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Sumoylation and the function of CCAAT enhancer binding protein alpha (C/EBP α)

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Abstract

CCAAT enhancer binding protein alpha (C/EBP α) is the founding member of a family of basic region/leucine zipper (bzip) transcription factors and is a master regulator of granulopoiesis. It is expressed at high levels throughout myeloid differentiation and binds to the promoters of multiple myeloid-specific genes at different stages of myeloid maturation. Profound hematopoietic abnormalities occur in mice nullizygous for C/EBP α including a selective early block in the differentiation of granulocytes. Mutations in C/EBP α are present in a subset of patients with AML presenting with a normal karyotype. These mutations can result in the expression of a 30kD dominant negative C/EBP α isoform, which contributes to loss of C/EBP α function. The molecular basis for this observation remains unknown. In addition to phosphorylation, C/EBP α is modified, post-translationally by a small ubiquitin-related modifier (SUMO) at a lysine residue (K159), which lies within the growth inhibitory region of the C/EBP α protein. Sumoylation at K159 in the C/EBP α protein prevents association of the SWI/SNF chromatin remodeling complex with C/EBP α , thereby hampering transactivation. In this review, the functional implications of post-translational modification, particularly sumoylation, of C/EBP α in normal granulopoiesis and leukemia are considered.

Keywords

transcription factor; myeloid leukemia; post-translational modification

Introduction

CCAAT enhancer binding protein (C/EBP) family of transcription factors

CCAAT enhancer binding proteins represent a family of basic region-leucine zipper (bZIP) class of transcription factors that recognize the consensus DNA-binding sequence **5' TKNNGYAANK3'** (Y=C or T, K=T or G) within the regulatory regions of target genes. C/EBP family proteins bind DNA either as homo- or hetero- dimers. This family of transcription factors, which plays a crucial role in hematopoiesis, includes C/EBP α , β , γ , δ , ϵ and CHOP-GADD 153, each of which contain highly homologous C-terminal dimerization (leucine-zipper) domains and DNA-binding (basic-region) motifs, but differ in their N-terminal transactivation domains, with the exception of CHOP-GADD 153, which lacks this domain

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altogether [1,2]. CHOP-GADD 153 can dimerize with and inhibit transactivation by C/EBP α , β and ϵ , and is found at a breakpoint in the gene associated with liposarcomas [3]. With the exception of C/EBP ϵ , which is expressed at high levels mainly in the late stages of granulopoiesis and in T-lymphocytes, the other C/EBP members are expressed in a wide variety of cells including liver, adipose tissue, lung, intestine, adrenal gland, blood mononuclear cells and placenta [2]. The C/EBP family members exert pleiotropic effects in the tissues in which they are expressed. This may be due to their tissue and stage-specific expression, their ability to dimerize with members of their own family and of the Fos/Jun and ATF/CREB families of transcription factors, and their ability to interact with other transcription factors such as NF- κ B and Sp-1 [4,5]. The C/EBP factors regulate the differentiation of a variety of cellular systems.

CCAAT enhancer binding protein alpha (C/EBP α)

C/EBP α is the founding member of a family of basic region/leucine zipper (bzip) transcription factors and is a master regulator of granulopoiesis.[6–8]. It is expressed at high levels throughout myeloid differentiation and binds to the promoters of multiple myeloid- specific genes at different stages of myeloid maturation [6,7][8]. C/EBP α also plays a role in adipocyte differentiation; inhibition of C/EBP α blocks adipocyte differentiation and overexpression of this C/EBP family member induces adipocyte differentiation[9]. Regulation of constitutive hepatic genes as well as acute phase response genes in the liver involves several C/EBP family members, in particular C/EBP α [10].

Profound hematopoietic abnormalities occur in mice nullizygous for C/EBP α [11]. C/EBP α –/– mice die perinatally due to defects in gluconeogenesis resulting in hypoglycemia [12]; they also have a selective early block in the differentiation of granulocytes. Since the expression of C/EBP α is reduced in many myeloid leukemias, this transcription factor has been hypothesized to serve, in some settings, as a tumor suppressor.

