REVIEW ARTICLE

CCAAT/enhancer-binding proteins: structure, function and regulation

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CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that all contain a highly conserved, basic-leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding. At least six members of the family have been isolated and characterized to date (C/EBPα–C/EBPζ), with further diversity produced by the generation of different sized polypeptides, predominantly by differential use of translation initiation sites, and extensive protein–protein interactions both within the family and with other transcription factors. The function of the C/EBPs has recently been investigated by a number of approaches, including studies on mice that lack specific members, and has identified pivotal roles of the family in the control of cellular proliferation and differentiation, metabolism, inflammation and numerous other responses, particularly in hepatocytes, adipocytes and haematopoietic cells. The expression of the C/EBPs is regulated at multiple levels during several physiological and pathophysiological conditions through the action of a range of factors, including hormones, mitogens, cytokines, nutrients and certain toxins. The mechanisms through which the C/EBP members are regulated during such conditions have also been the focus of several recent studies and have revealed an immense complexity with the potential existence of cell/tissue- and species-specific differences. This review deals with the structure, biological function and the regulation of the C/EBP family.

Key words: differentiation, inflammation, metabolism, proliferation, transcription factors.

INTRODUCTION

The dramatic change in gene expression that accompanies processes such as differentiation, proliferation and the execution of specialized cellular function is controlled mainly at the transcriptional level. Such control is achieved through specific transcription factors that interact with regulatory sequences present in the promoter and enhancer regions of target genes. Studies in the last two decades on the regulation of numerous such genes have led to the identification of key transcription factors that act as master regulators of many cellular responses. Transcription factors belonging to the CCAAT/enhancer-binding protein (C/EBP) family fall in this category, with many pathophysiological conditions associated with their defective function.

The first C/EBP protein was identified in the laboratory of Steve McKnight as a heat-stable factor in rat liver nuclei that was capable of interacting with the CCAAT box motif present in several cellular gene promoters and a 'core homology' sequence found in certain viral enhancers [1]. The C/EBP gene was cloned in 1988 [2], and detailed studies on it led to the discovery of the basic-leucine zipper (bZIP) class of DNA-binding and dimerization domain [2–5], which is now known to be present in a battery of other transcription factors [6]. By 1992, five other members of the C/EBP family had been identified, all of which contain a conserved bZIP domain at the C-terminus [7–14]. The function of the C/EBP family has been investigated in detail over the last decade and has identified pivotal roles for the proteins in numerous cellular responses, including the control of cellular growth and differentiation, immune and inflammatory processes and various diseases. The expression of the C/EBPs has also been found to change markedly during a number of physiological and pathophysiological conditions through the action of extracellular signals. This review summarizes key recent findings in relation to the structure, function and regulation of the C/EBP family.

STRUCTURE, PROPERTIES AND EXPRESSION PROFILE

The genes for six C/EBP members have been cloned to date from several species, with many of them being characterized independently in different laboratories and given distinct names (e.g. [7–14]). However, in order to avoid confusion, a systematic nomenclature was proposed by Cao and co-workers [12] in which members are designated as C/EBP followed by a Greek letter indicating the chronological order of their discovery (C/EBPα–C/EBPζ). Table 1 lists the C/EBP genes that have been cloned from different species, along with the alternative

Abbreviations used: AP, activator protein; APR, acute-phase response; ATF, activating transcription factor; BAT, brown adipose tissue; BTEB, basic transcription element-binding protein; bZIP, basic-leucine zipper; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP-responsive element; CREB, cAMP-response-element-binding protein; CUP, C/EBP undifferentiated protein; EGF, epidermal growth factor; ER, endoplasmic reticulum; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte–macrophage colony stimulating factor; GRP, glucose-regulated protein; IFN-γ, interferon-γ; IL, interleukin; LAP, liver activating protein; LIP, liver inhibitory protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony stimulating factor; mim-1, myb-induced myeloid protein-1; MIP, macrophage inflammatory protein; mTOR, mammalian target of rapamycin; NF, nuclear factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-kinase, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PPAR, peroxisome-proliferator-activated receptor; Rb, retinoblastoma; RSK, ribosomal protein S-6 kinase; Runx, Runt domain factor; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TLS, translocated in liposarcoma; TNFα, tumour necrosis factor-α; TPA, tumour promoter activators; USF, upstream stimulating factor; uORF, upstream open reading frame; UVC, UV light band C; VIP, vasoactive intestinal peptide; WAT, white adipose tissue.

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Table 1 Nomenclature of C/EBP genes

Gene*	Alternative name	Source	References
C/EBPα	C/EBP, RcC/EBP-1	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Rana catesbeiana*, fish	[2,12,13,17–20]
C/EBPβ	NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Aplysia*, fish	[7–10,12,13,20–24]
C/EBPγ	Ig/EBP-1	Rat, mouse, human, chicken, fish	[11,20]
C/EBPδ	NF-IL6β, CRP3, CELF, RcC/EBP2	Rat, mouse, human, *Rana catesbeiana*, bovine, ovine, fish	[12,13,19,20,25–27]
C/EBPϵ	CRP-1	Rat, mouse, human, ovine, fish	[13,24,28–31]
C/EBPζ	CHOP-10, GADD153	Mouse, rat, human, hamster	[14,32,33]

* It has not yet been possible to classify a *Drosophila* and a novel myeloid-restricted zebrafish C/EBP protein as homologues of these genes [15,16].

nomenclature. The C/EBPα, -β, -δ and -γ genes are intronless, whereas C/EBPϵ and -ζ contain two and four exons respectively [7–14,28–33]. An optimal C/EBP binding site has been determined to be a dyad symmetrical repeat RTTGCGYAAY, where R is A or G, and Y is C or T [34]. However, substantial variations are tolerated, and most sites contain a conserved half-site paired with a more divergent sequence that contains at least 2 bp of the consensus [34].

All C/EBP isoforms share substantial sequence identity ($> 90\,\%$) in the C-terminal 55–65 amino acid residues, which contains the bZIP domain [2–14]. This domain consists of basic-amino-acid-rich DNA-binding region followed by a dimerization motif termed the 'leucine zipper' [2–14]. Figure 1 shows the structure of the C/EBPβ bZIP region homodimer bound to DNA. The leucine zipper of the bZIP domain consists of a heptad repeat of four or five leucine residues that assume an α-helical configuration, with two such repeats being able to interdigitate in a parallel manner to form a coiled-coil structure [3,4,6]. Electrostatic interactions between amino acids along the dimerization interface determine the specificity of dimer formation [4,6,37]. Dimerization is a prerequisite for DNA binding, which is mediated by the basic region, which also assumes an α-helical structure [4,6]. The basic region is in constant register relative to the leucine zipper, with changes in the spacing between the two domains leading to a loss of binding activity [5]. The specificity of DNA binding is dictated by the sequence of amino acids within the basic region [38]. According to a model for DNA binding by bZIP proteins [4,6], the dimer forms an inverted Y-shaped structure in which each arm of the Y is made of the basic region, which binds to one half of a palindromic recognition sequence in the DNA major groove like a fork or a pair of scissors (Figure 1).

