Regulation of the Erythroid Transcription Factor NF-E2 by Cyclic Adenosine Monophosphate–Dependent Protein Kinase

By Darren Casteel, Modem Suhasini, Tanima Gudi, Reza Naima, and Renate B. Pilz

Activation of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (A-kinase) promotes hemoglobin synthesis in several erythropoietin-dependent cell lines, whereas A-kinase-deficient murine erythroleukemia (MEL) cells show impaired hemoglobin production; A-kinase may regulate the erythroid transcription factor NF-E2 by directly phosphorylating its p45 subunit or by changing p45 interactions with other proteins. We have mapped the major A-kinase phosphorylation site of p45 to Ser169; Ala substitution for Ser169 resulted in a protein that was no longer phosphorylated by A-kinase in vitro or in vivo. The mutant protein formed NF-E2 complexes that bound to DNA with the same affinity as wild-type p45 and functioned normally to restore β-globin gene expression in a p45-deficient MEL cell line. Transactivation properties of the (Ser169 → Ala) mutant p45 were also indistinguishable from wild-type p45 when Gal4-p45 fusion constructs were tested with a Gal4-dependent reporter gene. Transactivation of the reporter by both mutant and wild-type p45 was significantly enhanced when A-kinase was activated by membrane-permeable cAMP analogs or when cells were cotransfected with the catalytic subunit of A-kinase. Stimulation of p45 transactivation by A-kinase required only the N-terminal transactivation domain of p45, suggesting that A-kinase regulates the interaction of p45 with downstream effectors.

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MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis. The complete murine p45 cDNA clone in pBluescriptKS was provided by N. Andrews.1 To replace Ser169 with Ala, we performed site-directed mutagenesis, changing the unique site elimination.24 The mutagenesis primer was 5′-GCCGGAGGGGAGATCGACGCTAG-3′ and a second silent mutation was included to introduce a new Sal I site; the selection primer was 5′-GCCGGCTCTAGAAGCGCTGGATCCTCCG-3′, changing the unique Spe I site in the pBluescriptKS polylinker to Mlu I. The mutant p45 plasmid was sequenced completely to confirm the presence of the desired mutation and to exclude the introduction of other unwanted...
mutations. To produce pMT2-p45(wt) and (mut), the wild-type or mutant p45 cDNAs were cloned downstream of the adenovirus major late promoter into the EcoRI site of the vector pMT2.25 For stable transfection of CB3 cells, the wild-type and mutant p45 cDNAs were cloned downstream of the chicken β-actin promoter of pRC/β-Act using HindIII and Xba I; pRC/β-Act contains a neo' transcription unit to confer G418 resistance.26 To test the transactivation properties of p45 independently of its DNA binding properties, the N-terminus of wild-type and mutant p45 was fused in-frame to the DNA-binding domain of the yeast transcription factor Gal4 in the vector pSG424 (provided by M. Ptashne27). The resulting vectors pGal4/p45(wt) and pGal4/p45(mut) were sequenced across the Gal4-p45 fusion; Western blots developed with a p45-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) detected full-length p45/Gal4 fusion proteins expressed in baby hamster kidney (BHK) cells (data not shown). Stepwise truncation of p45 from the C-terminus was performed by digesting pGal4/p45(wt) with Xho I plus Sal I, Sal I, or Sac I; the vector containing the remaining portion of p45 was blunted and religated, generating pGal4/p45(Δ269), pGal4/p45(Δ112), and pGal4/p45(Δ83) (see Fig 5; the numbers in parentheses designate the number of N-terminal amino acids of p45 present in the construct). Digestion of pGal4/p45(mut) with Xho I plus Sal I removed two C-terminal fragments because of the new Sal I site at Ser166 introduced during site-directed mutagenesis; religation of the vector containing the remaining portion of p45 generated pGal4/p45(Δ171) (see Fig 5). Partial digestion of Gal4/p45(wt) with Pst I was used to remove all but the first 108 nucleotides of the p45 coding sequence; religation of the 3.4-kb partial digestion product generated pGal4/p45(Δ36). Truncation of p45 in these vectors was confirmed by restriction analysis and partial DNA sequence analysis.

