MSKs are required for the transcription of the nuclear orphan receptors *Nur77*, *Nurr1* and *Nor1* downstream of MAPK signalling

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MSK (mitogen- and stress-activated protein kinase) 1 and MSK2 are kinases activated downstream of either the ERK (extracellular-signal-regulated kinase) 1/2 or p38 MAPK (mitogen-activated protein kinase) pathways *in vivo* and are required for the phosphorylation of CREB (cAMP response element-binding protein) and histone H3. Here we show that the MSKs are involved in regulating the transcription of the immediate early gene *Nur77*. Stimulation of mouse embryonic fibroblasts with PMA, EGF (epidermal growth factor), TNF (tumour necrosis factor) or anisomycin resulted in induction of the *Nur77* mRNA. The induction of *Nur77* by TNF and anisomycin was abolished in MSK1/2 double-knockout cells, whereas induction was significantly reduced in response to PMA or EGF. The MSK responsive elements were mapped to two AP (activator protein)-1-like elements in the *Nur77* promoter. The induction of *Nur77* was

also blocked by A-CREB, suggesting that MSKs control *Nur77* transcription by phosphorylating CREB bound to the two AP-1-like elements. Consistent with the decrease in *Nur77* mRNA levels in the MSK1/2-knockout cells, it was also found that MSKs were required for the induction of *Nur77* protein by PMA and TNF. MSKs were also found to be required for the transcription of two genes related to *Nur77*, *Nurr1* and *Nor1*, which were also transcribed in a CREB- or ATF1 (activating transcription factor-1)-dependent manner. Downstream of anisomycin signalling, a second ERK-dependent pathway, independent of MSK and CREB, was also required for the transcription of *Nurr1* and *Nor1*.

Key words: extracellular-signal-regulated kinase (ERK), mitogen- and stress-activated protein kinase (MSK), *Nur77*, *Nurr1*, *Nor1*, p38.

INTRODUCTION

MSK (mitogen- and stress-activated protein kinase)1 and MSK2 are dual kinase domain proteins that are activated by either the mitogenic stimulation of cells or by cellular stress. MSKs are direct substrates of the MAPK (mitogen-activated protein kinase) family members ERK (extracellular-signal-regulated kinase) 1/2 and p38 (stress-activated protein kinase 2, SAPK2), and the phosphorylation of MSKs by these kinases is required for MSK activity [1–4]. *In vivo*, both the ERK1/2 and p38 pathways are able to activate MSKs, although the relative contribution of each pathway depends on the stimulus and cell type in question. MSKs are constitutively localized to the nucleus suggesting that they may play a role in nuclear function [1]. Knockout mice for either MSK1 or MSK2, or both, are viable and fertile and show no apparent adverse phenotype [5]. Despite this, analysis of cells derived from these mice has shown that MSKs are required for the mitogen or stress induced phosphorylation of the transcription factors CREB (cAMP response element-binding protein) and ATF1 (activating transcription factor-1) [5,6], and the chromatin proteins histone H3 and high mobility group-14 *in vivo* [7–10]. In addition MSKs have also been suggested to phosphorylate other transcription factors including nuclear-factor- κ B-binding protein and ER81 [11,12] although genetic evidence for this is still lacking. Studies on cells from knockout mice have further shown a role for MSKs in the regulation of mitogen- or stress-induced transcription of the immediate early genes *c-fos* and *junB* [5,8,13,14]. MSKs

have also been suggested to be involved in the transcription of *Cyr61* [15] and *MUC5AC* [16]. However, MSKs are not involved in the regulation of all immediate early genes as they do not regulate mitogen- or stress-induced transcription of early growth response factor-1 and *c-jun* [5,8]. The NR4A proteins, *Nur77*, *Nor1* and *Nurr1*, form a subfamily of nuclear receptors [17–19]. *Nur77* [also called N10, NGFI-B (nerve-growth-factor-induced B factor), NAK1 or TR3] was the first NR4A protein to be identified, and was initially described as an immediate early gene induced by mitogens [20]. Since then transcription of *Nur77* has been shown to occur in response to a variety of other signals. The two other NR4A genes, *Nurr1* and *Nor1*, have also been shown to act as immediate early genes, and can be induced by similar stimuli [18,21,22]. NR4A nuclear receptors have been implicated in the regulation of cell survival and apoptosis. Both *Nur77* and *Nor1* have been shown to promote apoptosis and negative selection in T-cells [23–25] and more recently have also been shown to promote cell death in cancer cells [26–28] and macrophages [29]. In contrast *Nur77* has also been shown to promote cell survival and growth in some cancer cell lines [30,31]. *Nurr1* is required for the development and survival of dopaminergic neurons [32,33], and has been linked to Parkinson's disease [34,35].

Similar to other steroid receptors, *Nur77*, *Nurr1* and *Nor1* contain a DNA-binding domain, a transactivation domain, and a putative ligand-binding domain. NR4A proteins have been classed as orphan receptors as they have no known ligand, and there is

Abbreviations used: AP-1, activator protein-1; ATF1, activating transcription factor-1; CRE, cAMP response element; CREB, CRE-binding protein; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; MEF, murine embryonic fibroblast; MEF2D, myocyte-enhancer-binding factor 2D; NGF, nerve growth factor; NGFI-B, NGF-induced B factor; NBRE, NGFI-B response element; PKA, protein kinase A; PKI, PKA inhibitor; TCR, T-cell receptor; TNF, tumour necrosis factor α .

