Serine/Thrreonine Kinases 3pK and MAPK-activated Protein Kinase 2 Interact with the Basic Helix-Loop-Helix Transcription Factor E47 and Repress Its Transcriptional Activity*

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In the search for physiological substrates of MAPK-activated protein (MAPKAP) kinases, we identified the basic helix-loop-helix (bHLH) transcription factor E47 as an interaction partner of chromosome 3p kinase (3pK) and MAPKAP-K2 (MK2). The E2A protein E47 is known to be involved in the regulation of tissue-specific gene expression and cell differentiation. E47 is a phosphoprotein, and we identified 3pK and MK2 as E47 kinases in vitro. Furthermore, the expression of either kinase results in a repression of the transcriptional activity of E47 on an E-box containing promoter. In summary, the MAPK-activated protein kinases 3pK and MK2 were identified to form an assembly with the bHLH protein E47 suggesting that these kinases are regulators of E47 activity and E47-dependent gene expression.

Chromosome 3p kinase (3pK) and MK2 belong to a family of serine/threonine kinases that are activated by members of the mitogen-activated protein kinase (MAPK) family. 3pK, also known as MAPKAP kinase 3 (1) is unique in that it was shown to be activated by mitogenic inducers such as serum/TPA through the Raf/MEK/ERK cascade as well as by stress-inducing agents that are activators of stress-induced MAPK cascades, thereby leading to the phosphorylation of 3pK by either JNK/SAPK or p38 (2). Thus, the kinase is targeted by the corresponding cascade depending on the extracellular stimulus (2). Although the upstream activation pathways of 3pK are well documented, little is known about its downstream effectors.

A close homologue of 3pK, MK2, is involved in stress response mediated through p38. Among the substrates of MK2 are the small heat shock proteins (Hsp27/25) (3, 4) and transcription factors such as CREB (5) and SRF (6).

The basic helix-loop-helix (bHLH) transcription factors E12 and E47 are encoded by the E2A gene and are generated by differential splicing of E12- and E47-specific bHLH-encoding exons (7, 8). These E-proteins are characterized by their ability to bind to the consensus DNA sequence CANNTG, referred to as an E-box, either as homodimers in B-cells or as heterodimers with tissue-specific Class II HLH proteins in other cell types (9, 10). Initially identified in B-cells as immunoglobulin enhancer-binding proteins, the E2A gene products classified as Class I HLH proteins are also involved in cell differentiation, lineage commitment, and the expression of many tissue-specific genes (11). Through phosphorylation, DNA binding and transactivation of a variety of transcription factors are regulated (12). For example, the dimerization of myogenin with E2A products was shown to enhance phosphorylation of myogenin, thereby reducing the transcriptional activity of myogenin, suggesting that phosphorylation of this myocyte enhancer factor (MEF) negatively interferes with muscle-specific gene expression (13). The DNA-binding activity of Myod homodimers but not Myod-E12 heterodimers was inhibited by phosphorylation (14). Johnson et al. (16) demonstrated that overexpression of casein kinase II (CKII) increased the transcriptional activities of MRF4 and Myod in vivo via a mechanism involving phosphorylation of its binding partner, E47. A direct effect of E47 phosphorylation was reported by Sloan et al. (17), providing evidence that phosphorylation hinders binding of E47 homodimers but allows E47 heterodimer binding. This suggests a differential regulation of the E-protein in B-cell versus non-B-cell-specific gene regulation.

E47 contains more than 100 potential phosphorylation sites and is known to be phosphorylated in many cell types. However, there is only limited information with regard to E47 kinases. So far, CKII and PKA have been described to phosphorylate E47 in vitro (16–18).