The p18 expression vector pMT2-p18 was from N. Andrews, the expression vector for the catalytic (C)-subunit of A-kinase, pCMV-Cα, was from S. Taylor, and the reporter plasmid pGAL4-Luc and the expression vector for the catalytic (C)-subunit of A-kinase, pCMV-C were incubated with a p45-specific antibody (Santa Cruz Biotechnology) as described.32 Some experiments, cells were treated for 1 hour with 1 mmol/L 8-Br-cAMP to activate A-kinase phosphorylation studies in vitro and in vivo. Inspection of the amino acid sequence of p45 reveals a consensus sequence for A-kinase phosphorylation at residues 166 to 169 (Arg-Arg-Arg-Ser); we showed previously that Ser166 of p45 is the site phosphorylated by A-kinase in vitro, we incubated the transfected BHK cells with 32P-O4 and probed with a radioactively labeled oligodeoxynucleotide (oligodNT) probe encoding the NF-E2 recognition sequence from the human porphobilinogen deaminase promoter as described previously.18 Quality and equal loading of nuclear extracts was tested by incubation with a probe for the ubiquitous transcription factor SP-1.18

RESULTS

A-kinase phosphorylates p45 on Ser169 in vitro and in vivo. Nuclear extracts were prepared and equal amounts of nuclear extract proteins were incubated with a radioactively labeled oligodeoxynucleotide (oligodNT) probe encoding the NF-E2 recognition sequence from the human porphobilinogen deaminase promoter as described previously.31 Western blot analyses. Western blots were performed using a p45-specific antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence detection as described previously.18 Northern blots were prepared with 8 µg of total cytoplasmic RNA and probed with a radioactively labeled β-globin probe as described.19

For phosphoamino acid analysis, immunoprecipitates were hydrolyzed in 6N HCl, lyophilized, and resuspended with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.18 All immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.18

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apparent molecular weight induced by phosphorylation of single amino acid residues have been observed in other proteins. Total PO incorporation into p45 was determined by quantitating Cerenkov radiation of excised gel slices and increased 25% in the presence of 8-Br-cAMP (result of three independent experiments). Basal phosphorylation of mutant p45 was considerably lower than that of wild-type p45 and neither the mobility nor the total PO incorporation into mutant p45 was influenced by 8-Br-cAMP (Fig 1C, lanes 4 and 5 show mutant p45 from cells cultured in the absence and presence of 8-Br-cAMP, respectively). Similar results were obtained when p45 was immunoprecipitated from labeled wild-type MEL cells: treatment with 8-Br-cAMP resulted in a mobility shift in p45 immunoprecipitated from wild-type MEL cells, but not from A-kinase–deficient MEL cells (Fig 1D, compare lanes 2 and 3, wild-type MEL cells cultured in the absence or presence of 8-Br-cAMP, with lanes 4 and 5, A-kinase–deficient MEL cells cultured in the absence or presence of 8-Br-cAMP, respectively). Phosphoamino acid analysis of p45 from transfected BHK cells labeled with PO demonstrated PO in p45 associated with phosphoserine, but not with phosphothreonine or phosphotyrosine.

The lower PO incorporation into mutant p45 compared with wild-type p45 suggests that the mutation at Ser169 may influence the phosphorylation of neighboring sites by other serine/threonine protein kinases; since the phosphorylation of Ser169 by A-kinase resulted in a mobility shift on SDS-PAGE, Ser169 does not appear to be targeted by other protein kinases in unstimulated cells. Thus, Ser169 is the only site in p45 that is phosphorylated in vitro and in vivo by A-kinase; p45 phosphorylation in vivo in the absence of 8-Br-cAMP can be attributed to other serine/threonine protein kinases.