Because of the high conservation in the bZIP domain, the different C/EBP proteins are able to form heterodimers in all intrafamilial combinations and, with the exception of C/EBPζ, interact with an identical recognition sequence, at least *in vitro* [7–15,21–22,29]. C/EBPζ, on the other hand, contains two proline residues in the basic region that disrupts its α-helical structure [14]. As a result, C/EBPζ can readily dimerize with other members of the family, owing to the presence of an intact leucine zipper, but such heterodimers cannot bind to a C/EBP recognition sequence in the promoter of target genes [14]. However, C/EBPζ/C/EBP heterodimers can bind to a different DNA sequence [consensus PuPuPuTGCAAT(A/C)CCC, where Pu is a purine] in the promoter regions of a subset of genes under conditions of cellular stress, and activate gene transcription [39]. Thus, C/EBPζ can act both as an inhibitor of C/EBP function and as a direct activator of other genes, depending on the cellular state.

In contrast with the bZIP domain, the N-termini of the C/EBP proteins are quite divergent ($< 20\,\%$ sequence identity), except for three short subregions that are conserved in most members [40–45]. These three subregions have been shown variously to represent the activation domains (Figure 2) that interact with components of the basal transcription apparatus and stimulate transcription [40–44]. The major exception is C/EBPγ, which lacks an activation domain and, therefore, represses gene transcription by forming inactive heterodimers with other members [45]. The N-termini of some C/EBP proteins also contain negative regulatory regions (Figure 2) whose precise function remains to be elucidated in detail [40,42–44,46,47].

Despite the existence of six genes, the number of C/EBP proteins that can be present in any given tissue/cell type may be considerably higher (Figure 2). First, different sized polypeptides can be produced for C/EBPα and -β either by alternative use of translation initiation codons in the same mRNA molecule due to a leaky ribosome scanning mechanism, or regulated proteolysis [48–51], and for C/EBPϵ via alternative use of promoters and differential splicing [30,52]. Thus C/EBPα mRNA can give rise to two polypeptides, 42 kDa and 30 kDa, with the latter having a lower activation potential [49,50]. C/EBPβ mRNA can produce at least three isoforms, 38 kDa (LAP*), 35 kDa (LAP) and 20 kDa (LIP), with the LAP and the LIP forms being the major polypeptides produced in cells [48]. LAP contains both the activation and the bZIP domains, whereas only the latter is present in LIP (Figure 2) [48]. LIP can, therefore, act as a dominant-negative inhibitor of C/EBP function by forming non-functional heterodimers with the other members [48]. For C/EBPϵ, at least four isoforms can be produced (32 kDa, 30 kDa, 27 kDa and 14 kDa), of which, the activation potential of the 30 kDa form is lower than the 32 kDa form, and the 14 kDa form lacks an intact transcriptional activation domain (Figure 2) [30,52]. Secondly, as stated above, the different members are capable of forming heterodimers in all intrafamilial combinations [7–15]. Because the *trans*-activation potential of the various members differs [8,25,40,53], such heterodimerization is likely to have a profound effect on the regulation of target genes. Thirdly, the C/EBPs can form protein–protein interactions with other bZIP and non-bZIP transcription factors [54–56].

The tissue/cell expression profile of the different C/EBP isoforms has been determined at the level of both mRNA and protein [7–14,17,28–30,57]. However, some discrepancies between different reports on the expression pattern exist, which may be due to species-specific differences and/or the use of different techniques. In addition, for some members the expression of the protein does not correlate with that for mRNA (e.g. [9,13]), thereby indicating the extensive use of tissue-specific post-transcriptional regulatory mechanisms. In general, C/EBPα is

Figure 1 Structure of the C/EBPβ basic region/leucine zipper domain bound to DNA

The two α-helical basic regions (bottom) dimerize through the α-helical leucine zipper domain (top) to form an inverted Y-shaped structure. Each arm of the Y is formed by a single α helix, one from each monomer, which binds to one-half of a palindromic recognition sequence. See the text and [35,36] for more details. The structure was produced from the Protein Data Bank entry IH8A using the Rasmol program.

expressed at high levels in the adipose tissue, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta [12,13,17,57]. In the liver and in adipose tissue, the highest levels of mRNA are present in the terminally differentiated cells [12,13,57]. Constitutive expression of C/EBPβ is particularly high in the liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells [7–10,12,13,21,22], whereas that for C/EBPδ occurs in the adipose tissue, lung and intestine [12,13,25]. On the other hand, C/EBPγ and C/EBPζ are expressed ubiquitously [11,14], and C/EBPϵ mRNA and protein are restricted primarily to myeloid and lymphoid cells [28–30,52]. The expression profile of different members is also regulated during physiological and pathophysiological changes by a range of extracellular mediators, and this is described in more detail below in the relevant sections.

FUNCTION OF THE C/EBP FAMILY

The function of the C/EBP family has recently been investigated in detail using a number of approaches, including analysis of the promoter regions of target genes, overexpression or inhibition approaches in cell-culture-based model systems and studies on knockout mice. These have identified pivotal roles of the family in a number of processes, including differentiation, the inflammatory response, liver regeneration, metabolism and numerous other cellular responses. These aspects are addressed below in detail, with Table 2 listing examples of target genes for C/EBP action during some of these processes.

Cellular differentiation

Adipocytes

Three classes of transcription factors, peroxisome-proliferator-activated receptor-γ (PPARγ), C/EBPs and adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1c, have been implicated as key regulators of adipogenesis [65–67]. The roles of these factors in adipocyte differentiation have been the subject of several excellent recent reviews [65–67], and thus the importance of only the C/EBPs will be described briefly. The original observation that C/EBPα is expressed at high levels in terminally differentiated cells of the adipose tissue promoted numerous studies investigating the role of the family in adipogenesis. The pre-adipocyte 3T3-L1 and the related 3T3-F442A cell lines have been used extensively to study this process [65–67]. Differentiation of growth-arrested pre-adipocytes is initiated by the addition of adipogenic hormones, which includes a cAMP elevating agent, a glucocorticoid, a hormone that interacts with the insulin-like-growth-factor receptors, and fetal-calf serum [65–67]. This treatment causes the pre-adipocytes to synchronously re-enter the cell cycle and undergo approximately two rounds of mitosis (mitotic clonal expansion) [65–67]. Both C/EBPβ and C/EBPδ mRNA are induced during this phase in response to cAMP-elevating agents and glucocorticoid respectively [12]. The pre-adipocytes then exit the cell cycle and begin to express C/EBPα, which is then followed by the induction of adipocyte-specific markers [65–67]. Because the proximal promoter of the mouse C/EBPα gene can be activated by all three C/EBP isoforms (α, β and δ), owing to the presence of a C/EBP recognition sequence [88], it has been postulated that C/EBPα expression is activated by the binding of C/EBPβ and C/EBPδ, which are induced early in the differentiation programme [12,67,88]. These two C/EBP isoforms have also been implicated in the induction of PPARγ expression [89]. In addition, both C/EBPα and PPARγ are able to induce each other's expression via a positive-feedback loop, which then promotes and maintains the differentiated state [65,66].

The importance of the C/EBP family in adipocyte differentiation has been confirmed by a number of approaches. First, ectopic expression of C/EBPα and C/EBPβ in 3T3-L1 cells is able to initiate the differentiation programme in the absence of adipogenic hormones, whereas overexpression of C/EBPδ accelerates the process triggered by these agents [90–92]. Secondly, expression of antisense C/EBPα RNA in 3T3-L1 cells blocks differentiation [93]. Thirdly, embryonic fibroblasts lacking both C/EBPβ and C/EBPδ are unable to initiate the differentiation programme in response to hormonal stimulation [94]. Fourthly, C/EBPα-deficient mice, which die soon after birth due to hypoglycaemia (see below), have dramatically reduced lipid accumulation in the adipose tissue [61].