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now much evidence that they are true orphan receptors that do not require ligand binding for their physiological function. Nur77, as well as Nurrl and Norl, are able to bind as monomers to NBREs (NGFI-B response element) (AAAGGTCA) and as homodimers to NurRE (Nur response element) (TGATATTTX6AAATGCCA) in DNA [36]. Nur77 has been reported to bind to, and activate transcription of, NBRE and NuRE sequence reporter constructs, without the requirement for additional signalling inputs [36–38]. The function of the ligand-binding domain of Nur77 is unclear, but may be involved in dimerization or cofactor recruitment. The crystal structure of Nurrl has shown that the ligand-binding domain exists in a closed form with an inaccessible ligand-binding site, supporting the idea that these proteins act independently of ligand binding [39]. As the NR4A nuclear receptors appear to function in a ligand-independent manner, other mechanisms such as control of NR4A transcription or post-translational modification are likely to exist, to allow the control of these nuclear orphan receptors *in vivo*. For instance phosphorylation by ERK1/2 has been shown to affect Nur77 localization [40], while phosphorylation of Ser³⁵⁰ in Nur77 has been suggested to inhibit the transcriptional activity of Nur77 [41].

Transcription of Nur77 has been shown to be upregulated by a variety of stimuli including TCR (T-cell receptor) activation in T-cells, serum in fibroblasts [42], NGF (nerve growth factor) and membrane depolarization in PC12 cells [43]. The kinetics and control of Nur77 transcription vary depending on the stimuli and cell type studied. In T-cells TCR activation causes a prolonged upregulation of Nur77 transcription, which involves the binding of the transcription factor MEF2D (myocyte-enhancer-binding factor 2D) to the Nur77 promoter. In unstimulated T-cells Nur77 transcription is repressed by the association of MEF2D with histone deacetylases and the repressor protein cabin1. Stimulation of the TCR inhibits the association of MEF2D with these repressor complexes allowing Nur77 transcription [29,44–48]. In PC12 cells induction of Nur77 transcription by both NGF and membrane depolarization has been shown to require two AP-1 (activator protein-1) (TGCGTCA)-like elements adjacent to the transcriptional start site in the Nur77 promoter [43]. These AP-1-like elements were reported to bind JunD, but not CREB, in PC12 cells, and expression of dominant-negative *junD* was found to block Nur77 transcription in response to both NGF and membrane depolarization [49]. Nur77 transcription induced by T-lymphotropic virus type I-tax-protein [50] and prostaglandin F_{2α} [51] has also been reported to involve JunD binding to these AP-1-like elements. Induction of Nur77 by prostaglandin was further shown to require the phosphorylation of JunD by ERK1/2 [52]. Transcription of Nur77 has also been shown to be stimulated by treatments such as forskolin, which elevates the level of cyclic AMP and activates PKA (protein kinase A) [53,54], which has recently been linked to the phosphorylation and activation of CREB by PKA [22]. It has also been suggested that in some cell types both PKA and ERK1/2 are involved in the regulation of Nur77 transcription [55].

Nurrl and Norl also act as immediate early genes and their transcription has been reported to be upregulated by similar stimuli to Nur77. In contrast to Nur77, however, both Nurrl and Norl have one or more classical CRE (cAMP-response element) sites in their promoters and have been reported to be CREB dependent genes [28,56–58].

Here we show that in fibroblasts both mitogens and cellular stress are able to activate transcription of all three NR4A genes. We also demonstrate a role for MSKs and CREB/ATF1 in the regulation of Nur77, Nurrl and Norl transcription in response to these stimuli.

MATERIALS AND METHODS

Antibodies

Antibodies which recognize total ERK1/ERK2, phospho-ERK1/2, total p38 and phospho-p38 were from Cell Signalling. The antibody against phospho-CREB was from Upstate. The anti-Nur77 antibody was generated against a specific peptide (VRDHLTGDP-PLALEFGK) from this protein (A. D. Wingate, unpublished work).

Plasmids and promoters

The Nur77 promoter was amplified by PCR from mouse genomic DNA using the primers gctcgagTGCGCTCCGCTCCGCAGTC (bp + 66) and gcgagctcCTAGGTGTCCAGGACAGACTGGG (bp – 533). The fragment was cloned into the basic pGL3 R2.1 vector (Promega) as a SacI/XhoI fragment. Truncated fragments from this clone were used to generate fragments bp – 274 to bp + 66 and bp – 100 to bp + 66 of the promoter by PCR. To mutate the TGCGTCA AP-1-like elements in the promoter to TATATCA, mutagenesis was carried out using the Quickchange System (Stratagene). All constructs were confirmed by DNA sequencing using the Sequencing Service (School of Life Sciences, University of Dundee, Scotland, U.K.; www.dnaseq.co.uk) and Applied Biosystems' BigDye v3.1 kit on an Applied Biosystems model 3730 automated capillary DNA sequencer. The dominant-negative *junD* and A-CREB constructs have been described previously [49,59]. The MSK1 expression vectors have also been described previously [1].

