The Kinases MSK1 and MSK2 Are Required for Epidermal Growth Factor-induced, but Not Tumor Necrosis Factor-induced, Histone H3 Ser10 Phosphorylation*

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Phosphorylation of histone H3 protein at serine 10 is an important step in chromatin remodeling during transcriptional activation. IkB kinase-α (IKK-α) and Mitogen- and Stress-activated protein kinases 1 and 2 (MSK1/2) have been shown to play key roles in the transcriptional regulation of immediate early genes such as c-fos. Interestingly, IKK-α and MSK1/2 have also been implicated as histone H3-Ser10 kinases. In this work, we have shown that MSK1/2 are required for epidermal growth factor (EGF)-induced, but not tumor necrosis factor-induced, histone H3-Ser10 phosphorylation, both globally and at specific promoters. Consistent with this, MSK1/2 are required for optimal immediate early c-fos transcription in response to EGF potentially through control of both H3-Ser10 and promoter-associated cAMP-response element-binding protein phosphorylation. Furthermore, MSK1/2 control EGF-induced IkBa promoter H3-Ser10 phosphorylation in the absence of elevated transcription. These studies demonstrate the existence of pathway-specific mechanisms to control histone H3-Ser10 phosphorylation and gene expression.

The remodeling of nucleosomes is now accepted as an early, critical step in the inducible activation of transcription of certain genes. A number of posttranslational modifications of histone proteins H3 and H4 have been associated with transcriptional activity of gene promoters. These include methylation of arginine residues, acetylation of lysine residues, and phosphorylation of serine residues (1). Immediate early genes are characterized by rapid induction of transcription in response to a variety of stimuli including mitogens, stress, and cytokines (2–4). For example, nucleosomes associated with the c-fos promoter undergo a well characterized series of modifications in response to mitogenic stimuli (5). Similarly, certain cytokine-induced genes undergo similar chromatin modifications associated with inducible gene expression. Specifically, after mitogen or cytokine exposure of cells, these genes undergo rapid histone H3 phosphorylation at serine 10 and acetylation at lysine 9 (1, 5–7). The regulatory proteins and kinases involved in this rapid, induced nucleosomal modification have been a major focus in this field of study.

Mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2) are nuclear kinases that act downstream of mitogen-activated protein/extracellular signal-regulated kinase pathways (8, 9). Recently, these kinases have been shown to be required for stress-induced phosphorylation of histone H3-Ser10 and transcriptional activation of several immediate early genes (10). In those studies it was demonstrated that MSK1/2 possess in vitro H3-Ser10 kinase activity. Utilizing either targeted gene disruption or pharmacologic inhibitors, other phosphorylation events involved in the control of target gene expression have been found to depend on MSK1/2, including phosphorylation of CREB and the NF-κB subunit p65 (9, 11).

IKK-α is a kinase component of the IKK complex (12), originally discovered for its ability to phosphorylate the cytoplasmic inhibitor (IκB) of NF-κB in response to a variety of extracellular signals, including hormones and cytokines (12). This phosphorylation event directs ubiquitination and destruction of IκB, freeing NF-κB to translocate to the nucleus and activate transcription of target genes. Recently, we and others have shown that IKK-α also translocates to the nucleus and is needed for optimal NF-κB-mediated transcription and histone H3-Ser10 phosphorylation of NF-κB target genes in response to cell stimulation with the inflammatory cytokine tumor necrosis factor (TNF) (13, 14). Additional studies have revealed that NF-κB and IKK-α are required for immediate early gene promoter-associated histone H3-Ser10 phosphorylation and an optimal transcriptional response of certain immediate early genes in response to the mitogenic growth factor EGF (15).

We have provided data that indicate that MSK1/2 are important for EGF-induced phosphorylation of histone H3-Ser10 at immediate early response gene promoters and globally on bulk histones. However, MSK1/2 are not involved with the control of cytokine-induced histone H3-Ser10 phosphorylation. Furthermore, evidence has been presented for additional roles of MSK1/2 in regulating immediate early gene expression. Finally, we provided evidence that MSK1/2 control EGF-induced histone H3-Ser10 phosphorylation on the IkBa promoter in the absence of measurable gene induction.

