Ig/EBP (C/EBP γ) is a transdominant negative inhibitor of C/EBP family transcriptional activators

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ABSTRACT

Analysis of cDNA and genomic clones shows that the murine Ig/EBP (C/EBP γ) gene encodes a small protein with a predicted molecular weight of 16.4 kDa which contains C/EBP family basic and leucine zipper domains but lacks the transcriptional activation domains present in C/EBP (C/EBP α) and NF-IL6 (C/EBP β). In transfection assays Ig/EBP is neither an activator nor a repressor of transcription; however, Ig/EBP inhibits the transcriptional ability of NF-IL6 (C/EBP β) and C/EBP (C/EBP α), acting as a transdominant negative regulator. Thus Ig/EBP resembles LIP, another negative regulator of the C/EBP family, in both structure and transcriptional activity. Of the three known C/EBP family inhibitors, Ig/EBP, LIP and CHOP-10, only Ig/EBP is ubiquitously expressed. Therefore, Ig/EBP may act as a general buffer for C/EBP activators in many cell types.

INTRODUCTION

The CCAAT/enhancer binding protein (C/EBP) family of transcription proteins has been shown to be important in regulating many genes including several cytokine genes (1–5), liver-specific genes, including those involved in the acute-phase response (6–9) adipose-specific genes (10,11), the regulatory elements of many viruses (12) and regulatory elements in the immunoglobulin (Ig) heavy chain and kappa loci (13–17). C/EBP family members share homology in their basic-leucine zipper (bZip) domains and as a result recognize the same DNA sequences and can form heterodimers with each other (18–21). This family of transcription factors includes transcription activators, C/EBP (C/EBP α) (22), NF-IL6 (C/EBP β) (23–26), CRP1 (21) and CRP3 (C/EBP δ) (21) and the negative regulators LIP (27) and CHOP-10 (28). Most C/EBP family members display regulated and tissue specific patterns of expression. For example, C/EBP is expressed in high levels in only differentiated liver cells and adipose tissue (10,11,22,29). Furthermore, NF-IL6 and CRP3 appear to be induced in several tissues by a number of stimuli including LPS, IL-1, TNF α and IL-6 (2,24,30). Expression of C/EBP and NF-IL6 is associated with the differentiation of adipocytes and monocyte/macrophages respectively (2,10,11).

Only one C/EBP family member, Ig/EBP (C/EBP γ), is known to be ubiquitously expressed. Ig/EBP is expressed in all cell lines and adult tissues studied (16) and is also expressed in most tissues of 14 day mouse embryos (Y. Ren and K. Calame, unpublished). However, the transcriptional activity of Ig/EBP is not understood. Ig/EBP was originally identified by its ability to bind to the Ig heavy chain promoters and Ig heavy chain enhancer (16). In addition to heterodimerizing with various C/EBP proteins, Ig/EBP also heterodimerizes with ATF family members (16,20).

In this study, we show that the Ig/EBP gene encodes a small protein with a predicted molecular weight of 16.4 kDa which lacks the N-terminal transcriptional activation domains found in C/EBP and NF-IL6. Ig/EBP alone neither activates nor represses transcription; however, Ig/EBP is a transdominant negative inhibitor of other C/EBP family activators, such as C/EBP and NF-IL6. Thus, Ig/EBP activity is similar to that of LIP, an N-terminally truncated form of NF-IL6. The widespread presence of Ig/EBP suggests that it may play an important role in modulating gene activation resulting from induction of activators C/EBP, NF-IL6 or CRP3.

MATERIALS AND METHODS

RACE cloning of the 5' end of Ig/EBP cDNA

Poly A+ RNA was prepared from 22D6 proB cells by standard methods (31) for RACE PCR cloning (32). The clone with the longest insert was sequenced using the Sequenase (USB; Cleveland, OH) system.

Immunoblots

Nuclear extracts from 22D6 preB were prepared as previously described (33). For Western analysis, 300 μ g protein was separated on a 15% SDS-PAGE and transferred to small pore (0.2 μ) nitrocellulose filter by electroblot overnight. The filter was fixed for 1 min in isopropanol then blocked for 2 h in 5% nonfat milk (NFM)/0.2% Tween-20. After thorough washing with PBS containing 0.2% Tween-20 (PBST), the filter was divided: half was transferred to a solution of 2% NFM in PBS containing a 1:2500 dilution of a polyclonal rabbit anti-Ig/EBP antiserum while the other half was incubated in a similar dilution of preimmune serum. This antiserum was generated by immunizing rabbits with the 7.2 kDa C-terminal *Dde*I/*Bgl*II fragment of Ig/EBP and was tested

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Figure 1. The murine Ig/EBP gene encodes a 16.4 kDa protein. (A) 5' untranslated sequence of Ig/EBP cDNAs. New 5' sequence for the murine gene was obtained by RACE PCR and confirmed by analysis of genomic clones. The murine sequence (bold) is compared with human (GenBank accession no. U20240) and rat (26) sequences; dashes indicate identity to the murine sequence and spaces indicate gaps in the human and rat sequences relative to the murine sequence. The inframe stop codons and predicted start codons are boxed. (B) Immunoblot showing that endogenous Ig/EBP is expressed in 22D6 preB cells as an 18.5 kDa protein. Crude nuclear extract was analyzed by SDS-PAGE; the blot was developed with polyclonal anti-Ig/EBP serum (left lane), then stripped and reprobed with preimmune serum (right lane). Arrow indicates where Ig/EBP migrates. (C) Ig/EBP is similar in structure to LIP, the negative regulator that is translated from an internal AUG in NF-IL6 mRNA (48). White bars show relative size of each protein in kDa, hashed and black boxes represent the basic and zipper regions respectively and the greyed boxes (C/EBP α) are activation domains (lighter grey regions in the N-terminus of NF-IL6 are homologous to the mapped activation domains of C/EBP α , but the precise boundaries of these regions remain to be determined for NF-IL6).