In the present study, we show that 3pK and MK2 interact with E47 in vitro and are able to phosphorylate E47 in vitro. Furthermore, the expression of 3pK or MK2 results in repression of E47-induced transcriptional activity, suggesting a role for these kinases in the regulation of this transcription factor.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Plasmids expressing 3pK wild type, the ATP-binding site mutant 3pK K73M (3pK KM), and the constitutively active gene and are generated by differential splicing of E12- and E47-specific bHLH-encoding exons (7, 8). These E-proteins are characterized by their ability to bind to the consensus DNA sequence CANNTG, referred to as an E-box, either as homodimers in B-cells or as heterodimers with tissue-specific Class II HLH proteins in other cell types (9, 10). Initially identified in B-cells as immunoglobulin enhancer-binding proteins, the E2A gene products classified as Class I HLH proteins are also involved in cell differentiation, lineage commitment, and the expression of many tissue-specific genes (11). Through phosphorylation, DNA binding and transactivation of a variety of transcription factors are regulated (12). For example, the dimerization of myogenin with E2A products was shown to enhance phosphorylation of myogenin, thereby reducing the transcriptional activity of myogenin, suggesting that phosphorylation of this myocyte enhancer factor (MEF) negatively interferes with muscle-specific gene expression (13). The DNA-binding activity of Myod homodimers but not Myod-E12 heterodimers was inhibited by phosphorylation (14). Johnson et al. (16) demonstrated that overexpression of casein kinase II (CKII) increased the transcriptional activities of MRF4 and Myod in vivo via a mechanism involving phosphorylation of its binding partner, E47. A direct effect of E47 phosphorylation was reported by Sloan et al. (17), providing evidence that phosphorylation hinders binding of E47 homodimers but allows E47 heterodimer binding. This suggests a differential regulation of the E-protein in B-cell versus non-B-cell-specific gene regulation.

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mutant 3pK TT202/313EE (3pK TTEE) in bacteria and eukaryotic cells as a GST fusion protein were cloned as described recently (2). A GFP-tagged version of 3pK was cloned in the pRSPA vector background. A GFP-tagged version of MK2 was kindly provided by M. Gieste (Halle, Germany). pcDNA3HA-3pK was generated by inserting the NbCI-SpeI fragment of pC67-3pK into pcDNA3HA. The BamHI insert of pGEX KG-3pK was ligated in the yeast two-hybrid vector pAS2.1 (CLONTECH) to produce pAS2.1-3pK KM. pGAD10-E47 (amino acids 1–494) was constructed by ligating the 5’ 1492-base pair NcoI fragment of pGAD10-E47 into pGAD10. The 3’ 912-base pair XhoI fragment was inserted in pGAD10 to create pGAD10-E47 (amino acids 372–651). For pGAD10-E47 (amino acids 18–229), the original pGAD10-E47 was cut with SacI and religated. 5’-ccggctctgggtccgctgccgctgccgctgccgctgccgctgcctgg-3’ and 5’-tgccagcggctctgggtccgctgccgctgccgctgccgctgcctgg-3’ primers (E-boxes are underlined) containing four E-box sequences were annealed and cloned into the MluI and XhoI sites of the pGL3 promoter vector (Promega).

**Yeast Two-hybrid System**—The yeast two-hybrid screen was performed using a human heart MAPKAP kinase cDNA library cloned into pGAD10 (CLONTECH). Yeast strain Y190 was manipulated according the MATCHMAKER Library User Manual (CLONTECH). A sequential transformation protocol was used with pAS2.1-3pK KM as bait. Positive clones were identified by growth on SD-Trp−/−Leu−/−His−/− vector with 10% (v/v) fetal calf serum (FCS) at 37 °C in humidified air with 6% CO2. Production of rabbit anti-glutathione S-transferase (GST) antiserum was described earlier (2) and used in the yeast two-hybrid assay. The kinase buffer (30 min) was preincubated with 1 mM Pefabloc, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 20 m M sodium glycolate, 20 m M sodium pyrophosphate, 137 m M NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, and 25 m M magnesium acetate, pH 7.8, 10 mM dithiothreitil, and 2% Triton X-100. 50 μl of precleared cell extracts were added to 50 μl of luciferase assay buffer (25 mM MgCl2, 25 mM β-glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamidine, 0.5 mM dithiotreitol, 1 mM sodium vanadate). Immuno-precipitated versions of tagged HA-3pK, HA-MK2, or E47 were combined and washed twice in RIPA buffer supplemented with 0.5 mM NaCl before being subjected to the kinase buffer. The kinase assays (30 min) were performed at 30 °C with 5 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) and 0.1 mM ATP. Luciferase assays were performed 4 h after transfection, COS7 cells were harvested in 100 μl of lysis buffer (50 mM Na-MES, pH 7.8, 50 mM Tris-HCl, pH 7.8, 10 mM dithiothreitol, and 2% Triton X-100). 50 μl of precleared cell extracts were added to 50 μl of luciferase assay buffer (125 mM Na-MES, pH 7.8, 125 mM Tris-HCl, pH 7.8, 25 mM magnesium acetate, and 2 mg/ml ATP). Immediately after the injection of 50 μl of 1 mM D-luciferin (Applichem), luminescence was measured for 5 s in a lumimeter (Berthold). The luciferase activities were normalized on the β-galactosidase activity of cotransfected β-Gal vector. The β-galactosidase assay was performed with 20 μl of precleared cell lysate according to a standard protocol (19). Mean values and standard deviations from three independent experiments are shown.