EXPERIMENTAL PROCEDURES

Cells and Reagents—MSK wild-type and MSK1/2 double knock-out mouse embryo fibroblasts (MEFs) were provided by J. S. Arthur (Dundee, Scotland). An antibody against phospho-histone H3 (Ser10) was obtained from Upstate Biotechnology Inc. An antibody to RNA polymerase II was obtained from Covance. An antibody to actin was purchased from Cell Signaling Technology Inc. Antibodies to IkBα and NF-κB were purchased from Cell Signaling Technology. The monoclonal antibody to IgG was from Chemicon. An antibody to the c-fos promoter was purchased from Upstate Biotechnology Inc. Antibodies to IkBα were from Cell Signaling Technology. An antibody to IkB was from Santa Cruz Biotechnology Inc. An antibody to the c-fos promoter was purchased from Upstate Biotechnology Inc. Antibodies to the IkBa promoter were from Cell Signaling Technology. An antibody to the c-fos promoter was purchased from Upstate Biotechnology Inc. Antibodies to the IkBa promoter were from Cell Signaling Technology. An antibody to the c-fos promoter was purchased from Upstate Biotechnology Inc. Antibodies to the IkBa promoter were from Cell Signaling Technology. An antibody to the c-fos promoter was purchased from Upstate Biotechnology Inc. Antibodies to the IkBa promoter were from Cell Signaling Technology.

5 The abbreviations used are: MSK, mitogen- and stress-activated protein kinase; H3-Ser10, histone H3 serine 10; IkB, IkB kinase; EGF, epidermal growth factor; TNF, tumor necrosis factor; MEF, mouse embryo fibroblast; ChIP, chromatin immunoprecipitation; CREB, cyclic AMP response element-binding protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethy]lglycine.

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obtained from Santa Cruz Biotechnology. Antibodies to CREB and phospho-CREB (Ser133) were obtained from Cell Signaling. EGF (Upstate Biotechnology Inc.) was used at a final concentration of 50 ng/ml. TNF-α (Kamiya) was used at a final concentration of 10 ng/ml.

**Chromatin Immunoprecipitation Assay—**ChIP analysis was performed following a protocol provided by Upstate Biotechnology, Inc. under modified conditions as previously described (13). The following promoter-specific primer pairs were used: primer pair 5′-AGAGATGAGCCTCCTATAGTA-3′ and 5′-TGCCCATTTCCATTTATAATGTC-3′ were used to amplify the proximal region of the c-fos promoter; primer pair 5′-AGGGATTTCCAGCCACACTA-3′ and 5′-CTGGGATTTCTTCTCAG-3′ were used to amplify the proximal region of the IlkBa promoter. Amplified products were run on 2% agarose gels and visualized after staining with ethidium bromide. Quantitative real-time PCR was performed to determine the association of phospho-histone H3-Ser10, RNA polymerase II, and phospho-CREB with the c-fos and IlkBa promoters. The aforementioned oligonucleotide primers and SYBR Green Master Mix were utilized in conjunction with an ABI PRISM 7000 Sequence Detection System. Standard curves were generated by using dilutions of input DNA. Reactions to quantitate each DNA input, no antibody immunoprecipitation negative control, and experimental samples were performed in triplicate for each independent immunoprecipitation. Independent immunoprecipitations were performed four to six times. Cell lines were serum starved, and at least three independent stimulations with EGF or TNF-α were performed; representative experiments are presented in the figures.

**Quantitative Real-time PCR—**Total RNA was prepared from MSK wild-type and MSK1/2 double knock-out MEFs using TRizol (Invitrogen) as recommended by the manufacturer. For real-time PCR, the cDNA was prepared with random primer (Invitrogen) and analyzed with triplicate real-time PCR reactions. The TaqMan 2× Universal PCR Master Mix No AmpErase UNG (Roche Applied Science) was used with the following conditions: 15 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 30 s and 63 °C for 30 s. Applied Biosystems Taqman Assay-On-Demand primer-probe sets were used for c-fos and β-glucuronidase. The oligonucleotide primers used to amplify IlkBa transcripts were 5′-AGGATGAGGCTCCTATAGTA-3′ and 5′-TGCCCATTTCCACCATTAATGTC-3′. The oligonucleotide probe for detection of IlkBa amplification products was 5′-6-FAM-TGTGTTTGGAGGCGCA-36-TAMRA-Sp3′. Reactions were performed on an ABI PRISM 7000 Sequence Detection System. Quantification of transcripts relative to β-glucuronidase transcripts was determined using the ΔΔCt method.