Cell culture

Primary murine embryonic fibroblast (MEF) cells from wild-type or MSK1/MSK2 double knockout (MSK1/MSK2^{–/–}) mice were isolated and cultured as described previously [5]. Primary cells were used for most of the experimental work on MSK; however, for the luciferase reporter experiments, cells immortalized with simian-virus-40-large-tumour-antigen were used to allow for more efficient transfection. As knockout of p38α is embryonic lethal [60], the use of primary MEF cells was not feasible; instead, immortalized MEF cells from p38α knockout mice were used [61].

MEF cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% serum (Sigma), 2 mM L-glutamine, 50 units/ml penicillin G and 50 µg/ml streptomycin (Invitrogen). HeLa cells were grown in DMEM containing 10% serum (Sigma) and 1 mM sodium pyruvate (Invitrogen). Before stimulation, cells were serum-starved overnight. Where indicated, cells were pre-treated with inhibitors (2 µM PD 184352, 5 µM SB 203580, 5 µM Ro 318220, 10 µM H89 or 10 µM U0126), and then stimulated with either anisomycin (10 µg/ml), TNF (tumour necrosis factor) (10 ng/ml), EGF (epidermal growth factor) (100 ng/ml) or PMA (400 ng/ml) for the times indicated. When required, cells were transfected using FuGene 6 (Roche), in the presence of serum but in the absence of antibiotics. After 24 h, transfected cells were serum-starved for 16 h and then stimulated as described above.

Immunoblotting

Cells were lysed in 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, and then centrifuged at 13 000 g for 5 min to remove insoluble material. Then 20–30 µg

Table 1 Primer sequences used for real-time PCR

Primer	Sense	Antisense
Nur77 (mouse)	CCTGTTGCTAGAGTCTGCCTTC	CAATCCAATCACCAAGCCACG
Nurr1 (mouse)	GAAGAGAGCGGACAAGGAGATC	AAGGCATGGCTTCAGCAGAG
Nor1 (mouse)	GCCATCTCCTCCGATCTGTATG	GAGGCCGTCAGAAGGTTGTAG
18 S RNA (mouse)	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
Nur77 (human)	ACAGGAGAGTTTGACACC	AACTTGAAGGAGGCAGAG
β_2 Microglobulin (human)	TGCCGTGTGAACCATGTGAC	ACCTCCATGATGCTGCTTACA

of soluble protein extract was run on 4–12 % polyacrylamide gels (Novex, Invitrogen) and transferred to nitrocellulose membranes. Proteins were detected using primary antibodies in combination with horseradish peroxidase (Pierce) and chemiluminescent substrate (Amersham).

Kinase assays

For kinase assays, MSK1 was immunoprecipitated from 0.5 mg of pre-cleared cell lysate using 2 μ g of anti-MSK1 antibody coupled to Protein-G-Sepharose. Precipitates were washed twice in 0.5 M NaCl, 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol and once in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol. Precipitates were then resuspended in 35 μ l of reaction buffer [containing Tris/HCl, pH 7.5, EGTA, PKI (protein kinase A inhibitor) and Crosstide peptide, GRPRTSSFAEG], and the reaction was started by the addition of 10 μ l of 50 mM magnesium acetate, 0.5 mM [32 P]ATP and incubated at 30 °C for 20 min. Final concentrations of reagents in the assay were 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 2.5 μ M PKI, 30 μ M Crosstide peptide, 10 mM magnesium acetate and 0.1 mM [32 P]ATP [5]. Reactions were stopped by transfer on to P81 paper and washing in 75 mM orthophosphoric acid. One unit was defined as the incorporation of 1 nmol of phosphate into the substrate peptide in 1 min.

Real-time PCR

Cells were treated as indicated, then lysed and RNA isolated using the NucleoSpin RNA purification method (Macherey-Nagel). RNA was reverse-transcribed (iScript, Bio-Rad) and real-time PCR was carried out using Sybr Green based detection. Levels of 18 S rRNA were used as normalization controls and relative mRNA levels calculated using the equation:

$$\text{Relative mRNA level} = \frac{E_u^{(ct_{uc} - ct_{us})}}{E_r^{(ct_{rc} - ct_{rs})}}$$

where E is the efficiency of the PCR, ct is the threshold cycle, u is the mRNA of interest, r is the reference gene (18 S RNA or β_2 -microglobulin), s is the sample and c is the unstimulated control sample [62]. Primer sequences for the amplification of mouse and human Nurr1, Nor1 and Nur77, mouse 18 S RNA and human- β_2 microglobulin are shown in Table 1.

Luciferase assays

Cells were transfected with the appropriate *Nur77* promoter vectors along with a *Renilla* luciferase vector as a transfection control. After 24 h transfected cells were serum-starved for 16 h. Cells were then left untreated or stimulated for 60 min with 400 ng/ml PMA, 100 ng/ml EGF or 10 ng/ml TNF. Following stimulation cells were lysed and the activities of firefly

and *Renilla* luciferase measured using the Dual-Glo system (Promega). Luciferase activities were normalized to the *Renilla* control, and then fold stimulation was calculated relative to the bp – 533 (Figure 2B) or bp – 274 (Figures 2C and 2D) *Nur77* promoter in unstimulated cells.