The C/EBP α gene—The C/EBP α gene is intronless and generates two isoforms as a result the differential utilization of alternate translational start codons. The resultant p42kD (full length) and p30kD (truncated) C/EBP α proteins differ from each other at the N-terminus, which is abbreviated in the p30kD protein (see Figure 1). Three activation domains referred to as TE-I, TE-II and TE-III have been described in the full length p42 protein (see Figure 1). The p30kD C/EBP α isoform lacks the TE-I and TEII domains, which are known to bind to the basal transcriptional machinery (e.g., TATA box binding protein, TBP), thus making p30 a less efficient transactivator as compared to its full length counterpart. P30 is a dominant negative form of C/EBP α . This isoform heterodimerizes with p42 C/EBP α , thereby inhibiting the ability of the latter to transactivate target genes. Conditional ablation of C/EBP α in the marrow of mice results in a block at the transition from the common myeloid progenitors (CMPs) to granulocyte-monocyte progenitors (GMPs), resulting in the accumulation of immature myeloid blast cells in the marrow, similar to that observed in acute myeloid leukemia (AML). Additionally, mice in which the dominant-negative p30 kDa isoform is expressed but the p42 kDa isoform is disrupted, develop an AML-like disease (C. Nerlov, unpublished data). However, neither conventional nor conditional C/EBP α -knockout mice develop AML. The functional role of p30kD C/EBP α in myeloid cells is, therefore, more complex than previously thought, as the p30kD isoform rescues the p42kD C/EBP α null phenotype from early mortality, suggesting that an exclusive dominant negative role ascribed to this isoform may be an insufficient explanation[6].

Growth inhibition associated with C/EBP α —In addition to its ability to transactivate myeloid-specific genes, C/EBP α also arrests mitotic growth. This growth arrest is thought occur by three main mechanisms. First, C/EBP α can induce the expression of p21^{waf1/cip1}, a

CDK (cyclin-dependent kinase) inhibitor, and disrupt E2F transcriptional complexes during the G1 phase of the cell cycle[13–16]. Second, C/EBP α can mediate growth control through interaction with the cyclin-dependent kinases CDK2 and CDK4[17]. Third, C/EBP α can interact with the SWI/SNF chromatin-remodeling complex[8]. Thus C/EBP α acts to facilitate exit from the cell cycle as well as to upregulate gene expression specific to the terminally differentiated state. This transcription factor is thereby positioned to play a vital role in orchestrating normal differentiation.

C/EBP α and leukemogenesis—Several groups have reported mutations in the C/EBP α gene in a subset of patients with AML presenting with normal karyotypes [6]. These mutations can be broadly classified into two main categories. The first includes in-frame mutations clustered in the C-terminus of the C/EBP α protein. This domain harbors the highly conserved basic-region leucine-zipper responsible for DNA binding, as well as the dimerization domain. Often additional mutations in the second C/ebp α allele accompany these mutations resulting in functional loss of C/EBP α in the cells of these patients. The second category of mutations involves the N-terminus of C/EBP α . These frame-shift mutations are responsible for the premature termination of the full length p42 C/EBP α isoform while keeping the truncated p30C/EBP α protein intact. The remaining p42C/EBP α is rendered inactive by the dominant -negative activity of the p30 isoform[6]. Additionally, knock-in mice with a targeted mutation in the C/EBP α basic region that disrupts the C/EBP α –E2F association also develop myeloid leukemia.

No mutations in the C/EBP α locus have been reported in patients with FAB-M2 leukemia associated with either the t(8;21) (AML1-ETO) or inv(16) (CBF β -MYH11) translocation, despite the fact that leukemic cells derived from these patients express decreased levels of C/EBP α mRNA. Leukemias involving the internal tandem duplication (ITD) in FLT-3 (Fms-like tyrosine kinase 3) that constitutively activates FLT-3 kinase activity have been reported to block C/EBP α function via phosphorylation at Serine 21 (S21) in the C/EBP α protein. Hence, the increased FLT-3 activity appears to both increase cellular proliferation and increase cell viability and also prevent C/EBP α from inducing granulocytic differentiation [18]. Decreased expression of C/EBP α has also been described in AML with the t(8;21) translocation resulting in the AML1-ETO fusion protein. AML1-ETO is thought to have a negative effect on the positive autoregulation of C/EBP α . Other subtypes of AML including the t(3;21), resulting in AML-1-MDS-1-Evi-1 fusion protein and the inv(16) mutation are also associated with reduced expression of C/EBP α [8]. Downregulation of C/EBP α expression is a vital correlate in the development of some myeloid leukemias. Based on evidence from mouse models and from patients with AML, complete loss of C/EBP α is not necessary for AML development. However, mutations resulting in the loss of one or more vital function of C/EBP α , such as cell cycle exit or growth arrest, may contribute to the development of AML.