Fig 1. A-kinase phosphorylates p45 on Ser169 in vitro and in vivo. (A) BHK cells were transfected with increasing amounts of expression vector encoding wild-type p45 [lanes 1 to 3, 0.3 \( \mu \)g, 0.6 \( \mu \)g, and 1.2 \( \mu \)g of pMT2-p45 (wt), respectively] or mutant [Ser169 → Ala]p45 [lanes 4 to 6, 0.3 \( \mu \)g, 0.6 \( \mu \)g, and 1.2 \( \mu \)g of pMT2-p45 (mut), respectively]. Cell extracts were subjected to Western blotting with a p45-specific antibody as described in Materials and Methods. The p45 doublet, which is thought to result from alternative usage of two translational start sites, is indicated by a double arrow. (B) BHK cells were transfected with expression vectors encoding wild-type p45 [1.2 \( \mu \)g and 0.4 \( \mu \)g of pMT2-p45(wt), lanes 1 and 2, respectively] or mutant p45 [1.2 \( \mu \)g and 0.4 \( \mu \)g of pMT2-p45(mut Ser169 → Ala), lanes 3 and 4, respectively]; lane 5 shows mock-transfected cells. Cell extracts were subjected to immunoprecipitation with a p45-specific antibody; immunoprecipitates were incubated with \([\gamma-32P]ATP\) and purified C-subunit of A-kinase and applied to SDS-PAGE/autoradiography as described in Materials and Methods. (C) BHK cells were transfected with expression vectors encoding wild-type p45 [1.2 \( \mu \)g of pMT2-p45(wt), lanes 1, 2, and 8] or mutant p45 [1.2 \( \mu \)g of pMT2-p45(mut Ser169 → Ala), lanes 4 and 5]; lanes 3 and 6 show cells transfected with 0.2 \( \mu \)g of wild-type or mutant p45 vector, respectively, and lane 7 shows mock-transfected cells. Cells were incubated with PO and some cultures were treated with 1 mmol/L 8-Br-cAMP (lanes 2, 3, 5, and 6); cell extracts were subjected to immunoprecipitation with p45-specific antibody (lanes 1 to 7) or control rabbit serum (lane 8) and immunoprecipitates were applied to SDS-PAGE/autoradiography as described in Materials and Methods. (D) Wild-type MEL cells (lanes 1 to 3) and A-kinase–deficient MEL cells (lanes 4 to 6) were incubated with PO, and some cultures were treated with 1 mmol/L 8-Br-cAMP (lanes 3 and 5); cell extracts were subjected to immunoprecipitation with p45-specific antibody (lanes 2 to 5) or control rabbit serum (lanes 1 and 6) as described in (C).
DNA binding activity of mutant p45(Ser\textsuperscript{169} → Ala) in BHK cells. To examine whether A-kinase phosphorylation of p45 Ser\textsuperscript{169} influences NF-E2/DNA complex formation, we cotransfected BHK cells with increasing amounts of expression vectors for wild-type or mutant p45(Ser\textsuperscript{169} → Ala) and equimolar amounts of p18 expression vector. Equal amounts of nuclear extract proteins were incubated with a radioactively labeled oligodNT encoding the NF-E2 recognition site of the human porphobilinogen deaminase promoter and protein/DNA complexes were resolved on nondenaturing PAGE: the faster migrating protein/DNA complex contains the p45/p18 heterodimer and it is absent in mock transfected cells (Fig 2A, compare lanes 1 through 6, transfected cells, with lanes 7 and 8, mock-transfected cells).\textsuperscript{10,18} Mutant p45(Ser\textsuperscript{169} → Ala) formed the same amount of NF-E2/oligodNT complexes as wild-type p45 (Fig 2A, compare lanes 1 to 3, wild-type p45, with lanes 4 to 6, mutant p45). As a control for equal protein loading, we performed EMSAs with an oligodNT probe containing a SP-1 recognition site (Fig 2B). EMSAs performed with variable amounts of NF-E2 oligodNT probe demonstrated that the DNA-binding affinity of the NF-E2 complex containing mutant p45(Ser\textsuperscript{169} → Ala) was indistinguishable from that containing wild-type p45.

