CCAAT/enhancer binding protein ε: changes in function upon phosphorylation by p38 MAP kinase

Elizabeth A. Williamson, Ian K. Williamson, Alexey M. Chumakov, Alan D. Friedman, and H. Phillip Koeffler

C/EBPε, a member of the CCAAT/enhancer binding protein family, is a transcription factor important in neutrophil differentiation. We have determined that it is phosphorylated on multiple serine and threonine residues and can be a target for phosphorylation by a number of kinases. We identified a threonine at amino acid 75, part of a consensus mitogen-activated protein (MAP) kinase site within the transactivation domain of C/EBPε, as being phosphorylated only by p38 MAP kinase. Phosphorylation of this residue resulted in enhanced transcriptional activity on a myeloid-specific promoter in vitro transient transfection reporter assays. We also determined that phosphorylation at Thr75 yielded a protein that was more effective at binding its cognate DNA sequence compared with the wild-type nonphosphorylated C/EBPε. Stable expression of C/EBPε T75A in interleukin 3 (IL-3)-dependent 32Dc13 did not result in the up-regulation of expression of secondary granule genes compared with wild-type C/EBPε or C/EBPε T75D. Therefore we suggest that C/EBPε is a target for p38 MAP kinase activity. (Blood. 2005;105:3841-3847)

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Introduction

Gene expression in response to external stimuli is regulated by the activity of transcription factors. The activity of these transcription factors is tightly regulated by a variety of posttranslational modifications, including acetylation and phosphorylation. Phosphorylation is one of the major, and most extensively studied, mechanisms for modulating transcription factor activity.

Many studies on transcription factors have demonstrated them to be modular proteins with separate domains for DNA binding and either transactivation or repression. C/EBPε is a member of the CCAAT/enhancer binding protein family of transcription factors, which we previously demonstrated had both transactivation and repression domains.1,2 The CCAAT/enhancer binding protein family also includes C/EBPα, C/EBPβ, C/EBPδ, and CHOP/GADD153 (C/EBP homology protein/growth arrest and DNA damage-inducible gene 153).3-6 The members of the C/EBP family of transcription factors are phosphoproteins and the phosphorylation of these proteins has been shown to affect their function. C/EBPβ is the most extensively studied member of this transcription factor family with respect to phosphorylation.

C/EBPβ is phosphorylated by a number of protein kinases including protein kinase A (PKA), protein kinase C (PKC), glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinases (MAPKs), ribosomal S6 kinase, and calcium-calmodulin kinase II (CaMKII). Phosphorylation affects a number of the functions of C/EBPβ, including inhibition of DNA binding, intracellular relocalization to the nucleus, increased transcriptional activity, and inhibition of apoptosis.7-12 C/EBPα is a target for phosphorylation by PKC and GSK3.3,13,14 Although phosphorylation by PKC resulted in a decrease in DNA binding by C/EBPα, phosphorylation by GSK3 did not appear to have any effect on C/EBPα function.

In contrast to C/EBPα and C/EBPβ, phosphorylation in the DNA binding domain of C/EBPβ by casein kinase 2 actually increased DNA binding.15 C/EBPδ can also be phosphorylated by MAPKs resulting in the translocation of this protein to the nucleus.16 MAP kinases can phosphorylate another member of the C/EBP family, CHOP/GADD153. In this case CHOP/GADD153 showed increased transcriptional activity.

Members of the C/EBP family are expressed in myeloid cells but appear to play different roles in differentiating and mature cells. C/EBPε is expressed almost exclusively in myeloid cells with its expression reaching a peak in mature neutrophils and macrophages.17,18 Both C/EBPβ and C/EBPδ are regulators of cytokine gene expression in macrophages, whereas C/EBPα is critical for granulocytic differentiation.19,20 As well as being expressed in hematopoietic cells, C/EBPα, C/EBPβ, and CHOP/GADD153 also play an important role in adipocyte differentiation. The p38 MAP kinase can phosphorylate both C/EBPβ and CHOP/GADD153 with opposing effects on adipocyte differentiation. Phosphorylation of C/EBPβ by p38 MAPK is essential for adipocyte differentiation, whereas phosphorylation of CHOP/GADD153 by p38 MAPK results in an inhibition of adipocyte differentiation.21,22 Thus phosphorylation of transcription factors involved in the same pathway by the same kinase allows for tight regulation of that process.