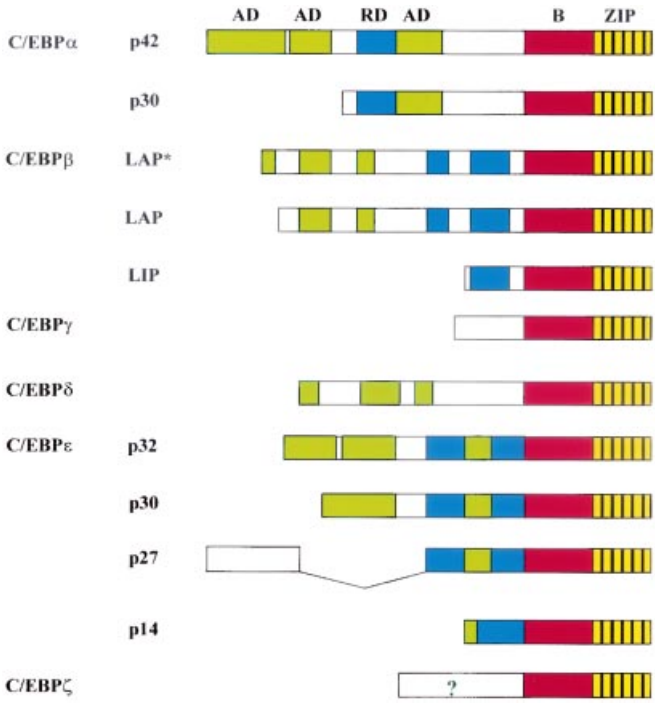

Figure 2 Schematic representation of the C/EBP family members

The leucine zipper is shown in yellow, with black vertical lines indicating the leucine residues, and the basic region is coloured red. The position of the activation domains (AD) and negative regulatory domains (RD) [40–44,47] are shown in green and blue respectively. ? indicates that the N-terminus of C/EBPζ contains an activation domain, although its exact position remains to be determined [39]. The mechanism for the formation of the different C/EBP α, β and ϵ polypeptides is described in the text and [30,48–52].

Despite the advances detailed above, several aspects of C/EBP function during adipogenesis remain unresolved and require further detailed investigation. The first concerns the precise role of individual C/EBP members in the differentiation of white adipose tissue (WAT) versus brown adipose tissue (BAT) (see [66]). Some progress in addressing this aspect, however, has been made recently. For example, C/EBPα-deficient mice rescued from early death by re-expression of the gene in the liver show an absence of WAT in many depots, whereas their BAT has minimal biochemical alteration [95]. On the other hand, mice that lack either the β or the δ isoform have normal WAT, but their BAT show reduced accumulation of lipids and expression of uncoupling protein-1 [94]. Mice that lack both these C/EBP genes have a high rate of mortality in the perinatal period, but those that survive have drastically reduced BAT, with only slight decreases in WAT [94]. Another area for further investigation relates to the different pathways that operate in adipogenesis. For example, the observation that the WAT and BAT of both C/EBPβ and C/EBPδ knockout mice express normal levels of PPARγ and C/EBPα [94] implicates the existence of an alternative pathway for the activation of these two genes. Progress in the identification of the relative roles of C/EBPα and PPARγ in adipogenesis has been made recently by the demonstration that whilst PPARγ is able to promote adipogenesis in C/EBPα-deficient cells, the converse is not possible [96,97]. This suggests that C/EBPα and PPARγ participate in a single pathway in adipogenesis, with PPARγ being the most proximal effector of the process.

Myeloid cells

Binding sites for the C/EBPs are present in the promoter regions of numerous genes that are expressed in myeloid cells (Table 2). Indeed, four members of the family (α, β, δ and ϵ) are expressed in myeloid cells and show a unique expression profile in tissue-culture-based myeloid-cell differentiation systems [30,98–100]. For example, the expression of C/EBPα is relatively high in early myeloid progenitors and decreases during granulocytic differentiation [98]. On the other hand, the myeloid-restricted member, C/EBPϵ, is preferentially expressed during granulocytic differentiation [30,99], whereas C/EBPβ is up-regulated during macrophage differentiation [100]. In addition, ectopic expression of the C/EBPs has been shown to activate a number of target genes in myeloid cells [52,71,73,74]. However, the most compelling evidence for a crucial role of the C/EBPs in myeloid-cell differentiation and maturation has come from studies on knockout mice. Thus, C/EBPα-deficient mice fail to undergo myeloid differentiation beyond the myeloblast stage and, therefore, lack mature neutrophils [77]. This defect correlates with a lack of receptors for granulocyte colony stimulating factor (G-CSF) or interleukin-6 (IL-6), and expression of these receptors in C/EBPα-deficient progenitors restores granulopoiesis [77,78]. C/EBPϵ-deficient mice, on the other hand, fail to produce atypical neutrophils, as indicated by morphological analysis, defective oxidative burst, delayed response to inflammatory challenge and impaired bacteriocidal responses [72,80]. In addition, these mice usually succumb to opportunistic infections by 3–5 months of age [72,80]. The phenotype of these mice is very similar to patients with a rare neutrophil-specific granulocyte deficiency [52]. Interestingly, two such patients have been shown to have defects in the C/EBPϵ gene, which leads to the production of a non-functional protein [101,102]. More recently, macrophage functional maturation and cytokine production was found to be impaired in C/EBPϵ-deficient mice [81]. Finally, the phenotype of C/EBPβ-deficient mice indicates a potential role in the activation and/or differentiation of macrophages [71,103,104]. These mice are highly susceptible to infections by *Listeria monocytogenes*, *Candida albicans* and *Salmonella typhi*, which, in part, may be due to defects in macrophage activation and the escape of phagocytosed bacteria from the phagosome to the cytoplasm [71,103,104]. Other defects include impaired nitric oxide production by splenic macrophages, low levels of IL-12 (produced normally by activated macrophages) and impaired Th1 immune response [71,103,104].

Other cells

The importance of the C/EBP family in cellular differentiation is not restricted to adipocytes and myelomonocytic cells, but also extends to other cell types, including hepatocytes, mammary epithelial cells, ovarian luteal cells, keratinocytes, neuronal cells and intestinal epithelial cells [57,85–87,105–107]. Binding sites for the C/EBPs are present in the promoter regions of numerous genes in hepatocytes that are involved in the maintenance of normal function and responses to injury (Table 2). In addition, C/EBPα-deficient mice show profound derangement in liver structure with acinar formation, resembling proliferative or pseudoglandular hepatocellular carcinoma [82,108]. These results, therefore, implicate a pivotal role of the C/EBPs in the regulation of terminal hepatocyte differentiation and function. Analysis of C/EBPβ knockout mice has shown that this isoform is required for the functional differentiation of mammary epithelial cells and the expression of milk protein genes [86,87]. In addition, the gene is required for the proper proliferation and

Table 2 Examples of target genes regulated by the C/EBP family

For brevity, further abbreviations have not been defined here; see the abbreviations footnote.