**RESULTS AND DISCUSSION**

**3pK Binds to E47 in Vivo in Yeast and Mammalian Cells**—To unravel the unknown physiological function of 3pK, we searched for binding partners in a yeast two-hybrid screen. The only in vitro and in vivo substrate for 3pK identified to date is the small heat shock protein Hsp27. Hsp27 phosphorylation may regulate actin filament dynamics, thought to be mediated by 3pK and MK2 (29). Because both kinases are found in the nucleus of cells (Fig. 2B and Ref. 22), 3pK and MK2 may also have nuclear targets. Indeed, MK2 was shown to phosphorylate the nuclear transcription factors CREB as well as SRF (5, 6) and to modulate their transcriptional activity. 3pK is also able to phosphorylate CREB in vitro and to interact with several nuclear proteins in vivo.

We used a human heart cDNA library and a kinase inactive version of 3pK as a bait, because wild-type 3pK autonomously led to the transactivation of the reporter genes. It has recently been demonstrated that substitution of a lysine residue by methionine in the putative ATP-binding site of a kinase allows a more stable binding with its substrates, thus leading to a stronger transcriptional activation of the two-hybrid reporter gene (20). In the two-hybrid screen, we found the full-length human basic helix-loop-helix transcription factor E47 to be a 3pK interaction partner (Fig. 1). To narrow down the interacting domain of E47 that binds 3pK, we cloned several deletion mutants of E47 for direct two-hybrid tests (Fig. 1).

Interestingly, deletion of 157 amino acids of the extreme C terminus including the acidic and the basic helix-loop-helix domain abolished the interaction, suggesting that this region is necessary for the binding of E47 to 3pK. The N-terminal deletion of amino acids 18–229 containing the first activation domain did not weaken the interaction, whereas a further N-terminal truncation of E47 up to amino acid 371 eliminated...
binding. This finding indicates that in addition to the loop-helix transactivation domain and the acidic domain located in the C-terminal half, an N-terminal region is also necessary for binding to 3pK.

To confirm independently the 3pK-E47 two-hybrid interaction, we performed coimmunoprecipitation experiments as well as colocalization studies. Transfected E47 specifically coprecipitates with HA-3pK (Fig. 2A, lane 4), whereas no coprecipitated proteins were detectable in the control samples (Fig. 2A, lanes 2 and 3).

Fig. 2B shows that GST- and GFP-tagged versions of 3pK and MK2 are located in the nucleus of transfected COS7 cells and GFP-tagged kinases colocalize with E47. It is noteworthy that neither GST nor GFP fusion affects kinase activity of 3pK (Ref. 2 and data not shown). These experiments indicate that 3pK and E47 interact in vivo in yeast as well as in mammalian cells.

3pK and MK2 Interact with E47 under High Stringency Conditions—We further analyzed whether MK2 also interacts with E47. Recombinant GST-tagged 3pK or GST alone was bound to glutathione-Sepharose and added to precleared HEK293 lysates containing either HA-3pK, HA-MK2, or no kinase. After the pull-down, the beads were washed twice either under moderate (high salt TLB) or high stringency conditions (RIPA) and then subjected to SDS-PAGE. Under both conditions, 3pK and MK2 were pulled down with GST-E47 (Fig. 3, lanes 2 and 6) but not when GST was bound to the Sepharose (Fig. 3, lanes 3 and 7) or when lysates of untransfected cells were used (Fig. 3, lanes 4). Thus, 3pK and MK2 overlap in their ability to bind to E47 even under highly stringent conditions, demonstrating a high binding affinity of the kinases to E47.