RESULTS

CREB is required for the transcription of *Nur77*, *Nor1* and *Nurr1*

CREB, and the related transcription factor ATF1, are phosphorylated in response to a range of mitogenic stimuli and cellular stresses and have been implicated in the induction of some immediate early genes. Using GALA prediction software, analysis of the promoter regions of *Nurr1* and *Nor1* reveals the presence of two potential CRE sequences that are conserved in the human, mouse, rat and canine promoters (Figure 1A). Analysis of the *Nur77* promoter did not identify a conserved CRE sequence but did reveal the presence of four conserved AP-1-like sequences. Three of these were found in the human, mouse, rat and canine promoters; however, the fourth was not conserved in the canine *Nur77* promoter region. The role of CREB or ATF1 in the induction of these genes was tested in HeLa cells using A-CREB. This is a dominant-negative form of CREB that lacks the DNA binding domain, but that can dimerize to endogenous CREB or ATF1 and prevent its binding to DNA. PMA, EGF, TNF and anisomycin were all found to induce the mRNAs for *Nur77*, *Nurr1* and *Nor1* in HeLa cells. In response to these stimuli, A-CREB either blocked or significantly reduced the expression of *Nur77*, *Nurr1* and *Nor1* (Figure 1B). As *junD* has also been suggested to play a role in the induction of *Nur77*, we also tested the effect of dominant-negative *junD* expression (Figure 1C). In response to PMA, dominant-negative *junD* did not prevent the induction of *Nur77*, *Nurr1* or *Nor1*. In response to EGF and TNF the dominant-negative *junD* was able to reduce the induction of *Nur77*, *Nurr1* and *Nor1*; however, this decrease was less marked than with A-CREB. In response to anisomycin, the dominant-negative *junD* did reduce the induction of *Nur77*, but not of *Nurr1* or *Nor1*. The dominant-negative *junD* did however completely block the induction of a known AP-1 target, *IL-6* (the gene encoding interleukin-6), in response to PMA (results not shown).

To map the response element in the *Nur77* promoter, various regions of the mouse *Nur77* promoter region were cloned into a luciferase reporter vector (Figure 2A), and transfected into immortalized MEF cells. A vector containing bp – 533 to bp +66 of the *Nur77* promoter was able to drive expression of the luciferase reporter, and this could be further stimulated by PMA and EGF and weakly stimulated by TNF. Truncation of the promoter to bp – 274 to bp +66 (a fragment still containing all four AP-1-like elements) did not affect this activity. Further truncation to a bp – 100 to bp +66 fragment decreased the basal activity of the promoter and prevented the induction by PMA, EGF and TNF, suggesting that the response elements lie between bp – 274 to bp – 100 (Figure 2B). This region contains two AP-1-like elements; mutation of either or both of these AP-1-like sequences prevented the induction of the reporter by PMA, EGF or TNF (Figure 2C). To confirm that this region was the region targeted by CREB, the bp – 274 to bp +66 vector was co-transfected with an expression vector for either A-CREB or dominant-negative *junD* (Figure 2C). A-CREB was found to significantly decrease the induction of the reporter by PMA, EGF and TNF. Consistent with the results obtained for the endogenous *Nur77* gene in HeLa cells, the dominant-negative *junD* vector was less effective at blocking the induction of the reporter than A-CREB.

Figure 1 NR4A gene transcription requires CREB

(A) The promoter region bp -2000 to bp +1000 of the transcriptional start of the *Nur77*, *Nor1* and *Nurr1* transcription factors were analysed by GALA prediction software (<http://gala.cse.psu.edu/>). Potential CRE (closed circle) or AP-1-like (grey rectangle in human, mouse, rat and canine promoters, black rectangle indicating a non-conserved region in the canine promoter) as well as potential sites for MEF cells (black diamond), Elk (star), USF (upstream stimulatory factor; black triangle) and nuclear factor κ B (grey triangle) were identified. Sequences predicted as CRE sites were TGACGTAG and TGACGTCT in *Nor1*, and TGACG.CA and TGACGTCA in *Nurr1*, against a consensus CRE site of TGACGT(A/C)A. (B) HeLa cells were transfected with either an empty vector (light grey bars) or A-CREB expression vector (dark grey bars). After 24 h transfected cells were serum-starved for 16 h and left unstimulated (control) or stimulated for 60 min with 400 ng/ml PMA, 100 ng/ml EGF, 10 ng/ml TNF or 10 μ g/ml anisomycin. Cells were then lysed and RNA isolated and analysed by real-time PCR. Levels of *Nur77* mRNA were determined relative to β_2 -microglobulin controls as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations. (C) As in (B) except a dominant-negative *junD* vector (black bars) was used in place of A-CREB.