A study of six patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), harboring the t(14;19)(q32;q13) translocation, demonstrated over expression of C/EBP α at the mRNA and protein levels suggesting that C/EBP α overexpression may also be leukemogenic. Here activation of C/EBP α is thought to result from the juxtaposition of the immunoglobulin gene enhancer upstream of the C/EBP α gene [19]. Together these data suggest that increasing or decreasing the level of C/EBP α expression can contribute to leukemogenesis.

Post-translational modifications of C/EBP α —Phosphorylation and sumoylation appear to be important post-translational modifications affecting C/EBP α activity.

Phosphorylation—C/EBP α is phosphorylated at several sites, indicating a significant role for posttranslational modifications in mediating C/EBP α activity (Figure 1, Table 1). At S21, phosphorylation is mediated by extracellular signal receptor kinase (Erk1/2).[20]. In leukemias

harboring the FLT-3-ITD mutation, constitutive FLT-3 kinase activity results in sustained phosphorylation at S21 in the C/EBP α protein. Phosphorylation at S21 in turn has been shown to interfere with the ability of C/EBP α to induce granulocytic differentiation, an observation that may explain the differentiation block associated with leukemia [18].

Phosphorylation of C/EBP α at S248 is thought to be mediated by activated Ras in a manner stimulating in vitro granulopoiesis by enhancing the ability of C/EBP α to upregulate the expression of the granulocyte-colony stimulating factor receptor(G-CSFR)[21] via the C/EBP binding site in its promoter.

The phosphorylation status of C/EBP α at S193, which is located within the growth inhibitory region or synergy control region (Figure 1), plays a role in liver cell regeneration. Phosphorylation at S193 blocks proliferation of hepatocytes as a result of C/EBP α binding to cyclin-dependent kinase-2 (cdk-2) and to the ATPase subunit of the SWI/SNF chromatin remodeling complex [22]. Dephosphorylation of S193 on the other hand, promotes proliferation by preferentially binding the retinoblastoma protein (Rb) in the repressive Rb-E2F complex, thereby freeing E2F, a transcription factor essential for cell cycle progression, to activate the expression of genes required for G1/S progression. In hepatic tumors, activation of the PI3K/Akt signaling pathway leads to the accumulation of the protein phosphatase PP2A, which in turn dephosphorylates C/EBP α at S193 thereby promoting proliferation [23] [22]. A role for S193 in modulating granulopoiesis has not been reported in the literature thus far.

Finally, phosphorylation at T222, T226 and S230 (numbering as per rodent sequence), by GSK3 kinase (glycogen synthase kinase 3) influences preadipocyte differentiation[24]. Studies addressing the in vivo function of these phosphorylation sites are now beginning to emerge.

Sumoylation: SUMO (small ubiquitin –like modifier) reversibly and post-translationally modifies target proteins thereby altering protein function and fate. The list of sumoylated proteins is increasing and includes several that play pivotal roles in processes as diverse as transcriptional regulation, nuclear targeting, sub-nuclear targeting, chromatin structure and genome stability.

The pathway leading to SUMO conjugation involves an evolutionarily conserved three step process similar to ubiquitin conjugation (Figure 2). The SUMO peptide is first activated by the formation of a thioester bond between its C-terminal glycine and the catalytic cysteine of the E1 activating enzyme heterodimer (Aos/Uba2) in a process requiring ATP hydrolysis. Next, activated SUMO is transferred to the catalytic cysteine of the E2 conjugating enzyme Ubc9. The final step involves the transfer of the SUMO moiety from E2 to the specific substrate in the presence of an E3 ligases. Thus, sumoylation results in the formation of an isopeptide bond between the C-terminal carboxy group of SUMO and the ϵ -amino group of a lysine residue in the target protein which generally harbors the consensus sequence ϕ KXE, where ϕ is a hydrophobic amino acid, K is the sumoylated lysine, X is any amino acid and E is glutamic acid. Three families of E3 ligases have been described thus far. These include the PIAS (protein inhibitor of activated STAT) family of proteins; RanBP2, which is localized in the nuclear pore complex; and Pc2, which is a component of the polycomb protein complex [25] and Mms1. In addition, SUMO peptidases, or members of the Sentrin specific proteases (SENP) family, complete the cycle by deconjugating SUMO from target proteins (see Figure 2).