Tissue/cell type	Target gene	References
Hepatocytes	Albumin, α_1-acid glycoprotein, bilirubin UDP-glucuronosyltransferase, C-reactive protein, complement C3, Factor IX, glucose-6-phosphatase, glycogen synthase, haptoglobin, haemopexin, ornithine-cycle-enzyme genes, PEPCK, serum amyloid A, tyrosine aminotransferase	[8–10,41,58–64]
Adipocytes	Fatty acid binding protein (aP2 or 422), insulin-responsive glucose transporter, leptin, obese, PPAR-γ, steroyl-CoA desaturase 1, uncoupling protein-1	[58,61,65–70]
Myeloid cells	CD14, cyclo-oxygenase-2, G-CSF, G-CSF receptor, GM-CSF receptor, IFN-γ, IL-1β, IL-6, IL-8, IL-12 p40, inducible nitric oxide synthase, lactoferrin, lysozyme, MCP-1, M-CSF receptor, mim1, MIP-1α, MIP-1β, myeloperoxidase, neutrophil elastase, TNF-α	[21,52,58,71–81]
Lung	Clara-cell secretory protein, surfactant proteins	[82–84]
Keratinocytes	Keratin 1 and 10	[85]
Mammary gland	Milk protein genes	[86,87]

morphogenic responses during mammary-gland development [86,87]. Adult female C/EBPβ-deficient mice also have been found to be sterile with the ovaries lacking the corpora lutea [105]. Further studies revealed that the isoform was essential for periovulatory granulosa cell differentiation in response to luteinizing hormone [105]. Finally, the C/EBPs have been implicated in the differentiation of neuronal cells, intestinal epithelial cells and keratinocytes on the basis of expression profile and/or presence of binding sites in the promoter regions of genes activated during the process, although the link needs to be investigated in detail [85,106,107].

Control of metabolism

Shortly after the discovery that C/EBPα was expressed at high levels in the liver, lung and the adipose tissue, McKnight and co-workers speculated that this isoform will be '… a central regulator of energy metabolism …' [109]. This proposition has been found to be true not only for C/EBPα, but also for C/EBPβ. As mentioned above, C/EBPα-deficient mice die soon after birth due to severe hypoglycaemia because of reduced expression of glycogen synthase, leading to undetectable levels of glycogen in the liver and decreased levels of three gluconeogenic enzymes [glucose-6-phosphatase, phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase], which impairs the ability to synthesize glucose *de novo* [61]. These mice also have impaired expression of ornithine-cycle enzymes, leading to higher blood concentrations of ammonia compared with the wild-type counterpart [63]. Conditional knockout of the C/EBPα gene specifically in the liver also leads to major metabolic derangement due to reduced transcription of several genes, particularly those involved in glycogen synthesis, gluconeogenesis, and bilirubin detoxification [59]. As detailed above, the C/EBPα-deficient mice also have reduced lipid droplets in the adipose tissue [61].

Investigation of the exact role of C/EBPβ in metabolism has been difficult because of at least two different phenotypes of knockout mice (A and B) [62]. Mice with the B-phenotype die soon after birth owing to hypoglycaemia, because of an inability to mobilize their hepatic glycogen and express PEPCK [110]. Mice with the A phenotype survive to adulthood, but display fasting hypoglycaemia, reduced blood lipids and impaired hepatic glucose production and adipose-tissue lipolysis in response to stimulation with hormones such as glucagon and adrenaline [110,111]. This has been attributed mainly to altered levels of hepatic cAMP production and the activity of protein kinase A [110,111].

Overall, these studies suggest that C/EBPα plays a more general role in energy metabolism, whereas C/EBPβ is involved in fine-tuning responses to nutritional changes.

Inflammation

The activity and/or expression level of three C/EBP members (α, β and δ) is regulated by a number of inflammatory agents, including lipopolysaccharide (LPS) and a range of cytokines [7,8,25,53,60,71,112–115]. For example, the expression of C/EBPβ and C/EBPδ mRNA is induced by inflammatory stimuli in a number of cell types, such as hepatocytes, macrophages, renal mesangial cells and astroglial cells, whereas that for C/EBPα is inhibited [7,8,25,53,60,71,112–115]. Indeed, C/EBPβ was first identified on the basis of its ability to regulate gene transcription in response to IL-1 and IL-6 [7,8]. Subsequent studies have led to the identification of binding sites for the C/EBPs in the regulatory regions of a battery of genes that are involved in the inflammatory response, including those coding for cytokines and their receptors, acute-phase plasma proteins synthesized by hepatocytes, and components of signal transduction pathways (Table 2). Further support for an important role of the C/EBPs in the inflammatory response has been provided by studies on knockout mice. For example, C/EBPϵ-deficient mice show delayed migration of neutrophils to an *in vivo* inflammatory challenge along with an impaired bactericidal response [80]. In addition, a number of genes that are involved in the immune and inflammatory responses are down-regulated in the macrophages of these mice [72,81]. C/EBPβ-deficient mice show impaired expression of serum amyloid A and P proteins, α_1-acid glycoprotein, complement C3 component and tumour necrosis factor-α (TNFα) [71,103,104]. However, not all the targets of C/EBPβ action during an inflammatory response are affected in these mice [71]. It is likely that this may be due to functional compensation by other members that are expressed at normal levels. In addition, because the induction of both C/EBPβ and C/EBPδ occurs relatively late following an inflammatory stimuli, it is possible that factors such as nuclear factor κB (NF-κB) and STATs (signal transducers and activators of transcription), whose activation during inflammation is much faster, and only transient, may be responsible for the primary induction of genes followed by the C/EBPs a few hours later [71].

Cellular proliferation

The initial observation that C/EBPα was expressed at high levels in terminally differentiated cells and down-regulated during proliferation (e.g. following partial hepactomy) suggested an

antiproliferative role of this isoform [57]. Indeed, C/EBPα is a strong inhibitor of cell proliferation when overexpressed in cultured cells [57,116]. In addition, hepatocytes from newborn C/EBPα-deficient mice display increased proliferative activity, and similarly, hyperproliferation of alveolar type II cells is seen in the lungs of the animals [82]. More recently, mutations in the C/EBPα gene have been described in patients with acute myeloid leukaemia [117]. Although C/EBPα is a transcription factor, its ability to arrest growth does not require its DNA-binding activity, but is mediated via protein–protein interaction [118]. C/EBPα has been found to interact with several proteins that are involved in the control of cell-cycle progression, thereby indicating the existence of multiple pathways through which this isoform mediates growth arrest [118–123]. For example, the C/EBPα-mediated growth arrest is accompanied by increased expression of a cell-cycle inhibitor, p21, and the isoform has been shown to interact with, and thereby stabilize, the protein [119]. More recently, C/EBPα has been shown to interact with the cyclin-dependent kinases cdk2 and cdk4 and arrest cell proliferation by inhibiting their activity [122]. In the case of cdk4, it has been shown further that this interaction leads to a proteasome-dependent degradation of the protein [123].

C/EBPβ, on the other hand, appears to play an important role in promoting proliferation, and its levels are increased in a number of tumours [124–127]. Partial hepactomy is associated with increased expression of C/EBPβ, and hepatocytes of mice deficient in this member do not proliferate normally during this process [124]. This abnormal regenerative response is associated with prolonged hypoglycaemia and altered expression of several genes that are important for hepatocyte gluconeogenesis and proliferation [124]. More recently, Zhu et al. [125] have shown that C/EBPβ-deficient mice also are completely refractory to skin tumour development induced by a variety of carcinogens and carcinogenesis protocols. In v-Ha-ras transgenic mice, C/EBPβ deficiency results in a significant reduction in tumorigenesis, thereby linking the proto-oncogene *ras* and C/EBPβ [125]. In addition, oncogenic *ras* stimulates C/EBPβ *trans*-activation function, with mutation of an extracellular-signal-regulated-kinase-1/2 phosphorylation site (Thr^{188}), abolishing this effect [125]. Indeed, phosphorylation of C/EBPβ is increasingly being found to be critical for its proliferative action [126,127]. For example, Buck et al. [126] have shown that an ribosomal protein S-6 kinase (RSK)-mediated phosphorylation of Ser^{105} (rat) or Thr^{217} (mouse) of C/EBPβ is required for hepatocyte proliferation induced by transforming growth factor-α (TGFα). More recently, they have shown that the hepatotoxin carbon tetrachloride activates RSK, phosphorylates C/EBPβ on Thr^{217} and causes proliferation of hepatic stellate cells in normal mice [127]. In contrast, the toxin causes apoptosis of these cells in C/EBPβ-deficient mice or transgenic animals expressing a non-phosphorylatable C/EBPβ Ala^{217} mutant [127]. They also showed that both [phospho-Thr^{217}]C/EBPβ and the phosphorylation mimic [Glu^{217}]C/EBPβ-, but not [Ala^{217}]C/EBPβ, are able to associate with procaspases 1 and 8, thereby providing a potential mechanism of action [127].