MSKs are required downstream of MAPK signalling for the transcription of *Nur77*, *Nurr1* and *Nor1*

PMA, EGF, TNF and anisomycin are all known to stimulate CREB phosphorylation downstream of either the ERK1/2 or p38 MAPK cascades. In MEF cells we found that TNF stimulation resulted in a transient activation of ERK1/2 and a more prolonged activation of p38. TNF also activated MSK1 and MSK2, resulting in the phosphorylation of CREB (results are shown in Supplementary Figure 1S at <http://www.BiochemJ.org/bj/390/bj3900749add.htm>). We have previously shown that in primary MEF cells, PMA stimulation resulted in a strong and sustained activation of the ERK1/2 cascade but did not activate p38 α . EGF both strongly activates ERK1/2 and weakly activates p38 α , although the activation of ERK1/2 was more transient than with PMA. In contrast anisomycin strongly activates p38 but only weakly activates ERK1/2 [5]; results are shown in Supplementary Figure 2S at <http://www.BiochemJ.org/bj/390/bj3900749add.htm>.

PMA and EGF were also found to activate MSK1 and MSK2, and phosphorylate CREB downstream of the ERK1/2 cascade in MEF cells, while anisomycin activated MSKs and phosphorylated CREB downstream of p38 [5]. We therefore examined the transcription of NR4A genes in these cells using real-time PCR. *Nur77*, *Nurr1* and *Nor1* mRNA levels were found to be induced by PMA, EGF, TNF and anisomycin (Figure 3). To determine the role of MAPK signalling in this process, the p38 α/β inhibitor SB 203580 [63] and the MKK (MAPK kinase) 1/2 inhibitor PD 184352 (which blocks the activation of ERK1/2 *in vivo* [63]) were used. In response to PMA and EGF, *Nur77*, *Nurr1* and *Nor1* induction were completely blocked by PD 184352, but unaffected by SB 203580, demonstrating a role for ERK1/2 but not p38 α/β in the transcription of these genes (Figures 3A and 3B). The induction of *Nur77*, *Nurr1* and *Nor1* mRNAs by TNF was partially inhibited by either PD 184352 or SB 203580 but completely blocked by a combination of both inhibitors (Figure 3C). The induction of *Nur77*, *Nurr1*

Figure 2 Reporter analysis of the murine *Nur77* promoter

(A) Fragments from bp -533, bp -274 and bp -100 to bp +66 of the murine *Nur77* promoter were cloned into pGL3 to give *Nur77* promoter luciferase reporters. Two proximal AP-1 sites were also mutated. Grey circles indicate AP-1-like elements while white circles indicate the mutated sites. (B) The 533 (cross-hatched bars), 274 (black bars) and 100 (grey bars) *Nur77* reporter vectors were transfected into wild-type immortalized MEF cells. After 24 h transfected cells were serum-starved for a further 16 h and then stimulated with PMA (400 ng/ml, 60 min), EGF (100 ng/ml, 60 min) or TNF (10 ng/ml, 60 min). Cells were then lysed and luciferase activity measured as described in the Materials and methods section. (C) The 274 (black bars), AP1-1 (cross hatched bars), AP1-2 (horizontal hatched bars) and AP1-1/2 (grey bars) vectors were transfected into wild-type fibroblasts. After 24 h transfected cells were serum-starved for a further 16 h and then stimulated with PMA (400 ng/ml, 60 min) EGF (100 ng/ml, 60 min) or TNF (10 ng/ml, 60 min). Cells were then lysed and luciferase activity measured as described in the Materials and methods section. (D) The bp -274 *Nur77* reporter vectors were co-transfected with either empty vector (black bars), A-CREB (white bars) or dominant-negative *junD* (cross-hatched bars) expression vectors into wild-type MEF cells. After 24 h transfected cells were serum-starved for a further 16 h and then stimulated with PMA (400 ng/ml, 60 min) EGF (100 ng/ml, 60 min) or TNF (10 ng/ml, 60 min). Cells were then lysed and luciferase activity measured as described in the Materials and methods section.

and *Nor1* by anisomycin was almost completely blocked by SB 203580 demonstrating a major role for p38 in this process (Figure 3D). As SB 203580 inhibits both p38 α and p38 β it does not distinguish between the roles of these two isoforms.

In vitro both p38 α and p38 β can phosphorylate and activate MSK; however, it is not known if both isoforms contribute to MSK activation and CREB phosphorylation *in vivo* [1]. We therefore used immortalized MEF cells from p38 α knockout and wild-type mice to determine the role of p38 α in MSK activation and NR4A transcription. As expected *Nur77*, *Nurr1* and *Nor1* transcription was induced in wild-type MEF cells and the induction was inhibited by SB 203580. No induction of these genes was seen in p38 α knockout MEF cells in response to anisomycin (Figure 4A). Knockout of p38 α was also found to prevent the activation of the CREB kinase MSK1 in response to anisomycin (Figure 4B). Consistent with this, CREB was phosphorylated in response to anisomycin in wild-type MEF cells, but not in p38 α knockout MEF cells (Figure 4C).

Interestingly PD 184352 partially inhibited *Nur77* induction and almost completely blocked *Nurr1* and *Nor1* mRNA induction in response to anisomycin, suggesting that ERK1/2 was involved in this process. This was unexpected as anisomycin is only a weak activator of ERK1/2 in MEF cells [5]. Inhibition of ERK1/2 by PD 184352 did not prevent p38 α activation, as judged by immunoblotting with an antibody against the phosphorylated TGY motif of p38. PD 184352 also did not affect the phosphorylation of CREB downstream of anisomycin in MEF cells (Figure 4D). Together this suggests that, for the transcription of *Nurr1* and *Nor1* in response to anisomycin, a second pathway, independent of CREB and p38, may be required. To confirm that the effect of PD 184352 was due to inhibition of the ERK1/2 cascade and not a non-specific effect, another MEK1/2 inhibitor, U0126, was tested. U0126 was able to reduce the *Nur77* transcription induced by anisomycin by half and the *Nurr1* and *Nor1* transcription by approx. 70% (Figure 4E).