From the Department of Medicine, Hematology/Oncology, Cedars-Sinai Medical Center, University of California—Los Angeles, Los Angeles, CA; and Oncology/Pediatrics, Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD.


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Reprints: Elizabeth A. Williamson, University of New Mexico, 1UNM/MSCO8 4630, Cancer Research Facility Room 117, Albuquerque, NM 87131; e-mail: ewilliamson@salud.unm.edu.

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demonstrates that C/EBP/H9280 binding of C/EBP/H9280 kinase. Phosphorylation of this residue results in increased transactivation on a myeloid-specific promoter accompanied by increased binding of C/EBP to its cognate DNA sequence. C/EBP phosphorylated on this residue was also able to up-regulate the expression of myeloid-specific secondary granule genes in vivo.

Materials and methods

CMV-C/EBPε constructs

Single point mutations were introduced into wild-type C/EBPε by polymerase chain reaction (PCR) using overlapping mutated oligonucleotides, followed by a second round of PCR using external primers containing appropriate restriction sites.24 The resulting PCR products were digested with the appropriate restriction enzymes and ligated into a cytomegalovirus (CMV)-expression vector, pCMV-SPORT (Invitrogen, Carlsbad, CA). The presence of these point mutations within C/EBPε was confirmed by sequencing.

Transient transfections and reporter assays

Transient transfections were performed in Jurkat cells using lipofectin (Invitrogen) with 3 μg promoter-luciferase reporter and 0.3 μg of the C/EBPε expression vectors, wild-type or mutants, as previously described.25 The internal control for transfection efficiency was 1 μg pCMV-β-galactosidase. At 24 hours after transfection, the Jurkat cells were activated with TPA (12-O-tetradecanoyl phorbol 13-acetate; 2 ng/mL) and the calcium ionophore A23187 (250 ng/mL) for 16 hours. After this time the transfections were harvested and the luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions (Promega, Madison, WI). In some assays the following inhibitors (Calbiochem, San Diego, CA) were added for 60 minutes prior to activation: SB203580 (5 μM) and SB202190 (2.5 μM) for inhibition of p38 MAP kinase; H-89 (10 μM), an inhibitor of PKA; and KN-62 (1.5 μM), an inhibitor of CaMKII.

GST-C/EBPε fusion proteins

C/EBPε, C/EBPεT75A, and C/EBPεT75D were subcloned into pGEX-5X-1 and the resulting constructs transformed into BL-21 cells. The glutathione-S-transferase (GST)-C/EBPε fusion proteins were isolated according to the manufacturer’s instructions (Amersham-Pharmacia, Piscataway, NJ), with specific modifications. Following overnight growth, the culture was divided 1:10 and then grown for a further 60 minutes. The GST-C/EBPε fusion protein was induced by 1 mM IPTG (isopropyl-B-D glucopyranoside) for 4 hours. The GST fusion proteins were then isolated by sonication and stored bound to the glutathione-sepharose beads in 50% glycerol. Expression of the GST fusion proteins was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting with a specific C/EBPε antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro kinase assays

Equal amounts (5 μg) of the GST-C/EBPε fusion proteins were incubated with the activated kinase (1-10 U) in kinase-specific reaction buffer containing 10 μCi (0.37 MBq) γ-32P–adenosine triphosphate. The kinases used for these assays were PKA (Roche, Indianapolis, IN), PKC (Roche), extracellular signal-related kinase 2 (ERK2; UBI, Lake Placid, NY), p38α MAP kinase (UBI), c-Jun N-terminal kinase (JNK2; UBI), casein kinase 2 (Roche), and CaMKII (UBI). The kinase reactions were incubated in appropriate kinase buffer at 30°C for 10 to 30 minutes and then stopped by the addition of Laemmli sample buffer (BioRad, Hercules, CA). The reactions were boiled and separated by SDS-PAGE. The gels were either dried for further analysis as described in the next section or the 32P-labeled protein was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) for immunoblotting with a C/EBPε-specific antibody. 32P-labeled protein was detected by autoradiography.

