

The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase

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The nucleosomal response refers to the rapid phosphorylation of histone H3 on serine 10 and HMG-14 on serine 6 that occurs concomitantly with immediate-early (IE) gene induction in response to a wide variety of stimuli. Using antibodies against the phosphorylated residues, we show that H3 and HMG-14 phosphorylation is mediated via different MAP kinase (MAPK) cascades, depending on the stimulus. The nucleosomal response elicited by TPA is ERK-dependent, whereas that elicited by anisomycin is p38 MAPK-dependent. In intact cells, the nucleosomal response can be selectively inhibited using the protein kinase inhibitor H89. MAPK activation and phosphorylation of transcription factors are largely unaffected by H89, whereas induction of IE genes is inhibited and its characteristics markedly altered. MSK1 is considered the most likely kinase to mediate this response because (i) it is activated by both ERK and p38 MAPKs; (ii) it is an extremely efficient kinase for HMG-14 and H3, utilizing the physiologically relevant sites; and (iii) its activity towards H3/HMG-14 is uniquely sensitive to H89 inhibition. Thus, the nucleosomal response is an invariable consequence of ERK and p38 but not JNK/SAPK activation, and MSK1 potentially provides a link to complete the circuit between cell surface and nucleosome.

Keywords: histone H3/HMG-14/MAP kinases/MSK1/nucleosome

Introduction

Cells respond to diverse stimuli, which include physiological agents such as growth factors (reviewed in Bravo, 1990; Marshall, 1994) and cytokines (Freshney *et al.*, 1994; Sluss *et al.*, 1994), pharmacological compounds such as anisomycin (Edwards and Mahadevan, 1992; Hazzalin *et al.*, 1998, and references therein), phorbol esters and okadaic acid (reviewed in Cohen, 1990; Cano *et al.*, 1995) and stresses such as UV radiation (Hibi *et al.*, 1993; Kyriakis *et al.*, 1994), hyperosmotic (Han *et al.*, 1994; Rosette and Karin, 1996) and heavy metal stress (Rouse *et al.*, 1994, and references therein) by initiating

intracellular signalling mechanisms that rapidly elicit transcription of a subset of genes in the nucleus (reviewed in Karin, 1994; Cano and Mahadevan, 1995; Treisman, 1996; Hazzalin *et al.*, 1998). These genes, called immediate-early (IE) genes, are activated directly and require no new transcription or translation for their induction (Greenberg *et al.*, 1986; Almendral *et al.*, 1988; reviewed in Bravo, 1990). Despite the very diverse nature of these stimuli, a striking observation is that they all activate MAP kinase cascades (reviewed in Cano and Mahadevan, 1995; Seger and Krebs, 1995; Denhardt, 1996; Cohen, 1997; Whitmarsh and Davis, 1998), although the exact extent and time-course of activation of each of the ERK, JNK/SAPK and p38 MAP kinase cascades by a particular stimulus is highly distinctive and characteristic for that stimulus (see Cano *et al.*, 1995; Hazzalin *et al.*, 1998, and references therein). The protein products of some IE genes, such as those of the *fos* and *jun* families (reviewed in Karin *et al.*, 1997), are themselves transcription factors which go on to activate and repress other genes, thereby producing secondary transcriptional reprogramming appropriate to the stimulus used.

The *fos* and *jun* genes are controlled by multiple upstream elements; for human *c-fos* these include an SIE (Hayes *et al.*, 1987; Wagner *et al.*, 1990), an SRE (reviewed in Treisman, 1992), a TCF site (Shaw *et al.*, 1989), an AP-1 site (Sassone-Corsi *et al.*, 1988; Fisch *et al.*, 1989), an AP-1/CRE (Wagner *et al.*, 1990), a DR (Fisch *et al.*, 1987) and a CRE (Sheng *et al.*, 1988; Fisch *et al.*, 1989). The human *c-jun* promoter is controlled by the *jun2* AP-1 site, an FP, an NF-*jun* site, two overlapping SP-1 sites, a CCAAT box, the *jun1* AP-1 site, an RSRF site (MEF2; Han and Prywes, 1995; Han *et al.*, 1997; Clarke *et al.*, 1998) and in the 5' UTR, two AP-2 sites and a weak AP-1 site (Angel *et al.*, 1988; Rozek and Pfeifer, 1993, 1995; Herr *et al.*, 1994). These regulatory elements are highly conserved in the murine *c-jun* promoter (Han *et al.*, 1992). Broadly, two types of signalling systems act through these elements. In one case, the DNA elements are constitutively occupied by sequence-specific transcription factors or, more usually, complexes of such factors (reviewed in Buckle *et al.*, 1995; Karin *et al.*, 1997). Activation is produced by translocation of a stimulus-dependent kinase, such as MAP kinases or their effector kinases, which bind to and phosphorylate these factors, effecting transactivation and transcription of the associated gene (reviewed in Karin, 1994; Treisman, 1996). A second type of upstream DNA element is unoccupied in quiescent cells, only becoming occupied by transcription factors after stimulation; these include STAT-binding and NF- κ B sites. For both STATs (reviewed in Darnell, 1997) and NF- κ B (reviewed in Baeuerle and Baltimore, 1996) the inactive transcription factor, located in the cytoplasm, is activated via specific kinase pathways upon stimulation

and translocates into the nucleus to effect transcription via the respective upstream regulatory elements. Two of the most complex aspects of these mechanisms are (i) that multiple signalling systems may be simultaneously utilized to control the same gene, and (ii) that there appears to be precise quantitative control of the transcription of each gene depending on the stimulus used.

In addition to signalling mechanisms targeted to transcription factors, there has been persistent interest in the role that chromatin structure might play in IE gene induction. As a rapidly and transiently inducible gene, *c-fos* has been a focus for these studies. The consensus arrived at by a number of approaches is that this gene undergoes transient relaxation in its conformation that correlates well with its induction, superinduction and shut-off (Feng and Villeponteau, 1992; reviewed in Buckle *et al.*, 1995). Furthermore, approaches aimed at isolating active genes, which include the active *c-fos* gene, yield nucleosomes with altered conformation (Allegra *et al.*, 1987; Chen and Allfrey, 1987). The targeting of MAP kinases to transcription factors controlling *c-fos* and *c-jun* has been very clearly established (reviewed in Karin, 1996; Treisman, 1996) and either the sites of phosphorylation or phosphorylation itself is implicated in recruitment of coactivators such as p300/CBP and pCAF, recently shown to be histone acetyltransferases (HATs, reviewed in Kuo and Allis, 1998; Kouzarides, 1999), to these promoters (Bannister *et al.*, 1995; Janknecht and Nordheim, 1996, and references therein). Finally, the direct demonstration by chromatin immunoprecipitation (CHIP) assays of inducible histone H4 acetylation of a chromatinized SRE-reporter gene concomitant with its induction has recently been presented (Alberts *et al.*, 1998). Through the direct or indirect recruitment of coactivators such as p300/CBP and pCAF, histone acetylation is now implicated in many inducible regulatory elements such as SRE, AP-1, STAT and NF- κ B sites (reviewed in Kuo and Allis, 1998; Kouzarides, 1999).

In investigating signalling to chromatin, we showed that two nucleosomal proteins, histone H3 and HMG-14, are both phosphorylated concomitant with IE gene induction by all the agents described above (hereafter called 'the nucleosomal response') (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994a; Hazzalin *et al.*, 1996). Most importantly in the context of the above, the population of histone H3 that becomes phosphorylated is minute and is also extremely susceptible to hyperacetylation (Barratt *et al.*, 1994b); this finding links the two types of modification, but the mechanism by which acetylation and phosphorylation are targeted to the same H3 tail remains unknown (see Discussion). Here, we have investigated further the signalling mechanisms by which diverse stimuli elicit histone H3 and HMG-14 phosphorylation concomitant with IE gene induction. We show that this response is mediated via MAP kinase cascades, but that alternative MAP kinase subtypes are utilized depending on the stimulus used in a manner that parallels the induction of IE genes. Furthermore, we report that the inhibitor H89 is a selective inhibitor of the nucleosomal response whilst not affecting MAP kinase activation or the phosphorylation of transcription factors. Finally, we provide evidence for the identity of the kinase downstream of the MAP kinases that mediates the nucleosomal response. These findings

provide alternative MAP kinase-mediated routes between the cell surface and the nucleosome and are discussed in relation to the proposed involvement of nucleosomal modifications in IE gene induction.

Results

Generation and characterization of antibodies against HMG-14 and phosphoepitopes on histone H3 and HMG-14

Previous studies have established that stimulation of quiescent C3H 10T1/2 cells with stress, mitogenic and pharmacological stimuli results in the rapid phosphorylation of HMG-14 on serine 6 and histone H3 on serine 10 concomitant with IE gene induction (Mahadevan *et al.*, 1991; Barratt *et al.*, 1994a; Cano *et al.*, 1995; Hazzalin *et al.*, 1996). To facilitate further study, three antisera have been raised which specifically recognize HMG-14, phospho-HMG-14 and phospho-H3; all three were extensively characterized against phosphorylated and non-phosphorylated peptides as well as by immunoprecipitation assays to prove their specificity (not shown). This is confirmed by Western blotting studies shown in Figure 1. Note that these blots contain high levels of other stainable proteins (Figure 1Ai and Bi), emphasizing the specificity of these antibodies.