We have shown previously that MSK1 and MSK2 phosphorylate CREB and ATF1 downstream of PMA, EGF and anisomycin in MEF cells. MSK1 and 2 are also required for the TNF-induced phosphorylation of CREB and ATF1 in these cells (results are shown in Supplementary Figure 2S at <http://www.BiochemJ.org/bj/390/bj3900749add.htm>). This suggests that MSKs may be required for NR4A transcription downstream of MAPK signalling. We therefore examined the induction of *Nur77*, *Nor1* and *Nurr1* in MSK1/MSK2 knockout primary MEF cell lines. In wild-type cells PMA strongly induced *Nur77*, *Nurr1* and *Nor1* mRNA levels up to a maximal time point of 60 min, and the induction of all three genes was significantly reduced in MSK1/MSK2 $^{-/-}$ cells (Figure 5A). EGF stimulated the transcription of all three genes in wild-type cells; however, *Nur77* induction was maximal by 30 min whereas induction of *Nurr1* and *Nor1* was maximal by 60 min. The induction of *Nur77* was decreased in MSK1/MSK2 $^{-/-}$ cells, while the induction of both *Nurr1* and *Nor1* was blocked in MSK1/MSK2 $^{-/-}$ cells (Figure 5B). TNF stimulation also induced all three genes in wild-type cells, reaching maximal levels after 60 min of stimulation, and this was significantly reduced in the MSK1/MSK2 $^{-/-}$ cells (Figure 5C). Anisomycin produced a more prolonged increase in *Nur77*, *Nor1* and *Nurr1* mRNA levels in wild-type cells, and this was abolished in MSK1/MSK2 $^{-/-}$ cells (Figure 5D). Consistent with the decrease in the mRNA levels for *Nur77* seen in the MSK1/MSK2 $^{-/-}$ cells, the increase in *Nur77* protein levels seen in MSK1/MSK2 $^{-/-}$ cells in response to PMA and TNF was much lower than in wild-type cells (Figure 5E).

To ensure that the loss of NR4A gene induction was due to the lack of MSKs, and not clonal differences between the different MEF cell preparations, we examined the effect of compounds that inhibit MSKs in cells, and also re-expressed MSK1 in immortalized MSK1/MSK2 $^{-/-}$ MEF cells. H89 and Ro 318220 are two compounds that have been shown to inhibit MSKs both

Figure 3 NR4A transcription requires MAPK signalling

Wild-type primary MEF cells were serum-starved for 16 h and then treated for 1 h with 2 μ M PD 184352 (PD), 5 μ M SB 203580 (SB), or no inhibitor as indicated. Cells were then left unstimulated (control) or stimulated (st) for 60 min with 400 ng/ml PMA (A), 100 ng/ml EGF (B), 10 ng/ml TNF (C) or 10 μ g/ml anisomycin (D). Cells were lysed and the RNA isolated. *Nur77* (grey bars), *Nurr1* (white bars) and *Nor1* (black bars) mRNA levels were determined by real-time PCR as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations.

in vitro and in cells [63,64]. Both of these compounds were able to reduce or block the induction of *Nur77*, *Nurr1* and *Nor1* mRNAs in immortalized wild-type MEF cells stimulated with either EGF or anisomycin (Figures 6A and 6B). Similar effects were also seen using PMA or TNF stimulation (results not shown). Transient expression of MSK1 in immortalized MSK1/MSK2^{-/-} MEF cells was tested for the ability to restore the induction of endogenous *Nur77*, *Nor1* and *Nurr1* transcription in knockout cells (Figure 6C). Consistent with observations in primary MSK1/MSK2^{-/-} cells, EGF stimulation caused modest induction of *Nur77*, but not of *Nor1* or *Nurr1*, whereas anisomycin

stimulation resulted in a modest induction (between 2–10-fold) of all three genes. In all cases expression of a wild-type MSK1 protein increased the induction of these genes. This change was dependent on the kinase activity of MSK1, as expression of a kinase-dead mutant of MSK1 had no effect on *Nur77*, *Nurr1* or *Nor1* induction. The fold induction was less than was seen for wild-type cells, however, this probably reflects the low transfection efficiency of these cells (15–25%). Finally the bp -274 to bp +66 *Nur77* promoter-reporter-vector was transiently transfected into MSK1/MSK2^{-/-} MEF cells with either empty vector or MSK1-expression vectors encoding either wild-type