Other functions

A novel role for the C/EBPs in long-term synaptic plasticity underlying memory was identified by studies on the *Aplysia* C/EBP homologue, ApC/EBP [23]. Blocking the function of ApC/EBP by use of either antisense oligonucleotides or specific antibody leads to a selective inhibition of long-term facilitation [23]. Several lines of evidence suggest that such a role of C/EBPβ in memory is not only likely to be conserved in mammals, but also extend to other members of the family. First, C/EBPα, C/EBPβ and C/EBPδ have been shown to be widely expressed in the mammalian nervous system [57,106,128–131]. Secondly, the expression and DNA binding activities of C/EBPβ and C/EBPδ are enhanced in hippocampal neurons, which are involved in long-term memory, by two crucial inducers of the memory process, cAMP and Ca^{2+} [129]. Thirdly, using specific model systems, Taubenfeld et al. [130] have identified a key role of C/EBPβ in mammalian long-term memory consolidation. Finally, C/EBPδ-deficient mice display a selectively enhanced contextual fear response, thereby implicating this isoform in learning and memory [131].

The expression of C/EBPζ is induced by a number of agents that cause cellular stress, particularly in the endoplasmic reticulum (ER) [39,132]. More recently, C/EBPζ has also been implicated in growth arrest and the control of apoptosis [133–135]. For example, C/EBPζ-deficient mice are defective in the development of apoptosis in response to ER stress, such as that produced by agents that cause destruction of the pancreatic β-cells, and thereby diabetes [133–135]. In addition, most, if not all, human myxoid and round-cell liposarcomas are associated with chromosomal translocations that lead to gene fusions that specify for chimaeric oncoproteins consisting of an N-terminus from the *FUS* or *TLS* gene and a C-terminus from the C/EBPζ gene [136,137]. In contrast with C/EBPζ, the chimaeric protein fails to cause cell-growth arrest [138].

Finally, C/EBPγ-deficient mice show impaired natural killer-cell cytotoxic activity and interferon-γ (IFN-γ) production, thereby indicating a novel role of this isoform in the functional maturation of these cells [139].

REGULATION OF THE C/EBP FAMILY

The expression of the C/EBPs is regulated under several physiological and pathophysiological conditions through the actions of a number of factors, including cytokines, mitogens, hormones, nutrients and agents that cause cellular stress (Table 3). Understanding the mechanisms that are involved in the regulation of the C/EBPs during such conditions has been the focus of recent research in a number of laboratories, including our own. It is hoped that such studies will not only contribute to increasing our knowledge of the molecular basis of these processes but also lead to the identification of potentially novel targets for therapeutic intervention of diseases associated with perturbations in C/EBP gene expression. For instance, targeted suppression of C/EBPζ expression in pancreatic β-cells has been recently proposed as a new therapeutic approach for preventing the onset of both type 1 and type 2 diabetes [134,135]. The C/EBPs have been found to be regulated at a number of levels, including gene transcription, translation, protein–protein interactions and phosphorylation-mediated changes in DNA-binding activity, activation potential and nuclear localization (see Table 3). In addition, the potential existence of tissue/cell- and species-specific differences in the mechanisms of regulation has been identified [57,58,113–115, 148,150,164–166]. For example, TNFα induces C/EBPβ mRNA expression in astrocytes and renal mesangial cells, but modulates nuclear–cytoplasmic translocation in hepatocytes [113–115,148, 150]. The initial part of this section will describe the different control points that operate to regulate the C/EBP family and will be followed by a summary of the mechanisms that are involved in the global modulation of C/EBP expression in three important processes: adipocyte differentiation, acute-phase inflammatory response in hepatocytes and liver regeneration.

Transcriptional regulation

This represents the main point of control in the regulation of mRNA expression by factors shown in Table 3 [57,112]. The promoter regions of C/EBP isoforms α, β, δ, ϵ and ζ have been analysed. The role of the different sequence elements in the regulation of gene expression during adipogenesis, inflammation and hepatocyte proliferation is described below under global gene regulation, with other aspects addressed here.

C/EBPα

The proximal promoter region of the mouse C/EBPα gene was first characterized and shown to contain potential binding sites for C/EBP, Sp1, nuclear factor (NF)-1, NF-Y, upstream stimulating factor (USF), basic transcription element-binding protein (BTEB) and NF-κB [88,167]. The promoter can be auto-activated in transfected cells by expression plasmids that specify for C/EBPα or C/EBPβ, which act via a C/EBP recognition sequence [88,167]. The proximal promoter region of the rat C/EBPα gene shows almost complete sequence identity with the mouse counterpart and also can be auto-activated through the C/EBP-binding site [141]. On the other hand, the promoter regions of the human, chicken and *Xenopus* C/EBPα genes show only limited sequence identity with the mouse/rat promoter (e.g. 53 % identity in the case of the human promoter) [164,166]. Nevertheless, both the *Xenopus* and the chicken promoters contain putative C/EBP recognition sequences and, in the case of the *Xenopus* promoter, we have shown that it can be auto-activated by C/EBPα or C/EBPβ [164]. On the other hand, the human promoter lacks a C/EBP recognition sequence, owing to a single base substitution [166]. However, it can still be auto-activated, although only by C/EBPα, which acts indirectly via stimulation of the DNA-binding activity of USF, which interacts with a site present in the proximal promoter region [166]. These studies, therefore, show that the C/EBPα gene is auto-activated in a species-specific manner.

The regulatory sequences that are involved in the transcriptional activation of the C/EBPα gene by thyroid hormones and suppression by overexpression of the proto-oncogene c-*myc* have also been investigated [168–170]. The action of the thyroid hormone is mediated via a responsive element present at position -602 to -589 of the rat C/EBPα promoter [168]. On the other hand, the c-Myc protein inhibits C/EBPα gene transcription through interaction with the core promoter region, although a discrepancy exists on the precise sequence that is involved in c-Myc action [169,170].

C/EBPβ

The promoters of the mouse, rat, chicken and *Xenopus laevis* C/EBPβ genes have been characterized to date, and all four are subject to autoregulation [165,171–173]. The transcription factor CREB (cAMP-response-element-binding protein) also controls C/EBPβ expression by interacting with two sites near the TATA box [174]. These cAMP-respone element (CRE)-like sequences are also required for the IL-6-mediated induction of C/EBPβ transcription during the acute-phase response (APR) through a novel pathway involving tethering of STAT-3 to a DNA-bound complex [175] (see below), and in conjunction with an Sp1 site for the activation of gene transcription during macrophage differentiation [176].