The anti-HMG-14 antibody, which recognizes the protein irrespective of its state of modification, was raised in rabbits using a synthetic peptide corresponding to the C-terminal of this protein (see Materials and methods). Because phosphorylation of HMG-14 causes retardation in its mobility on acid-urea gels (Figure 1Aiii and iv), these antibodies provide a means of assessing the extent of HMG-14 phosphorylation by Western blotting. For example, under superinducing conditions (Greenberg and Ziff, 1984; Edwards and Mahadevan, 1992), almost all the HMG-14 in the nucleus becomes phosphorylated (Figure 1Aiii). The phospho-specific HMG-14 antibody was raised in sheep using an N-terminal peptide of HMG-14 synthesized with phosphate on serine 6. The anti-phospho-HMG-14 antibody only recognizes the more slowly migrating form of HMG-14 (Figure 1Aii); this antibody shows no cross-reaction with non-phosphorylated HMG-14. Note that there is no Coomassie Blue-stainable band corresponding to HMG-14 (Figure 1Ai), showing that it is an extremely minor constituent of these extracts.

The anti-phospho-H3 antibody was raised in rabbits using a peptide corresponding to the N-terminal H3 tail synthesized with phosphate on serine 10. This antiserum shows high specificity for phosphorylated H3 (Figure 1Bii). We consistently observe a low level of signal in the control samples containing histone H3 extracted from unstimulated cells (Figure 1Bii). It is unlikely that this signal arises from cross-reactivity with non-phosphorylated H3 tails; first, because the antibody does not recognize synthetic non-phosphorylated H3 peptide; secondly, because it does not react with the lowest band of the H3 ladder on acid-urea gels corresponding to non-modified H3 (A.L.Clayton, S.Rose and L.C.Mahadevan, unpublished data); and finally, because this type of cross-reactivity might be expected to give a stronger signal due to the very large amounts of non-modified H3 on these blots (Figure 1Bi). The most likely explanation arises from the

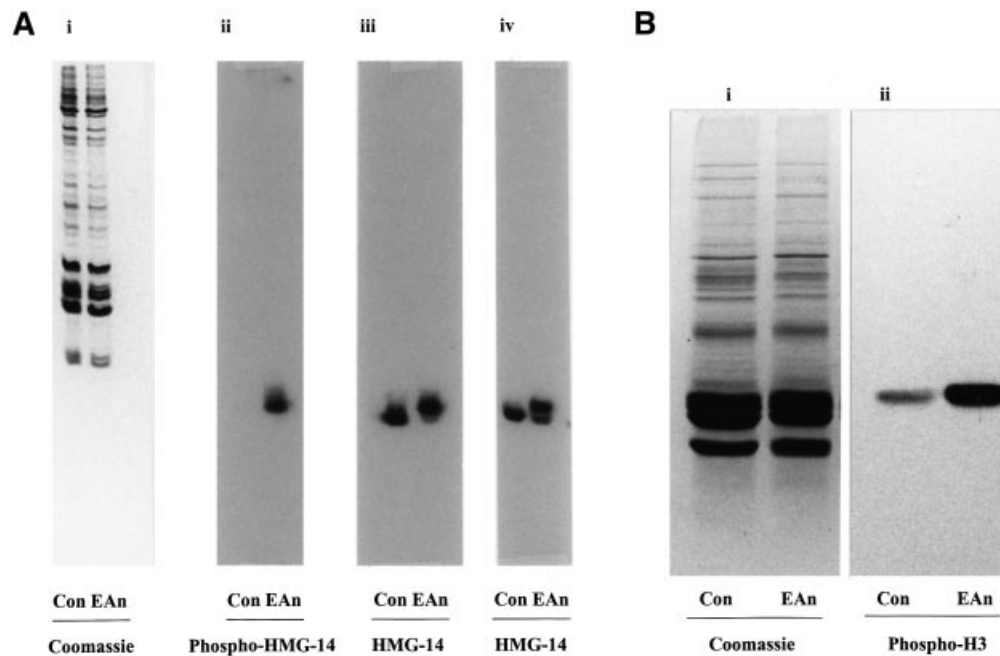


Fig. 1. Characterization of the antibodies by Western blot analysis. (A) Acid soluble nuclear proteins were prepared from quiescent C3H 10T1/2 cells (Control) or from cells stimulated with EGF (50 ng/ml) plus anisomycin (10 μ g/ml) (EAn) for 60 min. Samples were separated on 15% acid-urea gels and either stained with Coomassie (panel i) or transferred to PVDF membrane. The membranes were probed with either anti-phospho-HMG-14 (1:400 dilution, panel ii) or anti-HMG-14 (1:1000 dilution, panel iii) antibodies. HMG-14 purified by HPLC from Con or EAn-stimulated cells was run on acid-urea gels, transferred to PVDF membrane and probed with anti-HMG-14 antibody (panel iii). The antibodies are highly specific for HMG-14 and reveal that phosphorylation of HMG-14 after stimulation causes a reduction in its mobility on this gel system. (B) Acid soluble nuclear proteins were prepared as above and separated by 15% SDS-PAGE. Samples were either Coomassie stained (panel i) or transferred to PVDF membrane and probed with anti-phospho-H3 antibody (1:1000 dilution, panel ii). These antibodies are highly specific for phosphorylated H3, although a low background reaction to control samples is consistently observed. Note that these antibody dilutions were used in all subsequent immunoblots.

fact that dishes of quiescent cells do contain a very small number of cells that escape quiescence and carry condensed chromosomes, which contain a very high level of H3 phosphorylated on serine 10 (Paulson and Taylor, 1982; Hendzel *et al.*, 1997) detectable by immunocytochemical staining (P. Jeppesen, MRC Human Genetics Centre, Edinburgh; S. Thomson and L.C. Mahadevan, unpublished data).

These antibodies obviate the need for metabolic labelling with [32 P]phosphate, allowing detailed and site-specific assessment of histone H3 and HMG-14 phosphorylation by Western blotting. They have been used to reaffirm earlier observations that HMG-14 and H3 phosphorylation is induced by a wide variety of mitogenic, pharmacological and stress-related stimuli (see also below) and that this occurs in a number of different cell lines (data not shown).

The nucleosomal response and IE gene induction are both inducible via alternative MAP kinase pathways

We have previously characterized the relative activation of ERK, JNK/SAPK and p38 MAP kinase pathways elicited by physiological, pharmacological and stress stimuli in C3H 10T1/2 cells (Cano *et al.*, 1994, 1995, 1996; Hazzalin *et al.*, 1996). These data may be summarized as follows: first, and most strikingly in view of their extreme diversity, all these agents activate MAP kinase cascades. However, in the same way that the precise induction of five *fos* and *jun* genes are very characteristic of the stimulus used (Hazzalin *et al.*, 1998), the extent and time-course of activation of each of the three MAP kinase

cascades is specific to each stimulus. Thus, EGF strongly activates ERKs, but weakly activates JNK/SAPKs and p38, whereas UV radiation strongly activates JNK/SAPKs and p38 but is a very weak activator of ERKs. Relevant to data presented below, in C3H 10T1/2 cells TPA activates only the ERK pathway but not JNK/SAPKs or p38, whereas anisomycin strongly activates both JNK/SAPKs and p38 but does not activate ERKs (Cano *et al.*, 1994, 1996; Hazzalin *et al.*, 1996). This has been verified in several other cell lines, but cannot be extended to a generalization as some cell lines do show distinct characteristics of MAP kinase activation to these stimuli (E. Cano, R. LePense, K. Rutault and L.C. Mahadevan, unpublished). Inhibitors have been developed which are specific for ERK or p38 MAP kinase pathways. PD 98059 specifically inhibits the ERK pathway (Dudley *et al.*, 1995), whereas SB 203580 specifically inhibits the p38 pathway (Lee *et al.*, 1994; reviewed in Cohen, 1997).

TPA-stimulated c-fos/c-jun induction and histone H3/HMG-14 phosphorylation is mediated via ERK activation. The phorbol ester TPA activates the ERK pathway by an as yet uncharacterized route involving protein kinase C. Treatment of cells with PD 98059 blocked TPA-induced phosphorylation of HMG-14 and H3; however, treatment with SB 203580 had no effect on this response (Figure 2A, lanes 1–4). Similarly, the induction of the *c-fos* and *c-jun* genes by TPA is inhibited by PD 98059 (Figure 2B, lanes 1–3), showing that TPA-stimulated nucleosomal response and IE gene induction are both mediated via ERKs. As TPA does not activate either of the other two