3pK and MK2 Phosphorylate E47 in Vitro—E47 is phosphorylated in vivo at several different sites in many cell types (17, 18); however, there is only limited information about the identity of these E47 kinases. Several bHLH proteins, including E47, contain potential PKA and CKII phosphorylation motifs, which have since been confirmed in in vitro kinase phosphorylation studies (14, 16, 24). Interestingly, some of the PKA substrates such as the transcription factor CREB are also phosphorylated by MAPKAP kinases at the same sites, indicating that these kinases have overlapping sequence preferences. We therefore performed in vitro kinase assays to determine whether 3pK and MK2 are able to phosphorylate E47.

HA-3pK, HA-MK2, or E47 were separately transfected in HEK293 cells, immunoprecipitated, washed, and mixed prior to the kinase reaction (Fig. 4A). As a source of activated 3pK and MK2, cells were stimulated with arsenite resulting in a strong activation of both kinases, as shown by autophosphorylation. In the presence of active kinases, E47 was strongly phosphorylated (Fig. 4A, lanes 3 and 6).

Because the 3pK immunoprecipitates might contain additional kinases eventually responsible for this phosphorylation, we also used bacterially expressed and purified proteins for the experiments shown in Fig. 4B. A GST fusion protein of E47 (GST-E47) was incubated with active or inactive recombinant 3pK (Fig. 4B). In the presence of the active kinase, GST-E47 is phosphorylated, whereas the inactive version failed to phosphorylate E47. The negative control shows that the GST part is not a substrate. These results clearly demonstrate the ability of the MAPKAP kinases 3pK and MK2 to phosphorylate E47 in vitro.

The E47 amino acid sequence shows four potential minimal consensus motifs for phosphorylation by MAPKAP kinases, three in the extreme N terminus and one at Ser-529, phosphorylated not in B-cells but in non-B cells (17).

Phosphorylation of Ser-529 in concert with Ser-514, both phosphorylated not in B-cells but in non-B cells (17).
phosphorylation regulated DNA binding of Max homodimers (25), suggesting that phosphate modification is an important step in the regulation of bHLH transcription factors. It has been postulated that the overall negative net charge in close proximity to the basic DNA-binding region may weaken the contact of homodimers with DNA (17).

**3pK and MK2 Repress E47-induced Transcriptional Activity**—The colocalization and interaction of the two MAPKAP kinases with E47 suggest that the kinases are involved in the regulation of the transcriptional activity of the bHLH protein. Transcriptional activity of E47 was analyzed using a four E-box (4xE)-containing promoter gene construct that is strongly activated in the presence of the transcription factor (Fig. 5).

We observed that coexpression of both 3pK and MK2 repressed the transcriptional activity of E47 on the 4xE-box promoter (Fig. 5, black and gray bars) in a dose-dependent manner to levels near those observed in the absence of E47. Expression of the kinases alone had no effect on the activity of the 4xE-box promoter in the absence of E47 (data not shown), indicating that the effect we observed is due to a cooperative action between the kinases and the transcription factor. Repression of the promoter was not observed when increasing amounts of SAPKβ were coexpressed with E47 (Fig. 5, white bars), indicating that this phenomenon is specific for 3pK and MK2. We used SAPKβ instead of the dominant negative 3pK in this assay, because 3pK KM is aberrantly located in the cytoplasm of the cell and may artificially discern E47 from the nucleus.

The phosphorylation status of different bHLH proteins was found to be correlated with a reduced transcriptional activation of tissue-specific genes, including those involved in cell differentiation (13, 15, 16). This kinase-dependent transcriptional repression is consistent with a previous report showing that overexpression of CKII results in a dramatic reduction of E47 homodimer-directed transcription, suggesting that CKII may act as a positive regulator of myogenesis by preventing E-protein homodimers from binding to muscle gene regulatory elements (16). Thus, by binding and/or phosphorylating E47, MAPKAP kinases may also prevent E47 homodimer binding, thereby reducing homodimer-dependent transactivation.

In summary, we demonstrate that 3pK and MK2 are E47 kinases and that they bind to the bHLH transcription factor with high affinity and repress its transcriptional activity. These data link the E47 function to the MAPK signaling network and suggest that the MAPK-activated protein kinases are regulators of E47-controlled gene expression and differentiation.

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