C/EBPδ

This isoform is expressed constitutively in osteoblasts, where it has been shown to activate the synthesis of insulin-like growth factor I [177]. A binding site for the Runt domain factor (Runx) 2, which is essential for osteogenesis, is present in the 3′ proximal region of the C/EBPδ gene promoter ($-175/-147$) [177]. The promoter activity is reduced drastically by mutation of this Runx-binding sequence or by co-transfection of a Runx2 antisense expression plasmid into osteoblasts [177]. The transcription of the C/EBPδ gene is also induced in growth-arrested mammary epithelial cells and in the involuting mammary gland through the activation of STAT-3, and its subsequent binding to a recognition sequence present in the promoter region [178,179]. This STAT site is also required for the IL-6-mediated induction of C/EBPδ transcription in hepatocytes during the APR [180,181] (see below). Although the activation of STAT-3 under such conditions is transient, the C/EBPδ expression levels are maintained for a longer period of time [25,53,112,180,181]. This is achieved, at least in part, through autoactivation [27,182,183]. Interestingly, analysis of the mouse, rat and ovine C/EBPδ promoters has also suggested the potential existence of species-specific mechanisms for autoregulation [27,182,183]. For example, the autoregulation of the rat C/EBPδ gene could not be seen with up to 6 kb of upstream promoter region, but instead required two C/EBP recognition sequences present at the 3′ end of the gene [182]. In contrast, the 5′ ends of the mouse and the ovine C/EBPδ genes are sufficient for auto-activation [27,183]. In the case of the ovine promoter, no putative C/EBP recognition sequences could be identified [27], thereby suggesting that, similar to the human C/EBPα gene [166], it also may be subject to indirect autoregulation.

C/EBPε

The C/EBPϵ gene promoter is transcribed by two alternative promoters, pα and pβ, which, similar to those of several other myeloid-cell-specific genes, contains no TATAAA box, but have a number of purine-rich stretches with multiple sites for the ets family of transcriptional regulators [29–31]. The promoter also contains a functional retinoic acid-responsive element that mediates inducibility by the corresponding ligand [155].

C/EBPζ

C/EBPζ was first identified on the basis of its induced expression in response to growth arrest and DNA damage [32]. Such an inducibility was also seen in transfection assays using the $-778/+21$ promoter fragment from the hamster gene [32]. The activator protein-1 (AP-1) site present between -250 and -225 was found to be essential for this response and also played a role in the induction by oxidative stress [156]. Exposure of the cells to either oxidants or UVC irradiation was also shown to stimulate the binding of the c-Jun·c-Fos heterodimer to this element [156]. However, conflicting results have been obtained in relation to the induction of the endogenous C/EBPζ mRNA and protein by UVC irradiation [157]. Because the expression of the C/EBPζ gene is induced by a range of cellular stresses [39,133–135, 156–163], it is possible that differences in conditions for culturing of the cells may have been responsible for the discrepancies that exist. More recently, Schmitt-Ney and Habener [157] have re-examined the UVC response and shown that whereas the promoter is activated strongly in transfected cells, only a modest and transient increase in endogenous gene expression is observed. In addition, the induction of C/EBPζ expression in response to other cellular stresses is markedly attenuated by prior exposure of the cells to UVC [157]. The target of such an inhibitory effect of UVC on C/EBPζ expression is located in the first exon of the gene, a 5′-untranslated region that is highly conserved between different species [157].

Table 3 Regulators of the C/EBP family

Further abbreviations: DBA, DNA-binding activity; NL, nuclear localization; AP, activation potential. For any still-undefined abbreviations, see the abbreviations footnote.

Gene	Level	Activators	Repressors	References
C/EBPα	mRNA	Thyroid hormone, thiazolinediones	EGF, glucocorticoid, growth hormone, IFN-γ, IL-1, IL-6, insulin, LPS, TNF-α	[19,25,112,114,140–143]
	DBA		EGF, PKC activators	[140,141,144]
C/EBPβ	mRNA	cAMP, glucagon, glucocorticoid, growth hormone, IFN-γ, IL-1, IL-6, LPS, noradrenaline, NGF, VIP		[7,23,62,92,112–115,128,145–147]
	NL	cAMP, LPS, TNF-α, TPA	TNF-α	[21,148–150]
	AP	Ca^{2+}, IL-6, MAPK activators, PKC activators	Insulin	[8,53,125,151–153]
	DBA	TGF-β, EGF		[141]
C/EBPδ	mRNA	Glucocorticoid, growth hormone, IFN-γ, IL-1, IL-6, insulin, LPS, noradrenaline, PDGF, TNF-α, VIP		[25,53,92,112–115,145,147]
	NL	cAMP, TNF-α		[148,154]
C/EBPϵ	mRNA	Retinoic acid		[155]
C/EBPζ	mRNA	Amino acid deprivation, agents that cause ER-, nutrient- and oxidative stress, Ca^{2+}, nitric oxide, LPS, prostaglandin A_2, UV light		[32,33,39,133–135,156–163]
	AP	Cellular stress		[132]

The promoter region of the C/EBPζ gene contains a conserved C/EBP recognition sequence, located between positions -339 to -320, that plays an important role in its induction during the APR in hepatocytes [158] (see below), and in response to sodium arsenite treatment of rat pheochromocytoma PC12 cells [159]. C/EBPζ mRNA expression is induced by arsenite in a biphasic manner: a rapid increase during the first 4 h of treatment, a transient decline at 6–8 h, and a pronounced induction thereafter [159]. A closer examination of the C/EBP recognition site shows that it is a composite element with one half of each palindromic sequence comprising of an optimal C/EBP-binding motif and an activating transcription factor (ATF)/CRE variant site [159]. This C/EBP–ATF composite site interacts with both C/EBPβ and ATF family members [159]. In addition, time-dependent changes in the interaction of ATF members with the composite site are seen following exposure of the cells to arsenite: binding of the activator ATF-4 at 2 h, when C/EBPζ mRNA levels are increasing, and enhanced binding of the repressor ATF-3 at 6 h, when the levels are declining [159]. Thus the arsenite-induced biphasic induction of C/EBPζ expression in PC12 cells involves the sequential binding of multiple factors to the C/EBP–ATF composite site [159].

Amino acid limitation also induces the expression of C/EBPζ at both the transcriptional and post-transcriptional levels [160]. The decreasing amino acid concentration by itself can induce C/EBPζ expression independently of a cellular stress produced by an inhibition of protein synthesis [162]. This induction of C/EBPζ is mediated through the phosphoinositide 3-kinase (PI 3-kinase) and mammalian target of rapamycin (mTOR) signalling pathways that converge on the amino-acid-responsive element present between position -310 and -302 [161,162,184]. This regulatory sequence is related to the C/EBP–ATF-2 composite site and interacts with ATF-2 [162]. The precise mechanisms through which amino acid starvation regulates ATF-2 remains to be determined, but does not occur through any changes in its DNA-binding activity [162].

More recently, the -75 to -104 region of the C/EBPζ promoter has been shown to be essential for both the constitutive and the ER-stress-inducible expression [163]. The ER stress-responsive element contains two overlapping regions that share sequence homology with similar regulatory sites identified in the glucose-regulated protein 78 (GRP78), GRP94, protein disulphide-isomerase and calreticulin gene promoters [163]. The transcription factor NF-Y interacts with the two regulatory regions and mediates both constitutive and ER-stress-inducible activation of gene transcription [163].