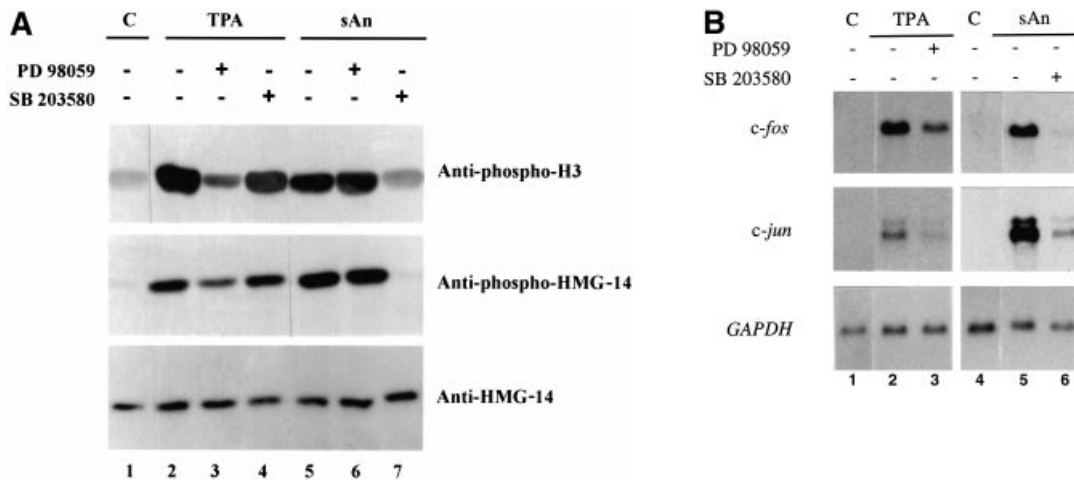


Fig. 2. Phosphorylation of HMG-14 and H3, as well as IE gene induction, is observed after activation of either the ERK or p38 MAP kinase pathways. (A) Quiescent C3H 10T1/2 cells were untreated (lanes 1, 2 and 5) or pre-treated with either PD 98059 (50 μ M, lanes 3 and 6) or SB 203580 (10 μ M, lanes 4 and 7) for 60 min. Cells were then left unstimulated (C, lane 1) or stimulated with TPA (100 nM, lanes 2–4) or sAn (50 ng/ml, lanes 5–7) for 30 min. Nuclear proteins were prepared, separated by 15% SDS–PAGE, transferred to PVDF and probed with the indicated antibody. Phosphorylation of HMG-14 and H3 is observed after stimulation with the ERK MAP kinase pathway activator TPA and is inhibited by PD 98059 but not by SB 203580. In contrast the sAn-stimulated phosphorylation is inhibited by p38 MAP kinase pathway inhibitor SB 203580 but not by PD 98059. The anti-HMG-14 immunoblot in the bottom panel controls for equal protein loading in each lane. (B) Northern blot analysis of *fos* and *jun* gene expression. C3H 10T1/2 cells incubated in the presence (+), or absence (–) of either PD 98059 (50 μ M) for 90 min, or SB 203580 (20 μ M) for 15 min, were then stimulated with TPA (100 nM), 45 min; or subinhibitory anisomycin (sAn; 25 ng/ml), 45 min. C, control (unstimulated). Blots were sequentially hybridized to *c-fos* and *c-jun* probes and then hybridized with a GAPDH probe as a loading control.

MAP kinase cascades and its effects are not affected by the p38-specific inhibitor, they cannot contribute to this response.

Anisomycin-stimulated *c-fos/c-jun* induction and histone H3/HMG-14 phosphorylation is dependent on p38 MAP kinase activation. Treatment of quiescent cells with concentrations of anisomycin that do not result in translational arrest (sub-inhibitory anisomycin, sAn; Edwards and Mahadevan, 1992) results in strong activation of both JNK/SAPK and p38 pathways (Cano *et al.*, 1996, and references therein; Hazzalin *et al.*, 1996). As shown previously, anisomycin strongly stimulates histone H3/HMG-14 phosphorylation (Figure 2A, lane 5) as well as *c-fos/c-jun* induction (Figure 2B, lanes 5). The anisomycin-stimulated nucleosomal response is inhibited by the p38-specific inhibitor SB 203580 but not by PD 98059 (Figure 2A, lanes 6 and 7). Similarly, anisomycin-stimulated IE gene induction is inhibited by the p38-specific inhibitor SB 203580 (Figure 2B, lane 6), proving that p38 activity is essential for both responses. Note that a role for JNK/SAPKs in anisomycin-stimulated IE gene induction is not ruled out by this finding; however, it is clear from these data that JNK/SAPK activation itself does not lead to H3/HMG-14 phosphorylation, because JNK/SAPKs remain highly active under these conditions whereas the nucleosomal response is not seen (Hazzalin *et al.*, 1996). Finally, ERKs cannot play a part in anisomycin-stimulated responses as they are not activated by this compound in C3H 10T1/2 cells.

These data show that the nucleosomal response and IE gene induction can be elicited via alternative routes; the TPA-stimulated nuclear responses require ERK activation, whereas anisomycin elicits the same responses via p38 activation. If anisomycin and TPA ultimately activate the same nucleosomal kinase/s for HMG-14 and histone H3

(see below), this kinase/s must be accessible by both ERK and p38 MAP kinase pathways and may therefore represent a point of convergence that integrates ERK and p38-mediated signals into a common nucleosomal response.

Phosphorylation of HMG-14 and H3 is inhibited by the protein kinase inhibitor H89

In the course of this study, we evaluated many kinase inhibitors for their effects on cytoplasmic kinases and nuclear responses; here, we describe the effects of one of these, H89, which we found to be a selective inhibitor of the nucleosomal response. The compound H89 is a member of the H-series of protein kinase inhibitors, which also includes H7 and H8. It shows a high level of specificity towards cAMP-dependent kinase *in vitro*, having an IC_{50} value of 40 nM (Engh *et al.*, 1996; see Discussion)

Pre-treatment of quiescent C3H 10T1/2 cells with 10 μ M H89 inhibits TPA-, anisomycin- and EGF-stimulated HMG-14 and H3 phosphorylation (Figure 3, lanes 4, 6 and 8). HMG-14 phosphorylation is virtually undetectable by Western blotting after pre-treatment with H89 whereas H3 phosphorylation is strongly, but not completely, inhibited. This effect is not due to the inhibition of cAMP-dependent kinase by H89, as a different inhibitor of this kinase, Rp-cAMP (Dostmann *et al.*, 1990), failed to inhibit the nucleosomal response even at very high concentrations (up to 1 mM, data not shown; see also Discussion). Dose-response studies show that 10 μ M H89 is the optimal concentration for inhibition of the nucleosomal response *in vivo*; at higher concentrations the compound begins to have non-specific inhibitory effects on other signalling events (described below).

MAP kinase activation is not inhibited by H89

Since MAP kinases mediate the nucleosomal response (Figure 2A), we asked whether the inhibitory effect of

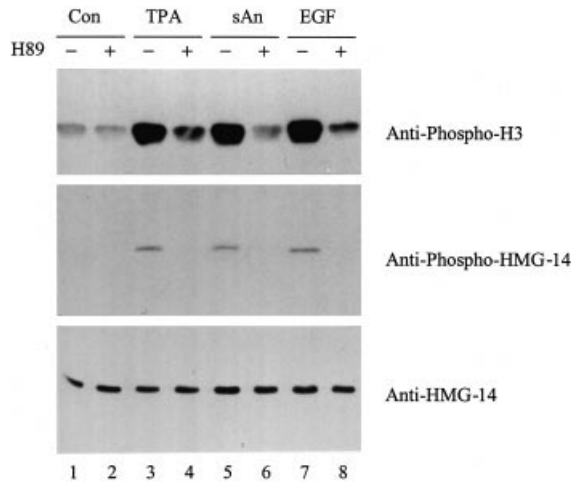


Fig. 3. Phosphorylation of HMG-14 and H3 is inhibited by H89. Quiescent C3H 10T1/2 cells were untreated (lanes 1, 3, 5 and 7) or pre-treated (lanes 2, 4, 6 and 8) with H89 (10 μ M) for 15 min. Cells were then left unstimulated (Con, lanes 1 and 2) or stimulated with TPA (100 nM, lanes 3 and 4), sAn (50 ng/ml, lanes 5 and 6) or EGF (50 ng/ml, lanes 7 and 8) for 30 min. Nuclear proteins were prepared and samples analysed by immunoblotting with the indicated antibody.

H89 was due to inhibition of MAP kinase activation. Activation of ERKs, detected by a mobility-shift assay, in response to TPA (Figure 4A, lanes 2–6) was unaffected by pre-treatment of the cells with 10 μ M H89 (Figure 4A, lanes 7–11). It should be noted however, that at 20 μ M H89, inhibition of EGF-stimulated ERK activation begins to be seen and virtually complete inhibition is seen at 50 μ M H89 (data not shown). The p38 pathway is activated by anisomycin resulting in phosphorylation and activation of p38 MAP kinase (Figure 4B, lanes 2–4). Pre-treatment with H89 had no effect on p38 phosphorylation (Figure 4B, lanes 5–7). Further evidence that p38 activation and activity in intact cells was not affected by H89 comes from the observation that its downstream kinase MAPKAP-K2 was fully activated in cells pre-treated with H89 (data not shown). Similarly the JNK/SAPK pathway, assessed by GST–Jun ‘pull-down’ and *in vitro* kinase assay, is strongly activated by anisomycin (Figure 4C, lanes 2 and 4). Again, no effect on JNK/SAPK activation was observed after pre-treatment with H89 (Figure 4C, lanes 3 and 5).

These data indicate that at the concentration used here, H89 does not have a non-specific inhibitory effect upon the activation of MAP kinases, implying that H89 must act at some point downstream of these kinases to inhibit the nucleosomal response.

Transcription factor phosphorylation is not inhibited by H89

There are two possible points downstream of ERKs and p38 through which H89 may act. The first is by inhibiting transcription factor phosphorylation, a critical prerequisite for IE gene induction which may also be required for the nucleosomal response, while the second is by inhibiting kinases downstream of ERKs and p38 (discussed further below).