Figure 4 ERK1/2 and p38 α are required for NR4A induction by anisomycin

Immortalized p38 α knockout (white bars) and wild-type (black bars) MEF cells (A–C) were serum-starved for 16 h, incubated with 5 μ M SB 203580 (SB) for 1 h where indicated and then left unstimulated (control) or stimulated with 10 μ g/ml anisomycin (Anis) for 1 h. After stimulation, total RNA was isolated and the expression of *Nur77*, *Nurr1* and *Nor1* determined by real-time PCR (A). Levels of MSK1 activity were determined by immunoprecipitation kinase assay as under the Materials and methods section (B). To determine if the ERK1/2 and p38 pathways were activated, lysates were run on SDS/polyacrylamide gels. The levels of phospho-CREB, phospho-p38, (p-p38), phospho-ERK1/2, total p38 α and total ERK1/2 were determined by immunoblotting with specific antibodies (C). (D) Primary wild-type MEF cells were serum-starved for 16 h, incubated for 1 h with 5 μ M SB 203580 (SB) and/or 2 μ M PD 184352 (PD) where indicated and then stimulated with 10 μ g/ml anisomycin (Anis) for 1 h. Cells were then lysed and the levels of phospho-CREB, phospho-p38, phospho-ERK1/2, total p38 α and total ERK1/2 were determined by immunoblotting with specific antibodies. (E) Primary wild-type MEF cells were serum-starved for 16 h, incubated for 1 h with 10 μ M U0126 (grey bars) or DMSO control (black bars) and then stimulated with 10 μ g/ml anisomycin for 1 h. The induction of *Nur77*, *Nurr1* and *Nor1* mRNA was then determined by real-time PCR as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations.

or kinase-dead protein. The luciferase reporter was expressed in these cells at similar basal levels to wild-type cells. Unlike in wild-type cells, however, where the reporter was further stimulated by PMA, the reporter was not significantly stimulated by PMA in MSK1/MSK2 $^{-/-}$ cells (Figure 6D). Co-transfection of the reporter with an expression construct for wild-type MSK1, however, restored the ability of the reporter to be induced by PMA in MSK1/MSK2 $^{-/-}$ cells. However, co-transfection with a kinase-dead version of MSK1 did not restore induction of the reporter vector.

DISCUSSION

Nur77, *Nurr1* and *Nor1* are a group of immediate early genes whose transcription has previously been shown to be induced by growth factors and mitogens, and this process is dependent on the activation of ERK1/2. Here we confirm these results and also demonstrate that MSK1 and MSK2 are involved in this process downstream of ERK1/2. We further demonstrate that in fibroblasts, transcription of *Nur77*, *Nurr1* and *Nor1* is also induced by the cellular stress-inducing anisomycin, and that this induction requires the activation of the p38 pathway and MSK1 and MSK2. The transcription of these genes was also induced by TNF in fibroblasts, and this required MSKs which acted downstream of both the ERK1/2 and p38 α cascades. The

induction of the NR4A genes by anisomycin or TNF was not seen in fibroblasts from MSK1/MSK2 $^{-/-}$ mice, suggesting that these kinases may act downstream of p38 α and ERK1/2 in controlling *Nur77* transcription. MSKs are essential for the phosphorylation of CREB and ATF1 in response to anisomycin or TNF stimulation of fibroblasts ([5], results are shown in Supplementary Figure 1S at <http://www.BiochemJ.org/bj/390/bj3900749add.htm>). MSKs have previously been shown to activate transcription of CRE-dependent genes, suggesting a possible mechanism by which they could control *Nur77* transcription [14]. The *Nur77* promoter in mice does not contain a consensus CRE sequence; however, the induction of *Nur77* by PMA, EGF, anisomycin and TNF was found to require the presence of two AP-1-like elements in the promoter. AP-1-like elements have a similar sequence to CRE elements and have been reported to be able to bind members of the CREB family of transcription factors [22]. Consistent with this, expression of a dominant-negative form of CREB, A-CREB, was able to completely block the expression of *Nur77* in response to cellular stress. Transcription of *Nur77* in response to PMA or EGF was also completely blocked by expression of A-CREB. In contrast, transcription of *Nur77* in MSK1/MSK2 $^{-/-}$ cells in response to PMA or EGF was decreased but not abolished. This may be because PMA is still able to induce residual phosphorylation of CREB and ATF1 in response to PMA in the MSK1/MSK2 $^{-/-}$ cells [5], and this is enough to promote transcription of these genes. Consistent with our findings

Figure 5 MSKs are required for the transcription of *Nur77*

(A–D) Wild-type (solid bars), MSK1/MSK2^{-/-} (hatched bars) and primary MEF cells were serum-starved for 16 h. Cells were then stimulated with (A) PMA (400 ng/ml), (B) EGF (100 ng/ml), (C) TNF (10 ng/ml) or (D) anisomycin (10 µg/ml) for 0, 30, 60 or 120 min. Cells were lysed and the RNA isolated. *Nur77*, *Nurr1* and *Nor1* levels were determined by real-time PCR as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations. (E) Wild-type (WT) or MSK1/MSK2^{-/-} cells were serum-starved (KO) for 16 h, and then stimulated with 400 ng/ml PMA or 10 ng/ml TNF for the times indicated. Cells were then lysed and Nur77 immunoprecipitated from 3 mg of cell lysate. Immunoprecipitates were then blotted for Nur77. Cell lysates were also blotted for total ERK.