Translation

Production of different C/EBPα and -β polypeptides by alternative use of initiation codons represents a major form of translational control in the regulation of the C/EBP family [18,48–50]. As detailed above, two distinct isoforms of C/EBPα, 42 kDa and 30 kDa, can be produced from a single mRNA (Figure 2) [49–50]. In contrast with the 42 kDa form, the 30 kDa polypeptide has a lower transactivation potential and lacks antimitotic activity [49,50]. The ratio of the two isoforms has been seen to change during both adipocyte differentiation and hepatocyte development [49,50], thereby raising the possibility of an important regulatory role. Similarly, in the case of C/EBPβ, the ratio of the activator LAP form to the repressor LIP polypeptide has been shown to increase under a number of conditions, and such changes are likely to be functionally important [48]. For example, the expression of LIP is elevated in breast cancer, and this is consistent with its ability to induce epithelial cell proliferation and the formation of mammary hyperplasia [185]. It has been suggested that a LIP-mediated growth cascade may be susceptible to additional oncogenic hits, which could then result in the initiation and the progression of neoplasia [185].

The C/EBPα mRNA contains an evolutionary conserved short upstream open reading frame (uORF) that is a fixed distance of seven bases from the initiation codon that specifies for the full-length protein [186]. A short conserved uORF is also present in the C/EBPβ mRNA [186]. Lincoln et al. [187] initially showed that such an uORF can act in *cis* to inhibit C/EBP translation as it is recognized readily by the translation machinery. It was proposed that, because of the potent differentiative and antimitotic activities of C/EBPα and, to a certain extent C/EBPβ, such an inhibitory mechanism could operate to restrict the expression of the protein in cells that are undergoing differentiation, and also could have been responsible for the previously noted differences in the tissue expression profile of C/EBPα/β mRNA and the corresponding proteins [187]. More

recently, however, Calkhoven et al. [18] have shown instead that the integrity of the uORF is essential for the leaky ribosome scanning mechanism that leads to the production of different sized polypeptides [18]. The ratio of the different isoforms was found to be controlled by both an RNA-dependent protein kinase and the mTOR signalling pathway through the regulation of the eukaryotic translation initiation factors eIF-2α and eIF-4E respectively [18].

Modulation of DNA-binding activity, transactivation potential and/or phosphorylation-mediated changes in nuclear localization

Phosphorylation plays a key role in the modulation of C/EBPβ function. This isoform is normally a repressed factor in which negative regulatory regions mask its transactivation domains [40,46]. Such a repression is abolished by phosphorylation of the repression domain by several signal transduction pathways, including those mediated by oncogenic proteins [46]. The transactivation potential of C/EBPβ is also induced by phosphorylation of Thr^{235} by a Ras/mitogen-activated protein kinase (MAPK) pathway [125,152], Ser^{105} via protein kinase C (PKC) [153], and Ser^{276} by Ca^{2+}/calmodulin-dependent protein kinase [151]. Phosphorylation can also suppress the activation potential of C/EBPβ. For example, insulin inhibits transactivation by C/EBPβ via the PI 3-kinase pathway [188]. In contrast, the PKA-mediated *in vitro* phosphorylation of a region between Ser^{173} and Ser^{223}, and Ser^{240}, results in a suppression of C/EBPβ DNA-binding activity [189]. Similarly, a PKC-mediated phosphorylation of Ser^{240} leads to a decrease in DNA binding [189]. On the other hand, the forskolin-induced activation of the cAMP/protein kinase A (PKA) signal-transduction pathway in rat PC12 cells results in the translocation of C/EBPβ into the nucleus, where it then activates the transcription of the c-fos gene [149]. Antioxidant-induced nuclear translocation of C/EBPβ in a colorectal cancer cell line DKO-1 is also mediated by a PKA-dependent phosphorylation, but of Ser^{299} [190]. Such phosphorylation-stimulated changes in the nucleo-cytoplasmic transport is also likely to be responsible, at least in part, for the regulation of C/EBPβ during activation of macrophages [21,191] and the TNFα-mediated regulation of gene expression in hepatocytes [148,150].

Several studies have also suggested a potential role of phosphorylation in the regulation of C/EBP isoforms α, δ and ζ under certain conditions. For example, PKC can phosphorylate C/EBPα at several sites (Ser^{248}, Ser^{277} and Ser^{299}), and leads to an attenuation of its DNA-binding activity [144]. In addition, insulin can reduce the expression of genes in adipocytes, in part, due to a dephosphorylation-mediated degradation of C/EBPα [192] (see below). Furthermore, during cellular stress, C/EBPζ undergoes an inducible phosphorylation at two adjacent serine residues (71 and 81) via the p38 MAPK pathway, which leads to enhanced transactivation potential of the factor [132]. Finally, studies on the regulation of the α_1-acid glycoprotein and the serum amyloid A genes during the APR have shown that dephosphorylation of C/EBPδ results in an inhibition of its DNA binding activity [64]. Additionally, the transactivation potential of C/EBPδ was also found to be increased when hepatocytes were treated with cellular phosphatase inhibitors, such as okadiac acid and sodium orthovanadate [64].

Protein–protein interactions

The transactivation potential of the different C/EBP members differs; for example, C/EBPα is a stronger transcriptional activator than C/EBPβ [8,9,53,60,71]. Because the different C/EBP members can form heterodimers under all intrafamilial conditions [7–15], such interactions are likely to have a profound influence on the regulation of gene transcription. In addition, the C/EBPs can form protein–protein interactions with other bZIP and non-bZIP factors. For example, C/EBPβ has been shown to interact with the p50 subunit of NF-κB, CREB/ATF, AP-1, glucocorticoid receptor, hepatitis B virus X protein, and the retinoblastoma (Rb) protein [54–58]. Such heterodimers often have different transactivation potential and/or DNA binding specificity or affinity compared with the corresponding homodimer [54–58].

Regulation of the C/EBPs during adipocyte differentiation

Both C/EBPβ and C/EBPδ mRNA is induced during the early phase of differentiation of pre-adipocyte cell lines in response to cAMP-elevating agents and glucocorticoids respectively [12,140]. The activation of these two members has been implicated, at least in part, in the later induction of C/EBPα gene transcription via a C/EBP recognition sequence present in the promoter region [65–67,88]. Once C/EBPα is induced, auto-activation ensures high expression levels, and thereby maintenance of the terminally differentiated state [65–67,88].

Although both C/EBPβ and C/EBPδ are expressed at high levels at the start of the differentiation programme and throughout mitotic clonal expansion, C/EBPα is expressed much later [67]. Such a delayed expression of C/EBPα is crucial, because the protein is anti-mitotic and its premature expression would block the mitotic clonal expansion that is vital for differentiation [67]. Several mechanisms have been identified that prevent the premature early expression of C/EBPα. First, although both C/EBPβ and C/EBPδ mRNAs are induced early in the differentiation programme, the DNA-binding activity is not acquired until the preadipocytes traverse the G_1-phase/S-phase checkpoint and initiate mitotic clonal expansion [193]. Concomitant with the acquisition of DNA-binding activity, C/EBPβ/δ localize to centromeres by binding to multiple consensus C/EBP-binding sites in centromeric setellite DNA [193]. The precise mechanism that is involved in such acquisition of DNA-binding activity is currently unclear. However, at least C/EBPβ has been shown to undergo phosphorylation concomitant with the acquisition of DNA-binding activity [67,193]. In addition, C/EBPζ, which is expressed by growth-arrested pre-adipocytes, transiently sequesters C/EBPβ/δ by heterodimerization [194]. As preadipocytes reach the S-phase, C/EBPζ is down-regulated, thereby releasing C/EBPβ/δ from the inhibitory constraint and, thereby, allowing activation of the C/EBPα gene [194]. In support of this proposition, up-regulation of C/EBPζ with a protease inhibitor *N*-acetyl-Leu-Leu-norleucinal prevents activation of C/EBPβ, expression of C/EBPα and adipogenesis [194]. Indeed, metabolic-stress conditions (e.g. low glucose) have been shown to inhibit adipogenesis by inducing the expression of C/EBPζ [195]. Secondly, nuclear factor CUP, an isoform of AP-2α, the expression of which is high in pre-adipocytes and declines drastically during differentiation, interacts with two repressive elements present upstream of the C/EBPα translation initiation codon, one located in the 5′ flanking region and another in the 5′ untranslated region [67,196,197]. Both these sites are required to maintain the promoter in an inactive state prior to differentiation [67,196,197]. Interestingly, the CUP regulatory element in the promoter region overlaps a Sp site (GT-element) to which Sp3 (or Sp1) interacts and, therefore, the binding by Sp3 and CUP/AP2-α is mutually exclusive [198]. Because Sp3 is a strong transcriptional activator and expressed throughout differentiation, it has been proposed that CUP/AP-2α may delay the access of Sp3 to the Sp regulatory element, and thereby