IE gene induction is preceded by phosphorylation of transcription factors such as c-Jun, ATF2 and CREB bound to regulatory elements at the promoters of these genes (reviewed in Karin, 1996; Treisman, 1996). We examined

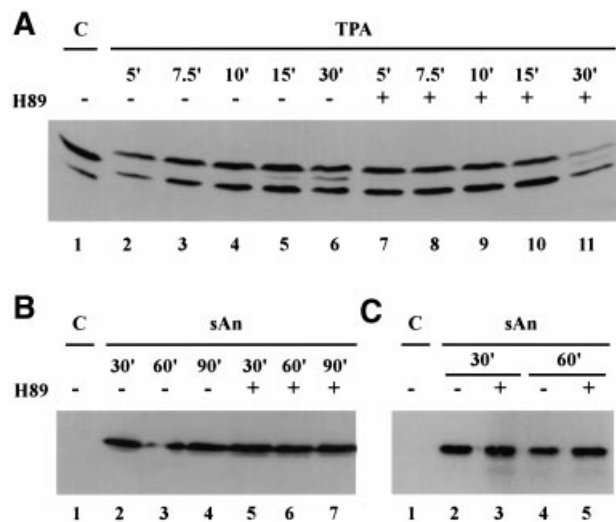


Fig. 4. H89 has no effect on the activation of MAP kinases. (A) Quiescent C3H 10T1/2 cells were untreated (lanes 1–6) or pre-treated with H89 (10 μ M) for 15 min (lanes 7–11). Cells were then left untreated (C, lane 1) or stimulated with TPA for the indicated times (100 nM, lanes 2–11). Cell extracts were analysed by immunoblotting with anti-ERK1/2 antibody, which recognizes the MAP kinases ERK1 and ERK2. The mobility of the ERKs is retarded upon activation. (B) Quiescent cells were untreated (lanes 1–4) or pre-treated with H89 as in (A) (lanes 5–7). Cells were then left untreated (C, lane 1) or were stimulated with sAn (50 ng/ml) for the indicated times (lanes 2–7). Cell extracts were analysed by immunoblotting with anti-phospho-p38 antibody. Activation of p38 MAP kinase results in its phosphorylation. (C) Quiescent cells were untreated (lanes 1, 2 and 4) or pre-treated with H89 as in (A) (lanes 3 and 5). Cells were then left untreated (C, lanes 1) or were stimulated with sAn (50 ng/ml, lanes 2–5) for the indicated times. Activation of JNK/SAPK kinase was assayed by its ability to phosphorylate GST–Jun after a ‘pull-down’ assay.

the phosphorylation of these transcription factors in response to TPA and anisomycin (Figure 5). ATF2 is activated by phosphorylation on Thr69 and Thr71 in its N-terminal domain (Livingstone *et al.*, 1995; van Dam *et al.*, 1995). We observe a transient phosphorylation of ATF2 in response to TPA, which is unaffected by H89 (Figure 5Ai, lanes 3–8). In contrast, anisomycin stimulation results in a more prolonged activation of ATF2, which is similarly unaffected by H89 treatment (Figure 5Aii, lanes 3–8). c-Jun is phosphorylated on Ser63 and Ser73 in response to stress-related stimuli and this is mediated directly by JNK/SAPKs (Hibi *et al.*, 1993; Kyriakis *et al.*, 1994). As expected, no c-Jun phosphorylation is observed in response to TPA, which does not activate JNK/SAPKs in these cells (Figure 5B, lanes 3–5). In contrast, c-Jun is strongly phosphorylated in response to anisomycin (Figure 5B, lanes 9–11), a response that is not inhibited by H89 (Figure 5B, lanes 12–14).

CREB is phosphorylated on Ser133 in response to both mitogenic and stress-related stimuli (Deak *et al.*, 1998; Xing *et al.*, 1998). Phosphorylation on Ser133 is detectable upon stimulation with TPA or anisomycin (Figure 5C, lanes 3–5 and 9–11, respectively). CREB phosphorylation elicited by TPA is inhibited by the MEK inhibitor PD 98059, whereas that elicited by anisomycin is blocked by SB 203580 (data not shown), corresponding exactly with inhibition of the nucleosomal response under these conditions. In contrast, H89 has relatively little effect on

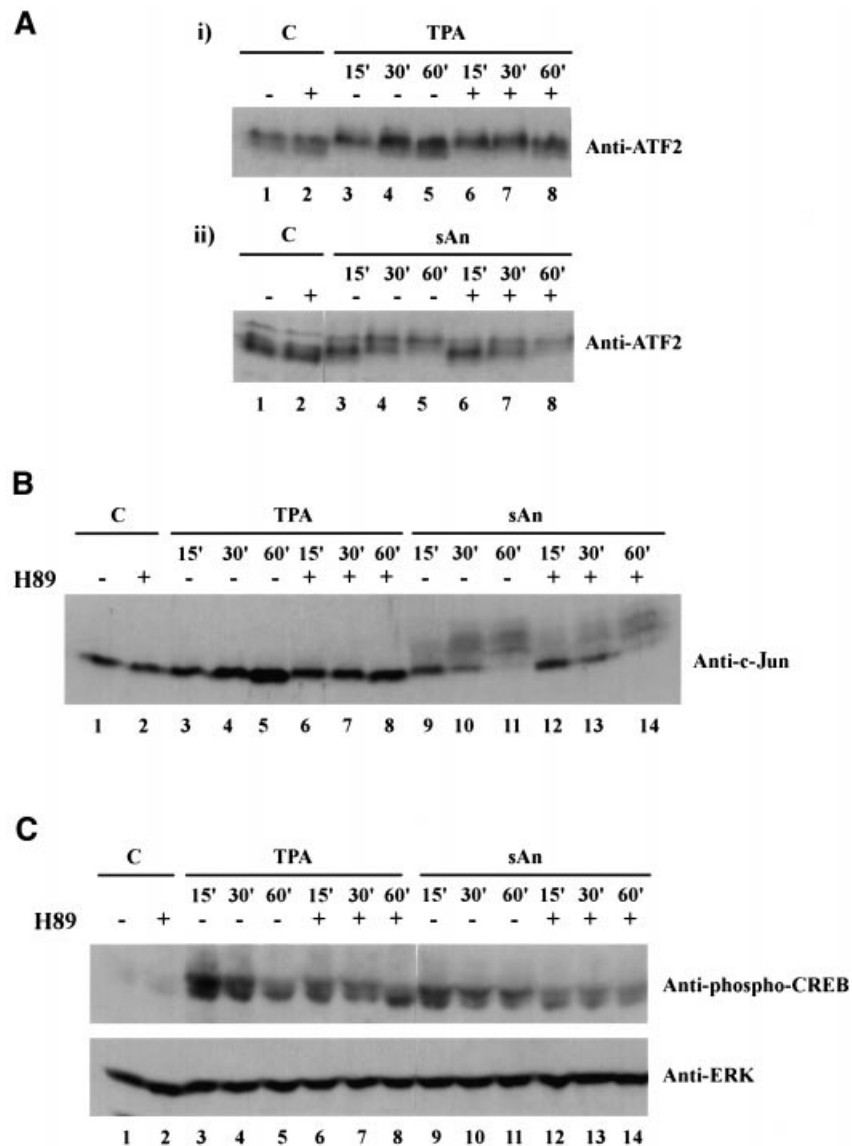


Fig. 5. H89 does not inhibit transcription factor activation. (A) Quiescent C3H 10T1/2 cells were untreated (lanes 1 and 3–5) or pre-treated with 10 μ M H89 for 15 min (lanes 2 and 6–8). Cells were then left untreated (C, lanes 1 and 2) or were stimulated with TPA (lanes 3–8, panel i) or sAn (lanes 3–8, panel ii) for the indicated times. Immunoblot analysis of nuclear extracts was carried out using an anti-ATF2 antibody. Stimulation of the cells with TPA results in a slight shift in the mobility of ATF2, whereas stimulation with sAn results in a more significant shift. Phosphorylation of ATF2 is unaffected by H89. (B) Quiescent C3H 10T1/2 cells were untreated (lanes 1, 3–5 and 9–11) or pre-treated with 10 μ M H89 for 15 min (lanes 2, 6–8 and 12–14). Cells were then left untreated (C, lanes 1 and 2) or were stimulated with TPA (lanes 3–8) or sAn (lanes 9–14) for the indicated times. Nuclear extracts were analysed with an anti-c-Jun antibody. Phosphorylation of c-Jun is only observed in response to sAn, giving rise to a ladder of bands. Treatment with H89 does not affect the phosphorylation of c-Jun. (C) Cells were treated as in (B). Extracts were analysed with an anti-phospho-CREB antibody. CREB is phosphorylated in response to both TPA and sAn and this phosphorylation is unaffected by H89. The membrane was reprobed with anti-ERK antibody to control for protein loading.

either TPA- or anisomycin-stimulated phosphorylation of CREB (Figure 5C, lanes 6–8 and 12–14, respectively).