implicating CREB or ATF1 in the transcription of *Nur77*, it has recently been reported that forskolin-induced transcription of *Nur77* involves CREB [22]. In addition it has also been shown that CREB is capable of binding to the endogenous *Nur77* promoter *in vivo* [65]. These results are, however, in contrast with earlier reports suggesting a role for *junD* in regulating *Nur77* transcription via the AP-1-like elements in response to NGF or

KCl in PC12 cells or prostaglandin F2α in ovarian cells [49,52]. In these systems, *junD* was observed to bind to an oligonucleotide based on the *Nur77* AP-1-like element in electrophoretic mobility shift assays and expression of a dominant-negative *junD* inhibited *Nur77* transcription. The dominant-negative *junD* acts by heterodimerizing with endogenous AP-1 proteins including JunD and c-Fos and preventing them from binding to DNA. Using

Figure 6 Expression of MSK1 in MSK1/MSK2^{-/-} cells rescues NR4A expression

(A) Wild-type immortalized MEF cells were serum-starved for 16 h and then incubated with 5 μ M Ro 318220 (RO) or 10 μ M H89 were indicated. Cells were then left unstimulated, or stimulated (St) with EGF (100 ng/ml) for 1 h. The induction of *Nur77*, *Nurr1* and *Nor1* mRNA was measured by real-time PCR as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations. (B) As in (A) except that cells were stimulated with anisomycin (10 μ g/ml) for 1 h. (C) Immortalized MSK1/MSK2^{-/-} MEF cells were transfected with either empty vector (white bars) or wild-type (light grey bars) or kinase-dead (black bars) MSK1 expression vectors. Cells were then serum-starved for 16 h and left unstimulated (control), or stimulated with either EGF (100 ng/ml) or anisomycin (10 μ g/ml), for 1 h. The induction of *Nur77*, *Nurr1* and *Nor1* mRNA was measured by real-time PCR as described in the Materials and methods section. Error bars represent the S.E.M. three individual stimulations. (D) Immortalized wild-type (wt) or MSK1/MSK2^{-/-} (ko) MEF cells were transfected with a bp -274 to bp +66 *Nur77* promoter luciferase reporter vector, together with either an empty vector, wild-type or kinase-dead (KD) MSK1 expression vector, as indicated. Cells were then serum-starved for 16 h and left unstimulated (white bars), or stimulated with PMA (400 ng/ml) for 1 h. Luciferase activity was measured as described in the Materials and methods section. Error bars represent the S.E.M. of three individual stimulations.

the same dominant-negative *junD* construct in MEF cells, we did observe a modest inhibition of *Nur77* transcription; however, this decrease was less marked than with A-CREB. It is unlikely that MSKs could regulate the activity of *junD* directly by phosphorylation, as *junD* does not contain any consensus MSK phosphorylation sites. It is possible that MSKs could regulate the *Nur77* promoter by controlling the transcription of AP-1 type transcription factors such as *c-fos* or *junB*. However, this also

seems unlikely, as MSK is required for anisomycin-stimulated *Nur77* transcription. As the concentration of anisomycin used inhibits protein synthesis [66], the induction of *Nur77* must be an immediate early response that does not require the synthesis of more AP-1 proteins. It is possible, however, that both *junD* and CREB or ATF1 act co-operatively at the level of the AP-1 sites in the *Nur77* promoter. The differences between effects of dominant-negative *junD* in the MEF cells reported here, and the

previous findings in PC12 and ovarian cells, could therefore be due to different occupancy of CREB or ATF1 and *junD* on the AP-1-like elements in the *Nur77* promoter in different cell types.

As MSKs were required for the induction of *Nur77*, the involvement of MSKs in the transcription of the *Nurr1* and *Nor1* was also examined. Both *Nurr1* and *Nor1* have been reported to be CREB-dependent genes [28,57,58], and we found their expression to be inhibited by A-CREB. The transcription of both of these genes was severely compromised in MSK-deficient cells and it is likely that MSK regulates these genes through its ability to phosphorylate CREB or ATF1. For *Nurr1*, an alternative distal promoter and first exon has also been described upstream of the proximal promoter studied here. The distal promoter is thought not to require CREB for its activity [67]; however, we could find no evidence using real-time PCR that this distal promoter was active in fibroblasts (results not shown).

Interestingly the transcription of *Nur77*, *Nor1* and *Nurr1* in response to anisomycin was inhibited by the MSK1/2 inhibitors PD 184352 and U0126, which block the activation of ERK1/2, as well as by the p38 α / β inhibitor SB 203580. In contrast, both MSK activation and CREB phosphorylation in response to anisomycin are blocked by SB 203580 but not PD 184352. This suggests that an additional input is required for *Nur77*, *Nurr1* and *Nor1* transcription, and that MSK activation and CREB phosphorylation are required but are not sufficient for transcription of these genes in response to anisomycin. As PMA, EGF and TNF phosphorylate CREB downstream of ERK1/2, it is not possible to tell if this second PD 184352-sensitive pathway also operates in NR4A induction by these stimuli. Further work will, however, be required in order to determine how ERK regulates these genes in response to anisomycin.

NR4A proteins have been described as playing roles in both dopaminergic neurons and T-cells [17,32,68]. It will therefore be interesting to examine if knocking out MSK affects the expression of NR4A proteins in these systems in mice, and if, as a consequence, the MSK knockout mice show defects in brain functioning and neuronal systems.

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