Two-dimensional (2D) phospho–amino acid analysis

The in vitro–kinased GST-C/EBPε fusion proteins were eluted overnight from the dried polyacrylamide gel in buffer P (25 mM N-ethylenolpholine, pH 7.7) and then precipitated. For 2D phospho–amino acid analysis the precipitated protein was treated with 6 M HCl for 60 minutes at 100°C and then lyophilized. The pellet was washed thoroughly and finally resuspended in 1.9 buffer (formic acid–acetic acid–water ratio, 1.31:3.59:9) and the liberated phospho–amino acids were separated on 100 μM thin-layer chromatography (TLC) plates (CBS Scientific, Del Mar, CA) on the Hunter thin layer peptide mapping electrophoresis system (HTLE-7000; CBS Scientific), with 1 μg cold phospho–amino acid standards. For 2D separation, the first dimension is electrophoresis in pH 1.9 buffer (formic acid–acetic acid–water ratio, 1.31:3.59:9) at 1.5 kV for 20 minutes and the second dimension is electrophoresis in pH 3.5 buffer (acetic acid–pyridine–water ratio, 10:1:189) at 1.3 kV for 16 minutes. Unlabeled phospho–amino acids were detected by ninhydrin staining of the TLC plate and the 32P-labeled phospho–amino acids were visualized by autoradiography.

Nuclear protein analysis

Jurkat cells were transfected with 5 μg of the CMV-C/EBPε expression plasmids, wild-type and mutated, as described above. The empty vector pCMV-SPORT was used as the negative control for these experiments. Nuclear proteins were prepared as previously described.25 The nuclear extracts were initially separated by SDS-PAGE and analyzed by Western immunoblot to confirm expression of the various C/EBPε proteins. DNA binding by the C/EBPε proteins was determined by electrophoretic mobility shift assay (EMSA) using double-stranded oligonucleotides containing the C/EBP consensus site and adjacent sequences of the main promoter.25 A standard reaction for the EMSA contained 2.5 ng 32P-labeled oligonucleotide, 10 μg Jurkat nuclear extract expressing either wild-type or mutated C/EBPε, 1.5 μg poly dIdC (poly-deoxyinosinic-deoxycytidylic acid), and 4.5 μg bovine serum albumin in a 20-μL volume. Competing cold oligonucleotides at 100-fold molar excess or antibodies (1 μg) were added where indicated. Electrophoresis was performed on a 4% polyacrylamide gel at 34 mA for 2 to 3 hours. The gel was dried and the results visualized by autoradiography.

Transduction of C/EBPε

The 32Dcl3 cells were cultured in Iscoves modified Dulbecco medium containing 10% fetal calf serum and 10% Wehi–3–conditioned medium. C/EBPε, C/EBPεT75A, C/EBPεT75D, C/EBPεS181D, and C/EBPεS188D were ligated into the pBabePuro retroviral vector to allow for retroviral infection of 32Dcl3 cells. Bosc23 packaging cells were transfected with 5 μg of the pBabePuro-C/EBPε constructs using GenePorter (GTS, San Diego, CA) as recommended by the manufacturer’s instructions. At 48 hours after transfection, 32Dcl3 cells were infected by incubation with medium removed from the Bosc23 cells for 48 hours in the presence of 4 μg/mL polybrene.23 After this time the 32Dcl3 cells were transfected to medium containing 2 μg/mL puromycin. Puromycin-resistant 32Dcl3-C/EBPε cells were expanded, and the expression of the wild-type and mutant C/EBPε proteins was examined by Western immunoblotting with an antibody specific for C/EBPε.