Thus, apart from CREB, which is slightly inhibited (Deak *et al.*, 1998), phosphorylation of transcription factors implicated in IE gene induction is not significantly affected by H89. This result agrees with data above showing that activation of MAP kinases, which are known to phosphorylate transcription factors, is not inhibited by H89. The important conclusion, however, is that in the presence of H89, MAP kinase activation and transcription factor phosphorylation are both not adversely affected, proving that signals continue to be delivered to IE genes under these conditions.

The effect of H89 on IE gene induction by TPA, EGF and anisomycin

We next analysed how IE gene induction was affected by H89 to establish if it correlated more closely with transcription factor phosphorylation in the presence of H89, in which case it should not be inhibited, or if it mirrored the inhibition seen in the nucleosomal response. Reminiscent of the quantitative activation of the three MAP kinase cascades, IE gene expression is quantitatively controlled depending on the stimulus used (Hazzalin *et al.*, 1997). Whereas TPA and EGF induce very rapid and transient expression of *c-fos*, anisomycin induces this gene more weakly, although transcripts remain detectable

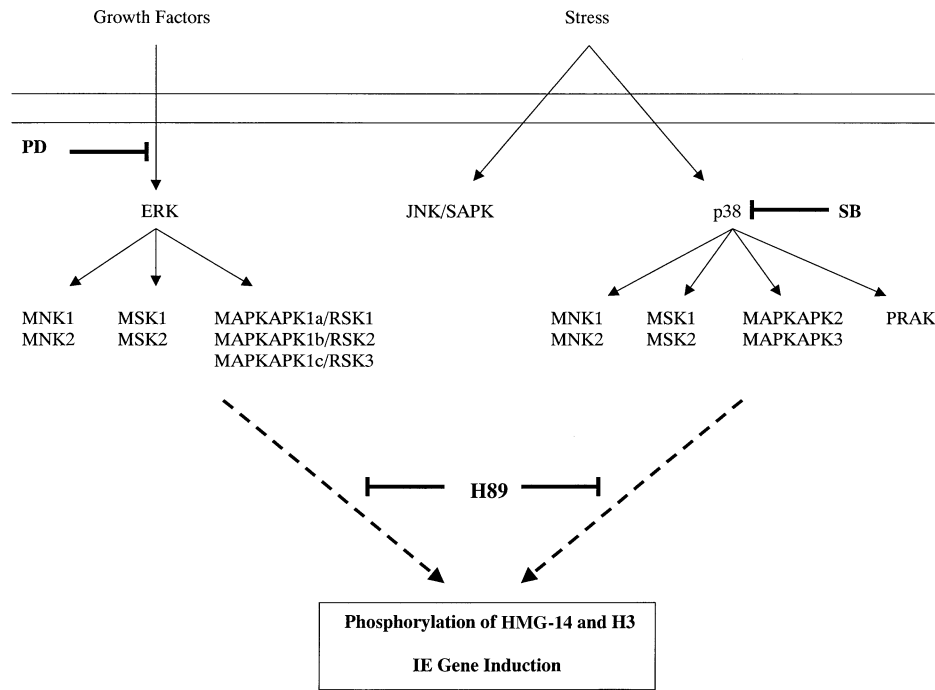


Fig. 7. The MAP kinase pathways involved in the nucleosomal response. The kinases which are known effectors of the ERK and p38 MAP kinase pathways are shown. Different signals activate each pathway but the signals converge to elicit similar nuclear responses, possibly through a common kinase. The signalling network upstream of the MAP kinases has been omitted for simplicity. The sites of action of the inhibitors used in this study are indicated. Solid arrows indicate a known route of activation, whereas the broken arrows indicate a hypothetical route.

IE gene induction is inhibited and its profile altered by this compound. It would appear that the effect of H89 is to both inhibit and delay accumulation of these transcripts, raising the possibility that phosphorylation of histone H3 and HMG-14 contributes quantitatively to influence the profile of expression of these genes (see Discussion).

H89 and effector kinases downstream of the MAP kinases

Data presented above indicate that the effect of H89 must be exerted downstream of ERK and p38 MAP kinases, as neither activation nor activity of these kinases are inhibited by H89 (Figure 4). We have eliminated the possibility that H89 acts by inhibiting transcription factor phosphorylation (Figure 5). Other potential points of H89 inhibition are at effector kinases that lie downstream of ERK and p38. There are at least six downstream effector kinases; their relationship to ERK and p38 MAP kinases is shown schematically in Figure 7 (reviewed in Cohen 1997; Thomson *et al.*, 1999). Of these, only MSK1, MSK2 (Deak *et al.*, 1998), MNK1 and MNK2 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997) are known to be downstream of both ERK and p38, a necessary criterion for the kinase mediating the nucleosomal response. Further, MSK1 is reported to be localized in the nucleus (Deak *et al.*, 1998), making it a good candidate for the nucleosomal kinase. However, previous work from Blenis and colleagues has shown that RSK kinases are also capable of phosphorylating histone H3 (Chen *et al.*, 1992). Additionally, RSK2 has recently been proposed to be the H3 kinase mediating the nucleosomal response (Mizzen *et al.*, 1998). We therefore compared the histone H3/HMG-14 kinase activity of MSK1 with that of RSK1 and RSK2.

In addition, because H89 blocks the nucleosomal response, we asked if any of these kinases were sensitive to H89.

Comparison of histone H3/HMG-14 kinase activity of MSK1, RSK1 and RSK2, and their sensitivity to H89

Kinase activities of the three enzyme preparations used here were normalized using the synthetic peptide 'cross-tide' as a standard, and H3 and HMG-14 peptide kinase assays corrected to ensure equal crosstide kinase activity in each assay. This showed that MSK1 is by far the best HMG-14 kinase, showing ~22-fold higher activity than RSK2 toward the HMG-14 peptide (Figure 8A). In addition, and most importantly in light of results presented above, MSK1 activity is severely inhibited by 10 μ M H89, whereas the activities of RSK1 and RSK2 are, if anything, slightly elevated in the presence of H89 (Figure 8A). Proof that MSK1 phosphorylates the HMG-14 peptide only on Ser6, the physiological site, derives from the complete lack of phosphorylation of an identical peptide with Ser to Ala substitution at position 6 (Figure 8B).

Similarly, phosphorylation of an H3 peptide by MSK1 was more efficient than by either RSK1 or RSK2 (Figure 8C); there was a 4-fold difference in activity between MSK1 and RSK2 for this substrate. As observed using the HMG-14 peptide, only MSK1 was efficiently inhibited by H89 in these assays (Figure 8C). Further, when a mixture of all four core histones was used as substrate for kinase assays, with activities again equalized against 'crosstide', H3 was the only histone significantly phosphorylated despite the abundance of the other three core histones (see stained gel, Figure 8D); MSK1 was much more efficient than either RSK1 or RSK2 as a kinase for

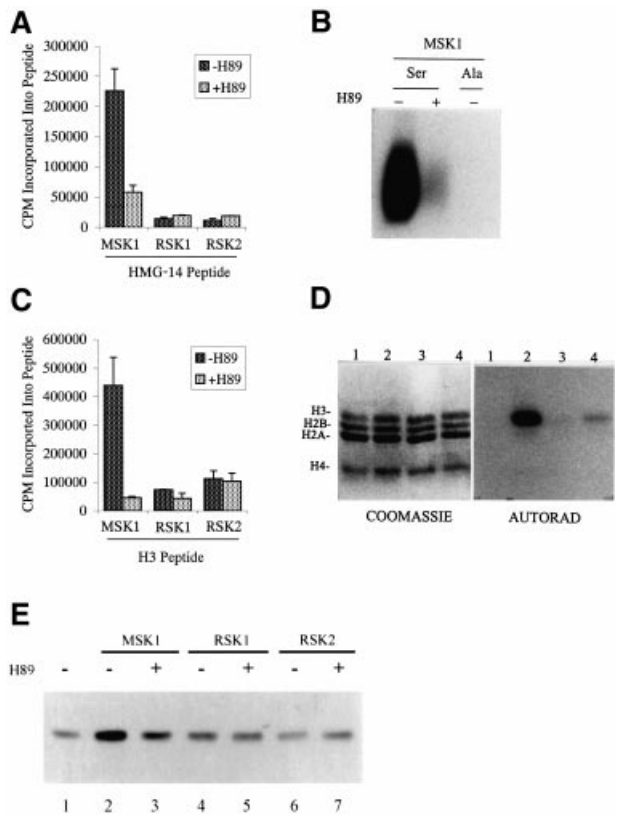


Fig. 8. MSK1 is a good candidate HMG-14/H3 kinase *in vitro* and can be inhibited by H89. (A) Phosphorylation of HMG-14 peptide by recombinant MSK1 was assayed *in vitro* using the phosphocellulose paper assay. For comparison, the activities of RSK1 and RSK2 towards the peptide were also determined. Each assay was performed in triplicate either in the presence or absence of 10 μ M H89. The counts incorporated were corrected to represent equal crossside kinase activity for each kinase. (B) An example of MSK1 activity toward HMG-14 peptide. Assays were carried out either in the presence or absence of 10 μ M H89, and with either the Ser peptide or Ala peptide. The absence of any phosphorylation on the Ala peptide indicates that it is Ser6 that is phosphorylated by MSK1. (C) Kinase activity toward H3 peptide was carried out as described in (A). (D) Phosphorylation of histones by these three kinases was assayed using acid soluble nuclear extracts as a substrate. Equal crossside kinase activities were used in each assay. The Coomassie-stained panel shows histones that were incubated with either no kinase (lane 1), MSK1 (lane 2), RSK1 (lane 3) or RSK2 (lane 4). The phosphorylated bands are shown in the adjacent panel, and indicate that H3 is the predominantly phosphorylated protein. (E) Assays were carried out essentially as described in (D), except that they were carried out either in the presence (lanes 3, 5 and 7) or absence (lanes 1, 2, 4 and 6) of 10 μ M H89 and without radioactive ATP. Phosphorylated H3 was visualized by immunoblotting with anti-phospho-H3 antibody and indicates that MSK1 phosphorylates H3 on Ser10 with much greater efficiency than either RSK1 or RSK2.