DNA binding and transactivation properties of mutant p45(Ser\textsuperscript{169} → Ala) in CB3 cells. To examine the DNA-binding and transactivation properties of the mutant p45(Ser\textsuperscript{169} → Ala) in an erythroid background, we transfected the variant MEL cell line CB3 with expression vectors encoding either wild-type or mutant p45 (Ser\textsuperscript{169} → Ala). CB3 cells are completely deficient in p45 expression, and unlike wild-type MEL cells, they show no increase in globin gene expression when treated with differentiation-inducing agents like hexamethylene bisacetamide (H MBA).\textsuperscript{9,10} Single clones of stably transfected CB3 cells were tested for NF-E2 DNA-binding activity and β-globin mRNA expression in response to H MBA. Several clones showed no detectable NF-E2/DNA complexes and little β-globin mRNA expression (Fig 3, clones W2, M2, and W4; some globin mRNA was detectable in these clones on long exposures of the Northern blot). In clones that expressed p45, the amount of NF-E2/DNA complexes correlated with the amount of β-globin mRNA expression observed in both wild-type and mutant p45-transfected CB3 cells (Fig 3, eg, clones M1 and W3, which expressed low amounts of NF-E2/DNA complexes, expressed low amounts of β-globin mRNA). On Western blots, the amount of p45 expressed correlated closely with the amount of NF-E2/DNA complexes found by EMSA (data not shown). Thus, in p45-deficient CB3 cells, mutant p45(Ser\textsuperscript{169} → Ala) could restore NF-E2 DNA-binding activity and β-globin mRNA expression to the same degree as wild-type p45. Only levels of p45 expression that were higher than those found in wild-type MEL cells restored β-globin mRNA expression in the variant CB3 cells to the amount of β-globin mRNA detected in wild-type MEL cells; this finding is in agreement with previous reports.\textsuperscript{9,10}

Effect of 8-Br-cAMP on the transactivation properties of p45. The NF-E2 binding site is recognized by a number of different bzip proteins, including members of the AP-1 family, which are known to be regulated by A-kinase.\textsuperscript{36} To study the transactivation properties of p45 independently of its binding to the NF-E2 recognition site, we fused p45 sequences with the DNA-binding domain of the well-characterized yeast transcription factor Gal4; this allows testing of the fusion protein’s activity on a test promoter bearing GAL4-binding sites.\textsuperscript{27} When we cotransfected BHK cells with the reporter pGAL4-Luc and increasing amounts of expression vector encoding Gal4 fusion proteins with either full-length wild-type (wt) or mutant (mut,
Ser169→Ala)p45, we observed the same modest degree of reporter gene transactivation by wild-type and mutant p45 (Fig 4, open squares and triangles). When cells were treated with 8-Br-cAMP to activate endogenous A-kinase, we observed a fivefold to sevenfold increase in the transactivation of pGAL4-Luc at each level of pGal4/p45 (wt) or (mut) expression (Fig 4, filled squares and triangles). When we transfected pGAL4-Luc with the parent vector pSG424, which contains only the Gal4 DNA-binding domain, luciferase expression was low and not significantly influenced by the activation of A-kinase (Fig 4, diamonds). When we cotransfected a Gal4-fusion protein containing the bzip transcription factor c-Fos, strong transactivation of pGAL4-Luc-1 was observed, which was not altered by 8-Br-cAMP (data not shown). Thus, the effect of A-kinase on p45 transactivation was specific for p45 sequences, but did not depend on the presence of the A-kinase phosphorylation site Ser169.