prevent the premature expression of C/EBPα [198]. Finally, the C/EBP site in the promoter region also overlaps with an Sp1 recognition sequence, with binding between the two factors being mutually exclusive [199]. Indeed, Sp1 has been demonstrated to block binding and activation by the C/EBPs both *in vitro* and in intact 3T3-L1 adipocytes [199]. Because Sp1 is expressed in pre-adipocytes and and its levels decrease during differentiation, this could represent an additional mechanism for the repression of C/EBPα expression [199]. The decreased expression of Sp1 during differentiation has been shown to be due to a cAMP-mediated phosphorylation of the protein, which triggers its degradation [199].

Several factors have also been shown to modulate the expression of C/EBP isoforms in adipocytes. For example, insulin produces both a rapid dephosphorylation of the C/EBPα protein and reduces its mRNA expression [65,192]. C/EBPα is phosphorylated on about six sites in fully differentiated adipocytes, of which two, Thr^{222} and Thr^{226}, are de-phosphorylated in response to insulin treatment through inactivation of glycogen synthase kinase 3 [192]. In contrast, insulin induces the expression of the C/EBPβ and C/EBPδ gene [65]. Glucocorticoids reciprocally regulate the expression of C/EBPα (decrease) and C/EBPδ (increase), mainly at the transcriptional level [140]. Finally, the expression of C/EBPα is attenuated by TNF-α, retinoic acid and TGF-β [142,200,201]. The precise mechanisms for cytokine action are currently unclear, with conflicting results being obtained with respect to mRNA expression [142,200]. However, retinoic acid blocks C/EBPα expression, and thus adipogenesis, by inhibiting C/EBPβ-mediated transcription [201].

Regulation of the C/EBPs during the APR and liver regeneration

Both the APR and liver regeneration are characterized by a decrease in C/EBPα expression and an increase in that of C/EBPβ and C/EBPδ [57,58,60,71]. Such reciprocal changes are mediated primarily through the actions of cytokines such as IL-6, IL-1 and TNF-α [57,58,60,71]. Analysis of the mouse C/EBPα promoter has shown that the LPS-mediated transcriptional suppression occurs through increased binding of the LIP isoform of C/EBPβ to a C/EBP recognition sequence present in the promoter region [202]. The increased production of LIP under such conditions is achieved through regulation of translation by binding of the CUG repeat binding protein, CUGBP1, to recognition sites present between the first and second AUG codons of C/EBPβ mRNA [202,203]. CUGBP1 binding activity is induced during the APR or liver regeneration, possibly in response to phosphorylation [202,203]. Although this model is potentially attractive and can explain the regulation of the mouse and the rat C/EBPα genes, it is not likely to be applicable to the human C/EBPα promoter, owing to the lack of a C/EBP recognition sequence [166]. In addition, Baer and Johnson [204] have shown recently that the truncated C/EBPβ isoforms can be generated by *in vitro* proteolysis during isolation from cells, thereby indicating that any findings on gene regulation based on LIP have to be interpreted with caution and need to be confirmed *in vivo*.

The regulation of C/EBPβ during the APR and liver regeneration is extremely complex, with control being exerted at both transcriptional and post-transcriptional levels [8,53,58,60,71]. Increased activity of CREB, which activates C/EBPβ expression via binding to CRE-like sites in the promoter region, represents one important mechanism for transcriptional regulation, especially since an increase in CREB activity has been seen during liver regeneration [174]. These CRE-like elements are also required for the activation of C/EBPβ transcription by IL-6, a major mediator of the APR, via a novel pathway in which tethering of STAT-3 to a DNA-bound complex contributes to activation [175]. Post-transcriptional mechanisms, however, play the most prominent role in the regulation of C/EBPβ during liver regeneration and the APR, and include increased production of LIP (see above), changes in nuclear–cytoplasmic transport, and phosphorylation-mediated modulation of DNA-binding activity and/or transactivation potential [8,53,60,71,202].

The transcription of the C/EBPδ gene is also activated by IL-6 and a number of pro-inflammatory factors during the APR [25,53,71,112]. The IL-6 induced expression is mediated via STAT-3, which interacts with a recognition sequence present in the proximal promoter region [180,181]. The activation of STAT-3 is transient, whereas the expression of C/EBPδ is more prolonged and therefore likely to be due to autoregulation [182]. Changes in the phosphorylation status of C/EBPδ also play a role in the regulation of at least some genes during the APR [64]. The expression of C/EBPδ mRNA is also induced during liver regeneration [57], although the mediator or the transcription factors that is responsible for such activation remains to be determined.

The C/EBPζ gene is also induced during the APR, although its expression is delayed with respect to C/EBPβ and C/EBPδ [158]. Such activation is mediated through the binding of C/EBPβ or C/EBPδ to a recognition sequence present in the promoter region [158]. The precise reason(s) for the activation of C/EBPζ during the APR is currently unclear, but may play a role in the curtailment of the response.

CONCLUDING REMARKS

Research carried out mainly in the last decade has revealed a pivotal role of the C/EBPs in numerous cellular responses, including proliferation, differentiation, apoptosis, control of metabolism and maintenance of specific functions. Studies on mice that are deficient in individual C/EBP members have clearly been instrumental in advancing our understanding of C/EBP function. However, the early demise of some knockout mice and functional redundancy between family members has clearly precluded the identification of the full range of different functions carried out by a specific gene. Conditional knockouts and analysis of mice that are deficient in more than one member will be necessary to resolve these issues. An important role of the C/EBPs in a number of disease conditions has also been determined, including the recent identification of specific gene mutations in patients (see, e.g. [101,102,117]). Thus further studies on the C/EBP family are essential to address fundamental questions in a number of areas, such as oncology, diabetes and haematopoiesis, and for the development of novel therapeutic strategies. We are only beginning to understand the transduction pathways that lead to the transcriptional and post-transcriptional regulation of the C/EBPs by specific signals. It is, therefore, important that further studies on this aspect are carried out using the full range of model systems that are available from different organisms given that species- and cell-type-specific differences in C/EBP regulation have been identified (e.g. [166]). Finally, the different C/EBP members have been shown to bind to an identical recognition sequence in the promoter regions of target genes, at least *in vitro* [57,58]. It is, however, possible that differences in the specificity and/or affinity to various binding sites may exist *in vivo*. This aspect needs to be addressed through the use of the chromatin immunoprecipitation technique [205], which has been employed recently to investigate the interaction of specific factors to recognition sequences in intact cells in the normal chromatin context.

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