Four SUMO family members, SUMO1,2,3 and 4 have been described, all of which are approximately 100 amino acids in length. SUMO 2 and 3 are 95% identical, whereas SUMO 1 is approximately 45% identical to the other two family members. SUMO 2 and 3 probably share similar functions. In addition, seven mammalian SENP family members have been recognized thus far [25].

RanGAP1 was the first protein found to be modified by sumoylation. While unmodified RanGAP1 is found to be dispersed in the cytoplasm, sumoylation of this protein targets it to the nuclear pore complex[26]. Additionally, sumoylation targets promyelocytic leukemia (PML) and the homeodomain-interacting protein kinase 2 (HIPK2) to specific domains within the nucleus[27]. By knocking out the essential E2 conjugating Ubc9 enzyme, the sumoylation pathway was shown to be essential for nuclear integrity and normal segregation of chromosomes[28].

Sumoylation and Transcription: In general, sumoylation of transcription factors leads to inhibition of transcriptional activity. However, there are exceptions to this rule. For example, sumoylation of T-cell factor 4 (Tcf-4) and Ikaros results in enhanced transcriptional activation [29,30]. A number of mechanisms have been proposed to explain the impact of sumoylation on target proteins. SUMO likely competes with other post-translational modifications such as ubiquitination and acetylation, which compete for specific lysine residues within target proteins. This competition may lead to an alternate fate for the target protein as is the case with the inhibitor of NF κ B and murine double minute 2. Both these proteins are targeted for degradation by ubiquitination. However, competition for the target lysine residue by SUMO stabilizes these factors. Sumoylation of transcription factors is also thought to confer new protein-protein interaction properties, which in turn can significantly alter transcription activity. Histone deacetylases (HDACs) are generally correlated with repression of transcription and can be recruited to sumoylated transcription factor complexes. For example, SUMO modification of the transcription factor Elk-1 has been correlated with increased association with HDAC2 [31]. Additionally, sumoylation of the co-activator p300 is associated with increased levels of HDAC6[32]. HDACs are thus thought to contribute a broad, but context dependent role in SUMO-associated repression. Proteins which themselves do not undergo SUMO modification but are capable of interacting with sumoylated substrates do so via a SUMO-interacting motif (SIM). Deletion of the SIM in the SUMO E3 ligase PIASx disrupts the non-covalent binding of this protein to SUMO[33]. Thus, proteins with SIMs may contribute to the repressive effects of SUMO-modification via non-covalent interactions, which are most likely context dependent.

Sumoylation of C/EBP α : C/EBP α is modified by SUMO-1 at lysine residue 159, which lies within a conserved negative subdomain within the TE-III domain (see Figure 1) referred to as the synergy control motif [34]. Synergy control motifs have been described in the negative regulatory domains of several transcription factors, and have been found to be sites of sumoylation [35]. Sumoylation of C/EBP α dramatically decreases its ability to transactivate the liver specific albumin gene in the presence of BRG1, the core subunit of the ATPase dependent SWI/SNF chromatin remodeling complex. In addition, sumoylated C/EBP α fails to induce proliferation arrest because its interaction with BRG1 is inhibited. SUMO modification alters C/EBP α transcriptional activity by affecting its repertoire of protein-protein interactive partners[36].

Both C/EBP α p42 and p30 transactivate the myeloid-specific lactoferrin gene promoter harboring a C/EBP site, by approximately 2.5–3 fold above the activity of LF89 (a reporter gene plasmid harboring a C/EBP-responsive motif in the lactoferrin promoter) alone. Cotransfection of p42 with an expression plasmid for Sumo-1 however, significantly reduces LF89 driven luciferase activity compared to p42 alone. In comparison, Sumo-1 has a much less profound effect on the reduction of p30 driven LF89 luciferase activity (manuscript in preparation). Overexpression of the p30 isoform of C/EBP α in an erythroleukemia cell line results in upregulation of Ubc9, which is essential for sumoylation (Figure 2)(37). Upregulation of Ubc9 sumoylates the p42 isoform, thereby rendering it inactive with respect to transactivation of a C/EBP responsive reporter plasmid in transactivation assays. Sumoylation of p42 C/EBP α in AML may be the result of increased expression of the p30 isoform(37). It