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activities were determined as described in Materials and Methods; enzymous A-kinase (dashed lines and filled symbols). Reporter gene mmol/L 8-Br-cAMP for 8 hours before harvesting to activate endogenous A-kinase. Half of the cultures were treated with 1 ng of the reporter plasmid [pGal4/p45(wt), triangles; pGal4/p45(mut), squares; pSG424, diamonds]. The total amount of transfected DNA was kept constant for each sample to correct for transfection efficiencies. Results represent the mean ± SD of three independent experiments.

cotransfected C-subunit of A-kinase; similar results were obtained when endogenous A-kinase was activated with 8-Br-cAMP. When we transfected pGal4/p45(Δ83) into severely A-kinase–deficient MEL cells (clones R1 mut/C1 and R1 mut/C3, described previously31), we found that the transactivation potential of pGal4/p45(Δ83) was reduced by 50% to 70% compared with the activity of this construct in control MEL cells with normal A-kinase activity (clones R1 wt/C1 and R1 mut/C3); gene expression from the cotransfected A-kinase (data not shown). Moreover, when we cotransfected BHK cells with pGal4/p45(Δ83) and full-length CBP, transactivation of the Gal4-dependent reporter was stimulated twofold to threefold by CBP in the presence or absence of A-kinase; this effect on transactivation appeared to be nonspecific, since CBP also increased expression of the control plasmid pRSV-βGal twofold (data not shown). Thus, we could not demonstrate a convincing effect of CBP on p45 transactivation under our experimental conditions.

### DISCUSSION

The importance of NF-E2 for the transcriptional activation of erythroid-specific genes and in particular for the activity of the α- and β-globin LCRs has been recognized, but little is known about the regulation of NF-E2 activity.5,7,10,39 NF-E2 is expressed in multipotent hematopoietic progenitor cells before their commitment to the erythroid lineage and the globin LCR NF-E2 recognition sites are occupied before globin gene expression is activated.6,39,41 NF-E2 activity may be regulated by posttranslational mechanisms, such as phosphorylation, or by interactions with ancillary proteins. Other mechanisms of regulation may include changes in NF-E2 complex composition through changes in the expression and activity of maf-related proteins or competition of other bzip proteins for binding at the NF-E2 recognition site.4,13-15,37,42

In this study, we found that A-kinase significantly stimulated the transactivation properties of p45 in erythroid and nonerythroid cells; in a previous study, we had shown impaired NF-E2/DNA complex formation and decreased α-LCR enhancer activity in A-kinase–deficient MEL cells.19 Since we could not detect any effect of p45 phosphorylation at the A-kinase recognition site Ser169 on NF-E2 DNA-binding or transactivation properties, our results suggest that A-kinase regulates NF-E2 indirectly, through changes in the activity of ancillary protein(s) present in erythroid and nonerythroid cells. Since modulation of p45 activity by A-kinase required only the N-terminal transactivation domain of p45, the effect of A-kinase is not likely to be mediated through modification of maf-related or other bzip proteins.

Although the activity of many transcription factors is modulated by phosphorylation, there are many examples of changes in transcription factor phosphorylation that have no detectable influence on DNA binding or transactivation.36,43 The erythroid transcription factor GATA-1 is a prime example, because careful mapping of seven major phosphorylation sites demonstrated that phosphorylation at an A-kinase consensus sequence increases during erythroid differentiation of MEL cells, but phosphorylation at this site or at the other sites does not measurably alter GATA-1 functions.43

The positive or negative regulation of transcription factors by changing protein/protein interactions has been described for several classes of bzip proteins. An example of negative regulation is the interaction of AP-1 with steroid hormone and retinoic acid receptors, which results in repression of AP-1 activity dependent on ligand binding to the steroid or retinoic acid receptors.30,44,45 An example of positive regulation is the interaction of CREB with the transcriptional coactivator CBP, which requires several A-kinase–regulated events, including phosphorylation of CREB and CBP.46,47 Although CBP has been
recently shown to bind to the transactivation domain of p45 in vitro and appears to mediate the potentiation of nuclear hormone receptor action by p45,20 we were unable to demonstrate a significant effect of CBP on the transactivation potential of p45 under our experimental conditions. However, we cannot exclude that BHK cells express too much endogenous CBP to demonstrate a positive effect of transfected full-length CBP or a dominant negative effect of the isolated p45-binding domain of CBP.