In order to demonstrate changes in 32Dcl3 cells occurring with the stable expression of C/EBPε proteins, total RNA was isolated from
Results

C/EBPα can be phosphorylated in vitro by multiple kinases

Since other C/EBP family members have been shown to be the target of a number of kinases, we analyzed the protein sequence of C/EBPα and identified a number of potential phosphorylation sites. We then carried out a series of in vitro kinase assays to confirm that C/EBPα was a substrate for these kinases as suggested by the protein sequence. In vitro kinase assays demonstrated that GST-C/EBPα could be phosphorylated by ERK2, p38 MAP kinase, and PKA (Figure 1A-C) but was not phosphorylated in vitro by JNK2, casein kinase 2, or PKC (Figure 1D-E; data not shown). CaMKII phosphorylated C/EBPα weakly compared with the other kinases (Figure 1F). The appropriate positive controls were included in these kinase assays as verification of the activity of the kinase, and GST alone was not phosphorylated in any of these assays (Figure 1; data not shown).

Previously we had determined, by in vivo labeling, that C/EBPα was phosphorylated on multiple serine and threonine residues but not on tyrosine residues.2 Furthermore, in vivo labeling with a series of previously described1 C/EBPα deletion mutants demonstrated that only threonine residues were phosphorylated within the N-terminal transactivation domain of C/EBPα (data not shown). Potentially both serine and threonine residues in C/EBPα could be phosphorylated by PKA, ERK2, and p38α MAP kinase. Therefore we performed 2D phospho–amino acid analysis on the in vitro–phosphorylated GST-C/EBPα to determine the identity of the residues phosphorylated by each of these 3 kinases. This experiment demonstrated that both ERK2 and PKA kinase activity resulted in the phosphorylation of serine residues only (Figure 2A-B). However, phosphorylation of both threonine and serine residues was observed when GST-C/EBPα was in vitro kinased by p38α MAP kinase (Figure 2C).

Within C/EBPα, 4 potential MAP kinase phosphorylation sites occur, 1 threonine and 3 serine residues. The serine residues are at amino acids 109, 181, and 188 and are all minimal MAP kinase sites (SP). The threonine residue is at amino acid 75 and is part of a consensus MAP kinase site (PGTP) within the N-terminal transactivation domain of C/EBPα. Since this is the only potential site within C/EBPα for the threonine phosphorylation observed with p38 MAP kinase, we mutated this residue to the nonphosphorylatable alanine GST-C/EBPαT75A. This mutated GST-C/EBPα was then used as a substrate in an in vitro kinase assay with p38α MAP kinase. The 2D phospho–amino acid analysis of the kinased GST-C/EBPα confirmed that the mutation Thr75Ala did result in the loss of the threonine phosphorylation observed for the wild-type GST-C/EBPα and p38α MAP kinase (Figure 2D). Therefore, these results suggest that Thr75 within the N-terminal transactivation domain of C/EBPα is a target for the p38 MAP kinase signaling pathway.

Mutation of Thr75 alters the capacity of C/EBPα to activate gene transcription

Although Thr75 appears to be a target for p38 MAP kinase in vitro, this does not necessarily mean that phosphorylation or lack of it at this site will affect the function of C/EBPα in vivo. Studies of other C/EBP family members showed that some residues can be phosphorylated in vitro but not in vivo and vice versa.9,12 Therefore we wanted to determine whether mutation of this amino acid could
affect the ability of C/EBPs to transcriptionally activate a myeloid-specific promoter. Thus 2 C/EBP mutants, C/EBP\(\text{T}75A\) (nonphosphorylated) and C/EBP\(\text{T}75D\) (threonine to aspartate to mimic a phosphorylation event), were generated and subcloned into a CMV-driven expression vector. These 2 constructs were then used in transient transfection assays with a myeloid-specific promoter-luciferase construct and compared with the wild-type C/EBP activity on this promoter. The Jurkat cell line was used for these transient reporter assays since it does not express detectable levels of endogenous C/EBP.