**Western Blot Analysis—**Western blot analysis was performed after preparing whole cell extracts and separating proteins (50 μg/lane) by NuPAGE Novex 4–12% Bis Tris gels (Invitrogen) followed by immunoblotting with antibodies as indicated. Extractions of acid-soluble proteins were done according to the protocol described by Upstate Biotechnology, Inc. and resolved on 10–20% Tris-Tricine SDS-PAGE gels.

**RESULTS**

**MSK1 and MSK2 Are Required for Optimal EGF-mediated c-fos Transcriptional Activation—**Previously it was shown that MSK1/2 null cells have diminished stress-induced c-fos transcription as compared with wild-type cells (10). We sought to determine whether MSK1 and MSK2 were similarly required for transcriptional activation of c-fos in response to EGF. Utilizing a quantitative reverse transcription PCR approach, transcription of c-fos in response to EGF was measured in MSK1/2 null MEFs and their wild-type equivalents (Fig. 1A). The data indicate that MSK1 and MSK2 are important for the efficient induction of c-fos gene expression in response to mitogenic treatment. Because IlkBa gene expression had not been reported to be induced significantly by growth factors, we analyzed expression of this gene in response to EGF treatment. EGF stimulation resulted in little change in IlkBa mRNA levels in wild-type MEFs, and IlkBa mRNA levels were slightly higher in MSK1/2 null MEFs regardless of EGF stimulation status (Fig. 1B). Because changes in gene-specific mRNA levels are regulated by several mechanisms, including transcriptional activation and mRNA degradation, a chromatin immunoprecipitation assay was used to assess the level of RNA polymerase II associated with the c-fos promoter in response to EGF stimulation. The results demonstrate that MSK1/2 null MEFs have diminished levels of RNA polymerase II associated with the c-fos promoter over the time course of the experiment as compared with wild-type cells (Fig. 1C). Thus, MSK is required for maximal c-fos mRNA accumulation in response to EGF treatment, and this regulation occurs at the transcriptional level.

**FIGURE 1.** MSK1 and MSK2 are required for optimal EGF-mediated transcriptional activation. Real-time PCR analysis was performed to quantify the endogenous levels of c-fos (A) and IlkBa (B) mRNA. MSK wild-type MEFs (open bars) and MSK1/2 double knock-out MEFs (closed bars) were serum starved for 48 h, treated with EGF (50 ng/ml) at the indicated time points, and then harvested. The values are reported as fold increase (above wild type at zero time) in c-fos or IlkBa mRNA relative to β-glucuronidase mRNA. C, ChIP assay was performed with anti-RNA polymerase II on serum-starved MSK wild-type (open bars) and MSK1/2 double knock-out (closed bars) MEFs treated with EGF (50 ng/ml) at the indicated time points. The quantity of RNA polymerase II-associated promoter DNA for c-fos was assessed by quantitative real-time PCR. The data are presented as percentages of total input chromatin. Error bars represent the S.E. for reactions performed in triplicate. Results are representative of three separate experiments.
MSK1 and MSK2 Are Required for Optimal TNF-induced c-fos and IkBa Transcriptional Activation—To understand potential roles of MSK1/2 in controlling cytokine-induced transcriptional responses, TNF-stimulated c-fos and IkBa mRNA accumulation was assessed by quantitative reverse transcription PCR. TNF induced a reproducible, modest increase in c-fos mRNA levels, with a peak at the 20-min time point, and MSK1/2 null cells were found to have a significantly blunted response of the induction of this gene in response to TNF when compared with wild-type MEFs (Fig. 2A). As expected, TNF strongly induced IkBa gene expression, and this induction was suppressed ~50% in the MSK1/2 null cells (Fig. 2B). Thus, MSK1 and MSK2 play an important, but not absolute, role in the transcriptional response of certain genes induced in response to TNF exposure.