intact histone H3, just as it was for the peptide (Figure 8D, lanes 1–4). Finally, using our phosphorylation site-specific anti-phospho-H3 antibodies we confirmed that MSK1 phosphorylates H3 on Ser10, the physiologically relevant site, with a much higher activity than either of the other two kinases (Figure 8E). These three different assays for H3 phosphorylation provide conclusive evidence that MSK1 is the best H3 kinase tested and that Ser10 is the residue predominantly phosphorylated. We also tested MNK1 and MNK2 (kindly provided by Professor Chris Proud, Dundee, UK) for H3/HMG-14

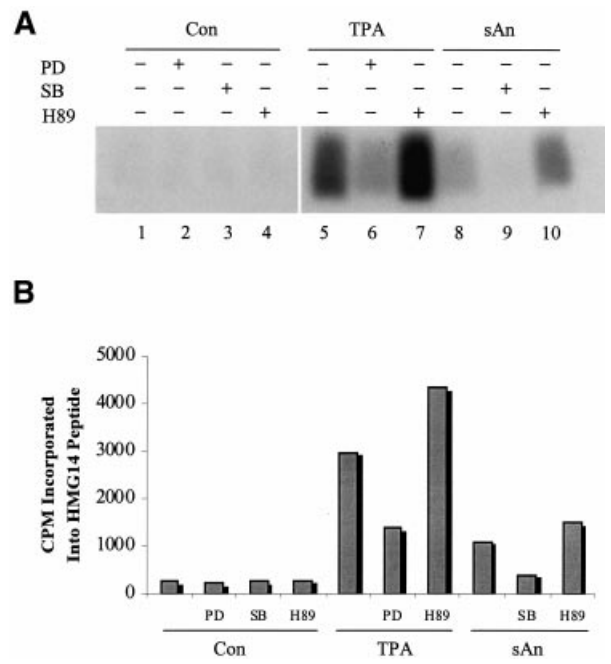


Fig. 9. MSK1 is activated by TPA and sAn in C3H 10T1/2 cells. (A) Quiescent cells were untreated (lanes 1, 5 and 8) or pre-treated with PD 98059 (50 μ M, lanes 2 and 6), SB 203580 (10 μ M, lanes 3 and 9) for 60 min or with H89 (10 μ M, lanes 4, 7 and 10) for 15 min. Cells were then untreated (lanes 1–4) or treated with TPA (100 nM, lanes 5–7) or sAn (50 ng/ml, lanes 8–10) for 30 min. MSK1 was immunoprecipitated from the cell lysates and the recovered kinase assayed for its ability to phosphorylate HMG-14 peptide. (B) The counts incorporated into HMG-14 peptide in (A) were quantified using a phosphorimager and the results represented graphically.

kinase activity but found that they were poor kinases for these substrates in similar assays (data not shown).

These data indicate that MSK1 phosphorylates both HMG-14 and H3 substrates *in vitro* very efficiently in comparison with the other kinases tested. Further, it phosphorylates these substrates on the physiological sites, and this is inhibited by H89 at concentrations that inhibit the nucleosomal response *in vivo*. This, in conjunction with the observations that MSK1 is activated by both ERK and p38, and that it is a nuclear kinase, strongly suggests that it is the kinase responsible for mediating the nucleosomal response *in vivo*.

MSK1 is activated by the ERK and p38 MAP kinase pathways in C3H 10T1/2 cells

To prove that it can fulfil the role of histone H3 and HMG-14 kinase, we sought to confirm that MSK1 is both present and activated by ERK and p38 in C3H 10T1/2 cells. The state of activation of MSK1 was assayed by immunoprecipitation from cell lysates followed by kinase assays using HMG-14 peptide as substrate (Figure 9A, lanes 5 and 8). MSK1 is activated by both TPA and anisomycin in C3H 10T1/2 cells and exactly as observed for the nucleosomal response (Figure 2), the pathway-specific inhibitors PD 98059 and SB 203580 inhibit MSK1 activation by TPA and anisomycin, respectively (Figure 9A, lanes 5, 6, 8 and 9). In agreement with Deak *et al.* (1998) who found that TPA activates MSK1 more strongly than stress-related stimuli in 293 cells, TPA elicits stronger activation than anisomycin in C3H 10T1/2 cells. Although H89 clearly inhibits MSK1 activity *in vitro* and the

nucleosomal response *in vivo*, treatment of intact cells with H89 did not cause any inhibition in the state of MSK1 activation elicited by anisomycin or TPA; in fact, its state of activation is enhanced. This is detectable by these assays because inhibition by H89 is reversible and the *in vitro* kinase assay on immunoprecipitated MSK1 is performed in the absence of H89; this assay therefore provides a measure of its state of activation in these lysates and not its activity *in vivo*. This implies that H89 may inhibit a negative feedback loop emanating from MSK1 itself, reminiscent of the effect of inhibiting p38 with SB 203580, which results in enhanced activation of its upstream kinase MKK6 (Hazzalin *et al.*, 1996).

The important conclusions here are first, that MSK1 is clearly activated by TPA and anisomycin in C3H 10T1/2 cells and could therefore fulfil the role of H3 and HMG-14 kinase *in vivo*; secondly, that its inhibition by PD 98059 and SB 203580 parallels exactly the effects of these compounds on the nucleosomal response; and finally, that signalling pathways leading to MSK1 activation are not inhibited by H89 verifying further the specificity of this compound. These data show that MSK1 possesses all the characteristics required for it to fulfil the role of histone H3 and HMG-14 kinase in intact C3H 10T1/2 cells.

Discussion

This study extends further the strong correlation between phosphorylation of nucleosomal proteins histone H3 and HMG-14 and induction of IE genes, focussing particularly on routes by which the nucleosomal responses are mediated. Using phospho-specific antibodies, we show that the nucleosomal response to mitogenic and/or stress-related stimuli can be mediated by either ERK or p38, but not by JNK/SAPK pathways. Finally, we identify the recently described kinase MSK1 as a strong candidate for the histone H3 and HMG-14 kinase, potentially completing the circuit between plasma membrane and nucleosome.

The nucleosomal response is mediated via alternative MAP kinase cascades depending on the stimulus used

We have shown that both nucleosomal response and IE gene induction in response to TPA are mediated via ERKs, whereas that elicited by anisomycin is mediated via p38, suggesting that both these kinase pathways can process diverse signals into a common response at the nucleosome. This is not without precedent as it is well established that the transcription factors CREB and Elk-1 can be phosphorylated via mechanisms utilizing either of these two pathways (Price *et al.*, 1996; Whitmarsh *et al.*, 1997; Deak *et al.*, 1998; Xing *et al.*, 1998). ERK and p38 themselves phosphorylate Elk-1 on proline-directed sites (Price *et al.*, 1996, and references therein), whereas the identity of the kinase that mediates CREB phosphorylation on serine 133, which is not proline directed, is more contentious. Like the nucleosomal kinase, CREB kinase must lie downstream of ERKs and p38 (Deak *et al.*, 1998; see also below). In anisomycin-stimulated cells in which p38 activity is ablated using SB 203580, strong JNK/SAPK activation continues to be observed but the nucleosomal response does not occur (Hazzalin *et al.*, 1996), showing that in contrast to the other MAP kinases, the JNK/SAPK

pathway is in itself not capable of delivering a signal to the nucleosome. There are many other systems of signalling to IE genes, e.g. via STATs or NF- κ B (Bauerle and Baltimore 1996; reviewed in Darnell, 1997; Introduction), which do not involve activation of the MAP kinases; data presented here would predict that these do not utilize the nucleosomal response as part of their mechanism for gene activation, whereas stimuli that do activate ERK and p38 MAP kinases would invariably result in a nucleosomal response. Note, however, that although ERK- and p38-equivalents are present in *Saccharomyces cerevisiae*, corresponding equivalents of their downstream kinases shown in Figure 7 and of HMG-14 do not exist. So far, there has been no report of rapidly inducible histone H3 phosphorylation in this organism. This suggests that the nucleosomal response is evolutionarily more recent and is possibly associated with more complex modes of gene regulation seen in multicellular organisms.