In the transient reporter assays, the wild-type C/EBP activated the promoter-luciferase reporter construct by 7.5-fold, whereas the C/EBP\(\text{T}75A\) mutant activated the promoter only 3.7-fold (Figure 3). In contrast, C/EBP\(\text{T}75D\) was even more effective at activating the promoter, giving a 10.5-fold increase in activity on the promoter-luciferase reporter (Figure 3). These results suggest that phosphorylation (or lack of it) at Thr75 does have an effect on the ability of C/EBP to activate gene transcription. Furthermore, when a series of protein kinase inhibitors was used in these transient reporter assays, only inhibitors of p38 MAP kinase phosphorylation (or lack of it) at Thr75 have an effect on the activity of C/EBP to activate gene transcription.

**Figure 4. Expression of wild-type and mutant C/EBP proteins.** Jurkat cells were transfected with either wild-type or mutant C/EBP expression vectors alone. Forty-eight hours after transfection cells were harvested for total and nuclear protein extraction. Western immunoblot analysis was carried out using an antibody specific for C/EBP. (A) Total protein extract. (B) Nuclear protein extract.

**Phosphorylation alters DNA binding affinity of C/EBP**

Since all but 1 of our mutant C/EBP proteins are correctly localized to the nucleus and expressed at similar levels, we were interested to examine their ability to bind their cognate DNA sequence. Therefore gel shift analysis was performed using nuclear extracts from Jurkat cells transfected with either wild-type or mutant forms of C/EBP and an oligonucleotide from the mim promoter containing a cognate C/EBP binding site and surrounding sequences.

The gel shift analysis demonstrated that wild-type C/EBP, C/EBP\(\text{T}75A\), C/EBP\(\text{T}75D\), C/EBP\(\text{S}181A\), and C/EBP\(\text{S}181D\) all bound the oligonucleotide containing the C/EBP binding site from the mim promoter (Figure 5A). In these gel shift experiments, the DNA-protein complex could be competed away by the inclusion of the same unlabeled oligonucleotide and it could be supershifted with a C/EBP-specific antibody (Figure 5A).

Although all C/EBP proteins bound to the cognate DNA sequence, there appeared to be an increase DNA binding by the nuclear extract containing C/EBP\(\text{T}75D\) compared with the wild-type C/EBP (Figure 5A). Therefore we wanted to determine whether there was a real change in DNA binding by the C/EBP\(\text{T}75D\) mutant protein. Therefore, further EMSAs were carried out using a constant amount of the C/EBP proteins and increasing dilutions of the oligonucleotide (Figure 5B). Wild-type C/EBP demonstrated good binding for its cognate DNA sequence at 10 nM of oligonucleotide (Figure 5B). However, this DNA-protein complex was very weak even when the oligonucleotide was diluted to only 5 nM (Figure 5B). In contrast, binding of C/EBP\(\text{T}75D\) was strong even when the oligonucleotide had been diluted to 0.25 nM (Figure 5B). Scatchard analysis determined that the DNA binding affinity for wild-type C/EBP is 7.69 nM. This value is about the same as the DNA binding affinity of C/EBP previously determined by our group.25 In contrast, the DNA binding affinity of C/EBP\(\text{T}75D\) is 0.48 nM. This demonstrates that C/EBP\(\text{T}75D\) binds its cognate DNA with 16-fold more affinity than wild-type C/EBP. Therefore, phosphorylation of Thr75 dramatically increases the DNA binding ability of the C/EBP protein.

**In vivo effects of C/EBP\(\text{T}75D\) expression**

The previous experiments are all in vitro analyses for effects on C/EBP function after phosphorylation. Therefore we were interested in investigating the biologic effects of expression of...
Discussion

The activity of transcription factors is regulated in a rapid and reversible manner by specific phosphorylations. This study demonstrates that C/EBP is phosphorylated in vivo on multiple serine and threonine residues and that it is a substrate in vitro for the protein kinases PKA, ERK2, and p38 MAP kinase. The combination of these in vivo and in vitro analyses suggested that p38 MAP kinase was the protein kinase responsible for the phosphorylation of a threonine residue within the N-terminal transactivation domain of C/EBP.

