knockout'.

The low level of phosphorylation of CREB and ATF1 seen in unstimulated cells, which is unaffected by inhibitors of the classical MAPK cascade (data not shown) was similar in WT and MSK1−/− cells, and must therefore be mediated by a distinct protein kinase(s). This could be the basal activity of PKA in unstimulated cells since forskolin and IBMX, which elevate cAMP and activate PKA induced a much stronger phosphorylation of CREB and ATF1 than EGF or TPA (compare Fig. 5D with B,C). This is different from human embryonic kidney fibroblast 293 cells [8] and murine RAW264.7 macrophages [18] that we have studied previously, where activators of the classical MAPK cascade and forskolin induce a similar degree of phosphorylation of CREB and ATF1. This may be due to the low level of MSK1 in WT ES cells, which is about 20-fold lower than in 293 cells or RAW264.7 macrophages.

The present findings raise the question of why mitogens do not apparently induce CREB/ATF1 phosphorylation in cells from patients with Coffin–Lowry syndrome, which carry inactivating mutations in the MAPKAP-K1b gene [10]. One possible explanation is that MAPKAP-K1b is required for the synthesis of MSK1, but this explanation is inconsistent with the normal growth factor-induced activation of CREB in the fibroblasts of MAPKAP-K1b 'knockout' mice [11]. A second possible explanation is that the inactive MAPKAP-K1b in the Coffin–Lowry cells acts as a dominant negative mutant binding to ERK1/ERK2 and thereby preventing them from activating MSK1/MSK2. Although the MAPKAP-K1b protein cannot be detected by immunoblotting of extracts prepared from Coffin–Lowry cells [10], it is possible that proteolytic fragments of MAPKAP-K1b are still present that cannot be detected immunologically but which nevertheless exhibit dominant negative effects. A third possibility is that Coffin–Lowry patients contain a second mutation that affects the synthesis or activity of MSK1. To our knowledge, the level and activity of MSK1 and MSK2 has not yet been examined in Coffin–Lowry cells. This is clearly critical in order to resolve this question.

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