MSK1 and MSK2 Are Required for EGF-induced, Promoter-specific Histone H3 Ser10 Phosphorylation—Previously, we and others (13–15) have shown that IKK-α is recruited to the IkBa and c-fos promoters in response to TNF-α or EGF and that IKK-α is important for efficient levels of H3-Ser10 phosphorylation and optimal stimulation of transcription from those promoters. MSK1 and MSK2 have also been shown to be required for maximally induced c-fos transcription and H3 Ser10 phosphorylation in response to anisomycin-induced cell stress (10). We sought to determine whether MSK1 and MSK2 were also important in both EGF- and TNF-induced, promoter-specific phosphorylation of histone H3 Ser10. First, induction of IkBa and c-fos promoter-associated phospho-histone H3 Ser10 was measured by chromatin immunoprecipitation assays after stimulation with EGF. The results demonstrate that MSK1/2 null MEFs have minimal induction of histone H3 Ser10 in response to EGF at the c-fos promoter as compared with wild-type cells (Figs. 3, A and B). Interestingly, despite no significant transcriptional response of the IkBa gene to EGF in either cell line (see Fig. 1B), EGF stimulation strongly induced histone H3 Ser10 phosphorylation at the IkBa promoter that was abolished in the MSK null cell line (Fig. 3, C and D). These results indicate that histone H3 Ser10 phosphorylation is not sufficient for the induction of gene expression and that this phosphorylation response can be uncoupled from transcription. Additionally, these data indicate that MSK1/2 are essential for growth factor-induced histone H3 Ser10 phosphorylation, at least as measured at two inducible promoters.

Interestingly, there was no appreciable difference in the TNF-induced phosphorylation of serine 10 of histone H3 at the c-fos (Fig. 4, A and B) and IkBa promoters (Fig. 4, C and D) in MSK1/2 null cells as compared with wild-type cells. These results demonstrate that MSK1/2 are not involved with the regulation of TNF-induced histone H3 Ser10 phosphorylation at certain known TNF-responsive promoters. The role of MSK1/2 in contributing to TNF-induced c-fos and IkBa gene expression may be explained through its proposed role in controlling p65 phosphorylation (11).
MSK1/2 in EGF-induced Histone H3 Ser\textsuperscript{10} Phosphorylation

**FIGURE 4.** MSK1 and MSK2 are not important in TNF-mediated histone H3-serine 10 phosphorylation at either c-fos or lck56 promoters. Chromatin was prepared from MSK wild-type (open bars) and MSK1/2 double knock-out (closed bars) MEFs that were serum starved for 48 h and treated with TNF (10 ng/ml) at the indicated time points. ChIP assays were performed using anti-phospho-Ser10-H3 as indicated. c-fos or lck56 promoter DNA sequences were detected by quantitative real-time PCR. The data are presented as percentages of total input. Error bars represent the S.E. for reactions performed in triplicate. Results shown are representative of two independent experiments.

**FIGURE 5.** MSK1/2 is required for global levels of EGF-induced, but not TNF-induced, H3-serine 10 phosphorylation. Acid-soluble proteins were extracted from MSK wild-type and MSK1/2 double knock-out MEFs that were serum starved for 48 h, treated with TNF (10 ng/ml) (top panel) or EGF (50 ng/ml) (bottom panel) at the indicated time points, and immunoblotted with an anti-phospho-Ser\textsuperscript{10} antibody. Coomassie staining of acid-extracted histones was used to visualize equal loading.

MSK1/2 Are Required for Global Levels of Phosphorylated Histone H3-Ser\textsuperscript{10} in Response to EGF but Not TNF—To address global aspects of EGF- and TNF-induced phosphorylation of histone H3-Ser\textsuperscript{10}, we analyzed total histone preparations for levels of this specific modification. Histones were extracted, electrophoresed on SDS-polyacrylamide gels, and probed with histone H3-Ser\textsuperscript{10} phospho-specific antibody. The results demonstrate that global levels of histone H3-Ser\textsuperscript{10} phosphorylation are strongly reduced in response to EGF, but not to TNF, in the MSK1/2 null cells as compared with the wild-type cells (Fig. 5). Thus, these data demonstrate that MSK1 and MSK2 play a vital role in EGF-mediated histone H3-Ser\textsuperscript{10} phosphorylation. They also demonstrate that kinases other than MSK1/2 play a role in histone H3 phosphorylation in response to cytokine stimulation.