Phosphorylation of this threonine residue resulted in an increase in the transactivation ability of C/EBP as determined by transient transfection reporter assays. EMSA analysis showed that this increase in activity on a myeloid-specific promoter was accompanied by an increase in the binding affinity of C/EBP for its cognate DNA sequence compared with the wild-type C/EBP protein.

Transcription factors are generally organized into discrete domains responsible for the functions of DNA binding and transactivation. However, phosphorylation within 1 domain may affect the function of other separate domains within the whole protein. This effect might occur due to a change in conformation of the transcription factor as a result of phosphorylation. Studies on the transcription factors Ets-like gene-1 (Elk-1) and sphingolipid activator protein-1 (SAP-1) have suggested that their activity is regulated in this manner.

For both of these transcription factors, phosphorylation of the C-terminal transactivation domain increased DNA binding via the N-terminal DNA binding domain. This suggests that the conformation change upon phosphorylation may unmask or alter the DNA binding surface of the transcription factor.

Therefore, we postulate that phosphorylation of C/EBP on Thr75 exerts such an effect on this transcription factor. It is possible that phosphorylation of Thr75 could change C/EBP from a “closed” conformation where it cannot bind DNA efficiently to a

![Figure 5. DNA binding affinity of C/EBP is altered by phosphorylation. Gel shift analysis was carried out on nuclear extracts derived from Jurkat cells transfected with wild-type and mutant C/EBP expression plasmids and an oligonucleotide containing the C/EBP site from the mim promoter. (A) Nuclear extracts from transfected Jurkat cells give a slow migrating band with this oligonucleotide. This retarded band can be competed by 100-fold excess of cold double-stranded (ds) oligonucleotide and supershifted by C/EBP-specific antisera. (B) EMSA was carried out using the same amount of nuclear extract with decreasing concentrations of hot oligonucleotides. The gel shifts were analyzed on a Phosphoimager (Molecular Dynamics, Piscataway, NJ) to obtain the radioactive counts of bound and free probe. Scatchard analysis determined the affinity of these C/EBP proteins for the C/EBP site in the mim promoter as follows: C/EBP 7.69 nM, C/EBP T75D 0.48 nM.

The C/EBP knockout mice are deficient in secondary granule gene expression. Therefore we were interested to examine whether these stably transfected 32Dc13 cells were expressing secondary granule genes, even when not undergoing differentiation. For this analysis RNA was isolated from exponentially growing 32Dc13 cells, parental and stably transfected, and Northern blot analysis was carried out (Figure 6B). The results from this experiment demonstrate that constitutive expression of C/EBP in 32Dc13 cells did affect endogenous secondary granule gene expression (Figure 6B). The secondary granule gene NGAL and B9 were both up-regulated in cells expressing wild-type C/EBP and C/EBP T75D (Figure 6B). However, more significantly, constitutive expression of C/EBP T75A did not result in the up-regulation of endogenous secondary granule gene expression (Figure 6B). This result demonstrates that phosphorylation of this threonine in the consensus MAP kinase site in C/EBP by p38 MAP kinase is important for the in vivo function of C/EBP.

Analysis of 32Dc13 cells expressing either C/EBP S181D or C/EBP S188D demonstrated that neither of these mutant proteins could up-regulate secondary granule gene expression. This lack of activity correlated with their level of activity in the luciferase-reporter assays (Figure 3).

![Figure 6. In vivo effects of constitutive expression of C/EBP on endogenous gene expression. (A) Expression of the C/EBP proteins from 32Dc13 stably transfected cells was analyzed by Western immunoblot on total cell lysate with an antibody specific for C/EBP. Results were visualized by ECL. GAPDH indicates glyceraldehyde 6-phosphate dehydrogenase. (B) Ten micrograms total RNA was examined by Northern blot analysis with probes for the secondary granule genes NGAL and B9. A probe specific for 18S was used as the loading control.
more “open” conformation allowing for much more efficient binding to its cognate DNA sequence.