A critical role for the N-terminal domain of p45 for globin gene expression in MEL cells has been shown previously.10,38 The main transactivation domain of p45 has been localized to the N-terminal 80 amino acids of the protein; this region is proline-rich and contains two PXXP motifs and a PPPSY motif that can mediate protein/protein interactions.8,38,48 We found that this N-terminal domain of p45 was sufficient to mediate the effect of A-kinase on p45 transactivation. Recently, direct interaction between this p45 domain and the TATA-binding protein-associated factor TAFII130, as well as other proteins, has been demonstrated.8,48 More work is necessary to determine whether the interaction of p45 with a component of the transcription initiation complex or other interactions between p45 and downstream effector molecules may be regulated by A-kinase.

The physiologic significance of A-kinase regulation of NF-E2 is supported by the effect of cAMP on the erythroid differentiation of various cell lines: stimulation of the cAMP signal transduction pathway promotes hemoglobin production in the

Fig 5. Transactivation properties of Gal4-fusion constructs containing truncated versions of p45: effect of A-kinase. The structure of p451 and the Gal4-fusion constructs containing variable amounts of N-terminal p45 sequences fused to the DNA binding domain of Gal4 are shown in (A); results of cotransfection experiments using these constructs in BHK cells are shown in (B). The indicated transactivator plasmid (5 ng) was cotransfected with the reporter pGAL4-Luc (100 ng), the control vector pRSV-β-Gal (50 ng), and either an expression vector for the C-subunit of A-kinase (pCMV-Cα, 50 ng, open bars) or empty vector (pRC/CMV, 50 ng, filled bars). Luciferase activity was normalized to β-galactosidase activity in each sample; the luciferase/β-galactosidase ratio obtained with the parent vector pSG424, which is lacking p45 sequences, was assigned a value of 1. Results represent the mean ± SD of three independent experiments.

Fig 6. Regulation of the p45 transactivation domain by A-kinase in MEL cells. MEL cells were cotransfected with 300 ng of pGAL4-Luc, 300 ng of pRSV-β-Gal, the indicated amounts of transactivator plasmid containing the N-terminal 83 amino acids of p45 [pGAL4/p45(Δ83)], and 200 ng of either A-kinase C-subunit expression vector (●) or empty vector (○) as described in Materials and Methods. Luciferase activity was normalized to β-galactosidase activity; results represent the mean ± SD of three independent experiments.
erythropoietin-responsive cell lines SKT6, TSA8, and J2E. \(^{21-23}\) While binding of erythropoietin to its receptor does not change intracellular cAMP concentrations, \(^{22}\) the effect of cAMP on hemoglobin production in these cell lines is consistent with a role of A-kinase in the regulation of erythroid gene expression, possibly via changes in the activity of NF-E2. In MEL cells, we have demonstrated that A-kinase activity is necessary for erythroid gene expression, \(^{18,19}\) although prolonged treatment of MEL cells with pharmacologic doses of cAMP analogs results in inhibition of erythroid differentiation. \(^{49}\) We have recently shown that this paradoxical effect is due to upregulation of c-myb, mediated at least in part by NF-κB (p50/relB), which is induced by prolonged activation of A-kinase. \(^{49}\) Thus, in MEL cells, A-kinase not only produces signals expected to promote differentiation (increased NF-E2 activity), but prolonged activation of the kinase can also produce signals that are incompatible with differentiation (upregulation of c-myb).

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Regulation of the Erythroid Transcription Factor NF-E2 by Cyclic Adenosine Monophosphate–Dependent Protein Kinase

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