is unclear if upregulation of Ubc9 by the p30 isoform of C/EBP α acts exclusively through sumoylation of C/EBP α p42, or whether other sumoylation targets also come into play. In this context, sumoylation of other C/EBP family members also occurs and the functional impact on these transcription factors is documented. Also, the issue of sumoylation of p30 itself was not addressed. Our laboratory has demonstrated that C/EBP α p30 can be sumoylated. We have shown that a) mutations of the sumoylated lysine residue in both p42 and p30 (lysine to alanine) renders both C/EBP α isoforms resistant to Sumo-1 mediated repression of LF89 and b) that sumoylated levels of C/EBP α decrease upon neutrophil maturation and that expression of the lactoferrin gene in the developing neutrophil correlates with loss of binding of sumoylated C/EBP α p42 to the LF promoter (manuscript in preparation).

Since the ratio of p42:p30 is altered in acute myeloid leukemias (AML) we hypothesize that the differential effect of Sumo-1 on p42 versus p30 may contribute to blocking preferentially the activity of p42, which in turn may contribute to the leukemic phenotype. In this context we have shown that sumoylated p42 remains persistently bound to the LF promoter in the acute promyelocytic leukemia cell line NB4 following ATRA-induction (manuscript in preparation). This observation is correlated with lack of lactoferrin expression in these cells. We also show that regardless of its sumoylation status, the p30 C/EBP α isoform binds poorly to the LF promoter in a C/EBP binding site pulldown assay, compared to its full length counterpart. This suggests that sumoylation of the two C/EBP α isoforms has functionally disparate effects, possibly due to differential protein-protein binding properties of each sumoylated isoform.

Thus, upregulation of p30 in leukemic cells may result in increased levels of Ubc9, which in turn may aid in sumoylating both C/EBP α p42 and p30, the functional implications of which add an additional layer of complexity to the altered function of C/EBP α in AML.

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Figure 1. A map of the structural features of the human C/EBP α protein

The transactivation domains TE-I, TE-II and TE-III are shown. Translation start sites for the two C/EBP α isoforms p42 and p30 are indicated by arrows. Phosphorylation sites (indicated by vertical arrows) and the single sumoylation site (indicated by a solid diamond) are shown. Protein-protein interacting partners of C/EBP α are indicated by horizontal arrows. Bzip marks the basic leucine zipper motifs.

Figure 2. The Sumoylation pathway

A general schema of the multi-step process that leads to sumoylation of a target protein. 1. The SUMO peptide is activated by the formation of a thioester bond between its C-terminal glycine and the catalytic cysteine of the E1 activating enzyme heterodimer (Aos/Uba2) in a process requiring ATP hydrolysis. 2. activated SUMO is transferred to the catalytic cysteine of the E2 conjugating enzyme Ubc9. 3. The SUMO moiety is next transferred from E2 to the specific substrate often harboring a lysine residue within the consensus sequence Ψ KXE, where Ψ is a large hydrophobic residue and K the modified lysine, in the presence of an E3 ligases. 4. Sumo-specific peptidases (SENPs) complete the cycle by removing the Sumo moiety from target substrates.

Table 1Effects of Phosphorylation on the function of C/EBP α .

Pathway	Phosphorylated residue	Biological effect	Refs
Flt-3 (activated) via ERK1/2 kinase	S21	Blocks C/EBP α -induced granulocytic differentiation	[18][20]
Glycogen synthase kinase (GSK3)	T222, T226 (rodent residue numbers) S230	Effects preadipocyte differentiation	[24]
RAS signaling	S248	Increases transactivation of the G-CSF receptor thereby upregulating granulocytic differentiation	[21]
?	S193	Phosphorylation at S193 promotes binding of CDK2 and Brm1 to C/EBP α , thereby inhibiting proliferation in hepatocytes	[22]
PI3K/Akt activation increases PP2A	Dephosphorylation at S193	Accelerates proliferation of hepatocytes via Rb:E2F interactions	[23]