Although we focused mainly on Thr75, we also assessed the effect of other phosphorylations by MAP kinases on C/EBPα function. The effect of phosphorylation on 2 serine residues within the repression domain of C/EBPα was studied. For these, the effects were mixed and not as dramatic as for Thr75. Dephosphorylation of Ser181 appeared to relocalize the C/EBPα protein to the cytoplasm. Whereas phosphorylation of Ser181 gave a C/EBPα protein that could bind DNA as well as wild-type C/EBPα but was inactive on a myeloid-specific promoter.

We analyzed individually these 3 MAP kinase sites: Thr75, Ser181, and Ser188. However, in vivo distinct protein kinases and phosphatases exist that orchestrate the responses of a transcription factor to a variety of external stimuli. These transcription factors frequently contain both stimulatory and inhibitory phosphorylation sites, which allows for a dynamic process resulting in the fine tuning of the transactivation potential. Thus phosphorylation is a mechanism that provides for tight regulation of the activity of a transcription factor rather than being a simple switch for turning both on and off the activity of a transcription factor.

With respect to C/EBPα, we suggest that Thr75 is frequently phosphorylated under normal growth conditions but that this is elevated upon other external stimuli. In this case, a stoichiometric shift occurs toward phospho-Thr75 in any given population of C/EBPα molecules. Also, under these conditions the molar ratio between activating and repressing phosphorylation sites would determine the magnitude of transactivation function of C/EBPα.

We next investigated the in vivo biologic effects of phosphorylation of C/EBPα. The 32Dc13 cells were stably transduced to constitutively express these C/EBPα proteins, both wild-type and phosphorylation mutants. C/EBPα plays a role in neutrophilic differentiation. Neutrophils are responsible for protecting the host against bacterial and fungal infections and are activated rapidly in response to inflammatory signals. In C/EBPα knockout mice it was found that the cells of the granulocytic lineage have an impaired ability to migrate, a reduced capacity to produce superoxide, and do not express secondary granule proteins. The p38 MAP kinase is involved in mediating intracellular signals resulting in superoxide production and cell migration. In human neutrophils, inhibition of p38 MAP kinase results in decreased superoxide anion production, reduction in adhesion and chemotaxis in response to fMLP (formyl-methionyl-leucyl-phenylalanine), and inhibition of release of interleukin-8 (IL-8) in response to lipopolysaccharide (LPS). Therefore, p38 MAP kinase appears to be important for neutrophil function.

The defects observed for the C/EBPα knockout mice are also observed in a number of diseases as well as other mouse knockout models. These include humans with specific granule disease, for which mutations of C/EBPα have been identified, and chronic granulomatous disease, as well as Rac2 knockout mice. More recently a human neutrophil immunodeficiency syndrome has been identified resulting from a mutation of Rac2 (ras-related C3 botulinum toxin substrate 2). Rac2 is a member of the Rho family of guanosine triphosphases (GTPases) that is highly expressed in myeloid cells and is a regulator of the nicotinamide adenine dinucleotide phosphate (NADPH)—oxidase complex. Therefore, the phenotype observed in C/EBPα knockout mice represents a defect intrinsic to neutrophil function.

Neutrophils are rapidly activated and short-lived. Therefore, C/EBPα has to be rapidly activated in response to an external stimulus. We hypothesize that C/EBPα may be phosphorylated in nonstimulated neutrophils and bound to its cognate DNA sequence. Activation of p38 MAP kinase signaling pathways could then result in the rapid phosphorylation of the threonine residue at amino acid 75 such that C/EBPα more efficiently binds DNA and can rapidly activate the transcription of genes necessary for neutrophil function.

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**References**

CCAAT enhancer binding protein (C/EBP) and AML1 (CBFα2) synergistically activate the macrophage colony-stimulating factor receptor promoter. Mol Cell Biol. 1996;16:1231-1240.


CCAAT/enhancer binding protein ?: changes in function upon phosphorylation by p38 MAP kinase

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