The protein kinase inhibitor H89 as an inhibitor of nuclear responses

H89 at 10 μ M concentration inhibits both nucleosomal response and IE gene induction elicited via ERK or p38 MAP kinase pathways but does not significantly affect activation or activity of MAP kinases, nor phosphorylation of transcription factors. H89 is well-characterized as a potent ATP-competitive inhibitor of cAMP-dependent kinase (IC₅₀ *in vitro*: 40 nM; Engh *et al.*, 1996) and of PKA-mediated phosphorylation events *in vivo* (Chijiwa *et al.*, 1990; Engh *et al.*, 1996; Daaka *et al.*, 1997). This might suggest that the nucleosomal response is mediated by cAMP-dependent kinase; indeed, it can phosphorylate both histone H3 and HMG-14 on the physiological sites *in vitro* (Taylor, 1982; Walton *et al.*, 1982; S.Thomson and L.C.Mahadevan, unpublished data). However, there are strong arguments against this. First, stimuli used here do not significantly raise intracellular cAMP levels; in fact, cAMP inhibits activation of the ERK pathway in response to mitogenic stimuli by blocking activation of Raf-1 by Ras (Cook and McCormick, 1993; Hordijk *et al.*, 1993). Secondly, Rp-cAMPs, another cAMP-dependent kinase inhibitor which acts differently from H89 (Dostmann *et al.*, 1990), does not inhibit the nucleosomal response even at the highest concentrations tested (data not shown). Thirdly, although able to phosphorylate H3 peptide *in vitro*, cAMP-dependent kinase is much less efficient than MSK1 (data not shown). Finally, our demonstration that MSK1 is also inhibited by H89, taken together with other characteristics of MSK1 (see below), suggests that it, and not cAMP-dependent kinase, mediates the nucleosomal response.

MSK1 as the potential nuclear kinase for histone H3 and HMG-14

The fact that ERK and p38-mediated signalling culminates in a common nucleosomal response suggests that there may be common nuclear effectors. MAP kinases themselves can be ruled out as the sites of phosphorylation are not proline directed; thus, kinases further downstream are implicated. Several downstream effector kinases have been identified (see Figure 7). Some, such as RSKs, are activated solely by ERKs, while others such as MAPKAP kinase 2 lie exclusively downstream of p38. However, two effector

kinases, MNK1/2 and MSK1/2, can be activated by either ERK or p38; these represent points of convergence for signals from both pathways. Of these, MSK1 is reported to be localized in the nucleus, prompting us to ask whether it might be responsible for phosphorylating histone H3 and HMG-14. Our data show that MSK1 is an excellent candidate for mediating the nucleosomal response. First, it is a nuclear kinase activated by both ERK and p38 MAP kinase pathways in C3H 10T1/2 cells. Note that MSK1 is not activatable by JNK/SAPKs (Deak *et al.*, 1998), in agreement with the observation that JNK/SAPK activation is insufficient for H3/HMG-14 phosphorylation (discussed above). Secondly, it can efficiently phosphorylate HMG-14 and H3 substrates *in vitro* on physiologically relevant residues; it is much more efficient than RSK1 or 2 (Figure 8) or MNK1 or 2 (data not shown). Thirdly, H3 and HMG-14 phosphorylation in C3H 10T1/2 cells is highly selectively inhibited by H89 and the point of inhibition lies downstream of the MAP kinases; MSK1 satisfies this criterion and is inhibited *in vitro* by H89 at concentrations which inhibit the nucleosomal response *in vivo*. Most importantly, H89 does not significantly inhibit the activity of RSK1 and RSK2. This observation is important as it has been shown previously that RSKs can phosphorylate histone H3 *in vitro* and RSKs have been proposed as a possible mitogen-stimulated H3 kinase *in vivo* (Chen *et al.*, 1992; Mizzen *et al.*, 1998). The demonstration here that RSKs are much less efficient than MSK1 at phosphorylating H3 and HMG-14, that RSKs are insensitive to inhibition with H89 and that RSKs do not lie downstream of p38 argues strongly that the RSKs are unlikely to be the physiologically relevant H3/HMG-14 kinases in these cells, at least in response to stress-related stimuli. Finally, it is worth noting that while it inhibits MSK1 activity *in vitro*, H89 does not inhibit its activation in intact cells; in fact, an increase in TPA or anisomycin-stimulated state of activation of MSK1 is seen upon H89 treatment, alluding to negative feedback loops within these pathways (see Hazzalin *et al.*, 1996). The fact that activation of MSK1 *in vivo* is not inhibited by H89, whereas its kinase activity towards histone H3 and HMG-14 is inhibited by this compound strongly suggests that inhibition of the nucleosomal response in intact cells occurs because H89 acts directly on activated MSK1, blocking its ability to phosphorylate histone H3 and HMG-14.

Targeting of kinase activity within the nucleus

The process of IE gene induction involves interplay between differentially activated MAP kinase cascades, multiple transcription factor phosphorylation events and potentially also the phosphorylation and acetylation of histones (reviewed in Buckle *et al.*, 1995). Part of this process includes the establishment of initiation complexes at the relevant promoters, which requires activation of transcription factors occupying upstream regulatory elements. It is the phosphorylation of these factors that appears crucial to triggering gene induction (reviewed in Karin, 1996; Treisman, 1996); this also affords a model by which distinct enzymatic activities required to execute chromatin modifications may be brought together at IE gene promoters. First, active ERK, JNK/SAPK and p38 MAP kinases must co-locate with DNA-bound transcrip-

tion factors in order to phosphorylate them; there is considerable evidence of transcription factors being able to form stable complexes with MAP kinases (Gupta *et al.*, 1996; Yang *et al.*, 1998). Secondly, MAP kinases are themselves known to bind avidly to some downstream effector kinases; in fact, MNKs, PRAK and MAPKAP kinase 3 have all been cloned as MAP kinase-binding proteins using two-hybrid screens (Waskiewicz *et al.*, 1997; New *et al.*, 1998; Thomson *et al.*, 1999). It is conceivable therefore that MAP kinases may convey downstream effector kinases to specific promoters. This would not apply for MSK1, which is reported to be a nuclear kinase (Deak *et al.*, 1998). In this case, MSK1 may be pre-associated with specific promoters and only require arrival of its upstream kinase for activation. Both these models provide a mechanism for specific IE gene promoter-directed targeting of histone H3 and HMG-14 phosphorylation. Thirdly, these transcription factors can themselves recruit coactivator complexes, which include HATs such as p300/CBP and pCAF, providing a mechanism by which histone acetylation may also be targeted to nucleosomes associated with these promoters. Elk-1 and c-Jun are both capable of functioning in this way and in the case of c-Jun, phosphorylation by JNK/SAPKs is reported to enhance CBP-binding (Bannister *et al.*, 1995; Janknecht and Nordheim, 1996). An implication of this type of model where two distinct histone-modifying activities coalesce around the same promoter is that acetylation and phosphorylation might be targeted to a common subset of H3 molecules; this is in fact exactly what is observed experimentally (Barratt *et al.*, 1994b; data not shown). We are currently using formaldehyde cross-linking and chromatin immunoprecipitation (CHIP) assays with our phospho-specific antibodies in an attempt to investigate the protein and DNA associated with these complexes (A.L.Clayton, S.Rose, M.J.Barrett and L.C.Mahadevan, manuscript in preparation). These studies show clearly that DNA encoding IE genes is co-immunoprecipitable from stimulated cells using antibodies against modified histone H3 and HMG-14, and proves for the first time that the nucleosomal response is targeted to chromatin associated with IE genes (A.L.Clayton, S.Rose, M.J.Barrett and L.C.Mahadevan, manuscript in preparation).

Potential roles for the nucleosomal response

Correlations between the nucleosomal response and IE gene expression under diverse conditions of induction, superinduction and inhibition strongly suggest that the two processes are linked mechanistically. By analogy with transcription factors, the phosphoepitope on histone H3 and HMG-14 may provide binding sites for recruitment of coactivators such as HATs or chromatin remodelling complexes. Alternatively, as suggested for acetylation of histone tails, phosphorylation may mediate a change in nucleosome and chromatin accessibility which aids transcription. IE genes show highly characteristic and reproducible patterns of induction with regard to the precise extent and duration of expression in response to different stimuli (see quantitative data in Hazzalin *et al.*, 1998). In the presence of H89, when histone H3 and HMG-14 phosphorylation is blocked, IE gene induction is both inhibited and its pattern of expression altered, the reduced mRNA accumulation being more prolonged and

observable at later time points. In agreement with the fact that MAP kinase activation and transcription factor phosphorylation are unaffected by H89, this shows that signals continue to arrive at these genes, but that the efficiency of their expression is inhibited. The argument that inhibition of IE gene induction by H89 is causal to the inhibited nucleosomal response is not tenable because the latter remains unaffected in the complete absence of transcription (using DRB or actinomycin D; Mahadevan *et al.*, 1991). Thus, although the nucleosomal response may not be obligate for IE gene induction in the way that transcription factor phosphorylation is, it may participate in quantitatively influencing the rate of expression of these genes. The products of the *fos* and *jun* family of proto-oncogenes homo- and hetero-dimerize to form the AP-1 complex, and the precise amounts of each protein partner must determine the nature of AP-1 complexes that result. In this light, quantitative modulation of IE gene transcript levels by the nucleosomal response may play a role in ultimately influencing the nature of the AP-1 complexes created in response to the diverse stimuli that elicit this response.