EGF-induced Accumulation of Promoter-associated Phospho-CREB Requires MSK1 and MSK2 and Correlates with Transcriptional Activation of EGF-responsive Genes—Although histone H3-Ser\textsuperscript{10} phosphorylation is an important step in chromatin remodeling and inducible tran-
scription, we sought to determine whether other regulatory events controlled in an MSK-dependent fashion might relate to the EGF-induced transcription of the c-fos gene. The cyclic AMP response element-binding protein (CREB) is a known transactivator of c-fos gene transcription and also is a substrate of MSK1 and MSK2 (8, 9, 16). After stimulation with EGF, immunoblot analysis of whole cell extracts reveals that the MSK1/2 null MEFs have dramatically lower levels of phospho-CREB when compared with wild-type counterparts (Fig. 6A), as expected. Importantly this lack of phosphorylated CREB is reflected at the c-fos promoter where chromatin immunoprecipitation assay demonstrates induction of c-fos promoter-associated phospho-CREB was reduced in MSK1/2 null MEFs (Fig. 6, B and C). Phospho-CREB was not recruited to the IkBα promoter in either wild-type or MSK1/2 null cells after EGF stimulation (Fig. 6D), suggesting that the absence of phosphorylated CREB and perhaps other MSK-phosphorylated transcription factors at the c-fos promoter may play a significant role in the control of c-fos gene expression. Consistent with reports of others (17, 18), CREB was found constitutively associated with its regulatory element. Thus the loss of histone H3-Ser10 phosphorylation seen in the MSK1/2 null cells at the c-fos promoter may account only partially for the loss of the gene expression response (Fig. 1A). Furthermore, the strong induction of H3-Ser10 phosphorylation observed at the IkBα promoter in response to EGF treatment without significant transcriptional activation is likely to reflect the broad control of MSK1/2 in regulating EGF-induced H3-Ser10 phosphorylation and also the lack of recruitment of a sequence-specific EGF- and/or MSK1/2-regulated transcription factor to this promoter.

DISCUSSION

Our current studies have revealed three interesting results regarding the role of MSK1 and MSK2 in transcriptional regulation and nucleosomal remodeling in response to extracellular stimuli. First, as had been previously noted for stress signaling pathways (10), MSK1 and MSK2 are also required for in vivo histone H3 phosphorylation at serine 10 in response to EGF. These conclusions are based on both promoter-specific and global experiments. Second, optimal TNF-mediated transcriptional activation of IkBα also requires MSK1 and MSK2, although these kinases do not play a role in histone H3 phosphorylation at the promoters of certain TNF-responsive genes. Finally, our data indicate that MSK1/2 induce H3-Ser10 phosphorylation at the IkBα promoter in response to EGF stimulation in the absence of increased IkBα gene expression.

Phosphorylation of histone H3-Ser10 is a known early step in chromatin remodeling and has been shown to be important during chromosomal replication (1). More recently, this modification has been found to be associated with rapidly inducible promoters. Both IKK-α and MSK1/2 have been implicated as kinases responsible for targeted H3 phosphorylation preceding transcriptional activity (10, 13–15). Our current data support a role for MSK1/2 in EGF-induced H3-serine 10 phosphorylation, but not in TNF induction of this modification. Our current studies are unable to establish whether MSK1 and MSK2 act directly as H3 kinases after EGF stimulation or whether these kinases simply lie in the kinase pathway that leads to this modification. Similarly, it is unclear what the role of IKK-α is in the EGF-induced pathway to control EGF-induced H3-Ser10 phosphorylation. One possibility is that IKK-α could regulate MSK1/2 downstream of EGF-induced, but not TNF-induced, signaling. Surprisingly, our current studies reveal that MSK-dependent histone H3-Ser10 phosphorylation occurs on both transcriptionally active and inactive promoters in response to EGF. This suggests that promoter-specific H3-Ser10 phosphorylation is not sufficient to induce transcription of the target gene. Thus, the data are consistent with the involvement of MSK1/2 in controlling inducible H3-Ser10 phosphorylation on certain available promoters. The distinction between a role for MSK1/2 in c-fos and IkBα gene expression is likely to relate to the MSK-dependent phosphorylation of CREB that contributes to the transcriptional transactivation of c-fos gene in response to EGF. Presumably the IkBα gene lacks a target sequence for an EGF-induced, MSK1/2-regulated factor, and consequently the gene is not activated. Although MSK1/2 have been implicated in controlling phosphorylation of the RelA/p65 subunit on Ser276 in response to cytokine treatment (11), it is possible that either EGF does not induce this response or this phosphorylation is not sufficient to induce IkBα transcription, explaining the uncoupling of H3-Ser10 phosphorylation from transcriptional activation. Additionally, the observed role for MSK1/2 downstream of TNF in contributing to c-fos and IkBα gene expression without affecting H3-Ser10 phosphorylation may relate to the reported ability of MSK1/2 to phosphorylate the RelA/p65 subunit, a factor known to regulate both c-fos and IkBα gene expression (12, 15).

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