Materials and methods

Materials

Peptides used for both antibody production and kinase assays were synthesized by Dr G.Bloomberg, University of Bristol, UK. Microcystin-LR, H89, PD 98059 and SB 203580 were purchased from Alexis Biochemicals (Nottingham, UK). TPA and anisomycin were purchased from Sigma and EGF was kindly provided by Dr G.Panayotou (Ludwig Institute for Cancer Research, London). GST-MSK1, GST-MAPKAP-K1a (RSK1), MAPKAP-K1b (RSK2) and crosstide peptide were kindly provided by Dr D.Alessi (MRC Protein Phosphorylation Unit, Dundee, UK), and GST-MNK1 and GST-MNK2 were kindly provided by Professor C.Proud (University of Dundee, UK). Anti-ERK MAP kinase antibodies were purchased from Zymed Laboratories, anti-phospho-p38 antibody was purchased from New England Biolabs, anti-phospho-CREB antibody was purchased from UBI and anti-c-Jun and anti-ATF2 antibodies were purchased from Santa Cruz Biotechnology.

Antibodies

Antibodies were raised at the Scottish Antibody Production Unit (SAPU), Lanarkshire, UK. Anti-HMG-14 and anti-phospho-H3 antibodies were raised in rabbits whereas anti-phospho-HMG-14 antibodies were raised in sheep. The sequence of the HMG-14 peptide (CNQSPASEEKEAKSD) corresponds to residues 81–96 of the C-terminus of mouse HMG-14 (Bustin and Reeves, 1996). The sequence of the phospho-HMG-14 peptide [RKVS(P)ADGAKVS(P)ADPC, where (P) indicates that the preceding residue is phosphorylated] corresponds to a repeat of residues 3–10 of mouse HMG-14. The sequence of the phospho-H3 peptide [ARKS(P)TGKAPRKQLC] corresponds to residues 7–20 of mouse H3. The peptides were conjugated to keyhole limpet haemocyanin (KLH) and injected into the animals. Antibodies were purified from serum using peptide affinity columns (Pierce).

Cell culture, stimulation and lysis

C3H 10T1/2 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (FCS). Confluent cell cultures were quiesced by incubation in DMEM containing 0.5% (v/v) FCS for 16–24 h. Cells were pre-treated with inhibitors and then stimulated as described. Cells were lysed and harvested in 200 μ l of ice-cold lysis buffer A [10 mM Tris-HCl pH 7.5, 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.5% (v/v) Triton X-100, 20 mM sodium β -glycerophosphate, 100 μ M sodium orthovanadate, 1 μ M microcystin-LR plus protease inhibitor mixes A and B] (Barratt *et al.*, 1994a).

Preparation of nuclear extracts

Lysates were centrifuged at 5000 r.p.m. for 3 min at 4°C in a Heraeus biofuge. The supernatant was discarded and the pelleted nuclei washed

with a further 1 ml of buffer A. In order to extract the HMG proteins the nuclei were resuspended in 150 μ l of 5% perchloric acid (PCA). The suspension was incubated on ice for 1 h and then centrifuged at 10 000 r.p.m. for 5 min. The supernatant was removed, 1 ml of acetone added and placed at –20°C for 2–12 h. The PCA insoluble pellet was resuspended in 150 μ l 0.4 M HCl and incubated on ice for 1 h to extract the histones. The suspension was centrifuged at 13 000 r.p.m. for 5 min, the supernatant removed, 1 ml of acetone added and the sample placed at –20°C for 2–12 h. The acetone precipitated proteins were pelleted by centrifugation at 13 000 r.p.m. for 20 min and the pellet dried under vacuum in a speed vac. The dried pellets were taken up in the appropriate buffer.

Electrophoresis and immunoblotting of protein samples

Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) as described previously (Cano *et al.*, 1995). Membranes were blocked with blocking buffer [5% Marvel in phosphate buffered saline (PBS)] for 1 h and then probed at room temperature with the indicated primary antibody diluted in blocking buffer for 1–2 h. The membranes were washed with four or five changes of wash buffer (PBS, 0.1% Triton X-100) and then incubated with horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer for 1 h. The membranes were then washed with a further four or five changes of wash buffer and proteins detected using the enhanced chemiluminescence reagent (Amersham).

Analysis of protein samples by acid-urea gel electrophoresis was essentially as described previously (Barratt *et al.*, 1994a). After electrophoresis the gels were washed in 5% acetic acid/10% methanol for 30 min and then transferred to PVDF membranes using 0.1% acetic acid/10% methanol as a transfer buffer. The membranes were then probed with antibodies as described above.

Analysis of transcription factor phosphorylation

Cells were treated as indicated and then lysed either in buffer A containing 0.4 M NaCl (ATF2 and c-Jun) or directly in SDS sample buffer (CREB). Extracts were separated on 10% acrylamide gels, transferred to PVDF and probed with the appropriate antibody. Anti-c-Jun, anti-ATF2 and anti-phospho-CREB antibodies were used at a 1:1000 dilution. The anti-c-Jun antibody recognizes residues 1–79 at the N-terminus of c-Jun. The anti-ATF2 antibody recognizes a 19 amino acid C-terminal epitope. The anti-phospho-CREB antibody recognizes CREB phosphorylated on Ser133.

Northern blot analysis of RNA

The expression of genes was analysed by Northern blot analysis essentially as described previously (Hazzalin *et al.*, 1997). The blots were hybridized with a GAPDH probe to ensure equal sample loading.

Kinase assays

ERK/MAP kinase and JNK/SAPK1 kinase assays were carried out as described previously (Cano *et al.*, 1995; Hazzalin *et al.*, 1997). p38 MAP kinase activation was assayed using a phospho-specific p38 antibody (New England Biolabs).

Peptide kinase assays were carried out essentially as described in Alessi *et al.* (1995). Briefly, 50 μ l kinase assays contained 50 mM Tris-HCl pH 7.5, 0.03% (v/v) Brij-35, 0.1 mM EGTA, 0.1% (v/v) β -mercaptoethanol, 10 μ M PKI, 1 μ M microcystin-LR, 10 mM MgCl₂, 20 μ M ATP, 10 μ Ci [γ -³²P]ATP (3000 Ci/mM) and 30 μ M peptide substrate. Assays were incubated at 30°C for 15 min and reactions stopped by pipetting 40 μ l on to a square of phosphocellulose paper. The papers were washed five times in 0.1% phosphoric acid, once in acetone and then left to dry. Incorporated counts were determined by scintillation counting. Alternatively, reactions were stopped by addition of HCl to 0.4 M and the samples acetone precipitated. The precipitated samples were pelleted by centrifugation and taken up in SDS sample buffer and loaded on to 15% SDS gels. The gels were Coomassie Blue stained, dried down and exposed to Fuji X-ray film. Assays of histone phosphorylation were carried out as above, except that for immunoblot analysis of phosphorylated substrates the radioactive ATP was omitted.

Kinase activities against the crosstide peptide were determined and these values used to normalize kinase activity and correct the obtained values for counts incorporated into the HMG-14 and H3 peptides (Figure 8A and C). Alternatively, equal aliquots of crosstide kinase activity were used in each assay (Figure 8D and E). The peptides used in the assays were as follows: crosstide (GRPRTSSFAEG); HMG-14 (PKRKVSAEG-AARQIKIWFQNRMRKWKK), residues 1–11 of human HMG-14 (Bustin and Reeves, 1996) conjugated to residues 43–58 of the antenna-

pedia protein (Derossi *et al.*, 1994). The Ala peptide was identical except for a Ser to Ala substitution at position 6. H3 (ARTKQTARKSTGGKAPRKQL): residues 1–20 of mouse histone H3.

Assay of MSK1 kinase activity in cells

Cells were treated as indicated and lysed in buffer B [50 mM Tris–HCl pH 7.5, 0.1% Triton X-100 (v/v), 1 mM EDTA, 1 mM EGTA, 20 mM sodium β -glycerophosphate, 100 μ M sodium orthovanadate, 1 μ M microcystin-LR, 0.1% β -mercaptoethanol plus protease inhibitor mixes A and B]. The lysate was cleared by centrifugation at 13 000 r.p.m. for 5 min in a Heraeus biofuge and the protein concentration of the supernatant determined by Bradford assay (Bradford, 1976). Cell extracts (500 μ g) were incubated with 3.5 μ g of MSK1 antibody coupled to 10 μ l of protein G–sepharose for 1 h at 4°C. The protein G beads were washed three times with 1 ml buffer B containing 0.5 M NaCl and twice with buffer C [50 mM Tris–HCl pH 7.5, 0.03% (v/v) Brij-35, 0.1 mM EGTA, 0.1% β -mercaptoethanol]. The beads were then resuspended in buffer C containing 10 mM MgCl₂, 10 μ M PKI, 1 μ M microcystin-LR, 10 μ g HMG-14 peptide. Reactions were started by adding 20 μ M ATP and 10 μ Ci [γ -³²P]ATP (3000 Ci/mM) and incubating at 30°C for 30 min. Assays were stopped and analysed by running the peptides on SDS–PAGE and quantifying the incorporated counts by phosphorimager analysis.

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Two recent papers published after this paper was accepted impinge on issues discussed here. Sassone-Corsi *et al.* (*Science*, 1999, vol. **285**, 886–891) suggest that Rsk2 is required directly or indirectly for histone H3 phosphorylation in response to EGF, serum or UV radiation. Ye Jin *et al.* (*Mol. Cell*, 1999, vol. **4**, 129–135) report the cloning of JIL-1, a *Drosophila* chromatin- and chromosome-associated kinase most closely homologous to human MSK-1 described here, which is also capable of phosphorylating histone H3.