for specificity by probing Western blots of recombinant C/EBP family proteins. After a 2 h incubation, the filter was thoroughly washed in PBST and transferred to 2% NFM/PBS containing a 1:20 000 dilution of peroxidase-conjugated anti-rabbit secondary antiserum (Boehringer Mannheim). After a final 2 h incubation, the filter was extensively washed in PBST then incubated for 1 min with the chemiluminescent ECL Detection System (Amersham). The filter was exposed to film for 30 min.

In order to detect the HA-tag NF-IL6, Ig/EBP and LIP, whole cell lysates were prepared by resuspending cells in 200 μ l of 2 $\times$ SDS running dye (0.2% bromophenol blue, 4% SDS, 100 mM Tris pH 6.8, 200 mM DTT, 3 ng/ml aprotinin, 2 ng/ml pepstatin A, 1 ng/ml leupeptin, 10 mM PMSF, 30% glycerol). Twenty microliters of each sample were loaded onto a 10% SDS-PAGE gel and blotted as described above. The 12CA5 epitope from the human influenza hemagglutinin protein was detected by using a 1:20 000 dilution of mouse-anti-HA (Boehringer Mannheim) as a primary antibody and 1:20 000 peroxidase-conjugated sheep-anti-mouse Ig as a secondary antibody (Boehringer Mannheim).

Transfections

Transient cotransfection assays were performed in 18-81 preB cells, M12 (mature B cell line), P3X plasmacytoma cells or 3T3

fibroblasts. Several reporter constructs were used in these experiments: (i) APLuc, a minimal alkaline phosphatase (AP) promoter driving luciferase gene expression (34); (ii) E₄APLuc, four tandem C/EBP sites cloned upstream of APLuc; (iii) TK200Luc, a 200 bp fragment of the thymidine kinase (TK) promoter driving luciferase gene expression (35); and (iv) E₄TK200Luc, four tandem C/EBP sites cloned upstream of the TK promoter in TK200Luc. The cotransfected DNAs were expressed from a cytomegalovirus (CMV) promoter and included: (i) CMV-LAP (23), a rat clone of NF-IL6 in which translation initiates from the second in-frame methionine so that only the LAP protein is made; (ii) CMV-LIP where translation initiates from the third in-frame methionine so that only the truncated LIP form is made; (iii) CMV-C/EBP; and (iv) CMV-Ig/EBP. Both CMV-LAP and CMV-LIP have engineered ribosome binding sites so that their level of translation cannot be taken to reflect the translational efficiency of the endogenous protein. The HA-tag NF-IL6, Ig/EBP and LIP were generated by sub-cloning the respective cDNAs in frame into the *Xba*I site of pGCN (36). Carrier DNA was used to equalize the total amount of DNA in each transfection and CMV β gal was used to equalize the total promoter concentration. Transfections were done with two or more DNA preparations.

Figure 2. Ig/EBP is not a transcriptional activator. (A) The activity of APLuc compared to E4APLuc after transfection into 18-81 cells. Values were normalized to a cotransfected human growth hormone control to correct for transfection efficiency and the mean activity of the APLuc reporter assigned a value of 1. (B) Comparison of equimolar amounts (25 fM) of cotransfected CMV-Ig/EBP and CMV-NF-IL6 expression vectors activity on APLuc (open bars) and E4APLuc reporters (filled bars). Bars indicate one standard deviation from three or more independent transfections.

RESULTS

Murine Ig/EBP encodes a 16.4 kDa protein which lacks N-terminal activation domains homologous to those found in NF-IL6 and C/EBP

The 1.2 kb murine Ig/EBP cDNA clone originally reported contained 3' untranslated sequence but did not contain the entire 5' coding sequence (16). Subsequently, we and others have isolated and characterized additional Ig/EBP cDNA and genomic clones (5,26, this paper). Additional 5' Ig/EBP murine cDNA was cloned using RACE PCR and was sequenced. The sequence revealed an in-frame stop codon in the 5' exon (Fig. 1A). In order to be certain that this stop codon was not the result of an error by reverse transcriptase or *Taq* polymerase, genomic DNA encoding this exon was also cloned and sequenced. This sequence verified that of the cDNA clone, establishing the existence of the stop codon. Translation initiating at the first in-frame AUG downstream of this stop codon would give rise to a protein with a predicted molecular weight of 16.4 kDa.

The known sequence 5' of the predicted translation initiation codon for murine, rat (26) and human (GenBank accession no. U20240) Ig/EBP is shown in Figure 1A. The three sequences are

highly conserved. The 5' portion of the human gene has two gaps (4 and 5 bp) compared to the mouse gene but has the same inframe stop codon (Fig. 1A). Therefore the human and murine genes are predicted to initiate translation at the same codon and to encode proteins of similar sizes although the human protein has not been characterized. A single 5 bp gap in the rat gene compared to the mouse gene causes their reading frames to diverge. The available rat sequence does not have a stop codon or an AUG in this 5' region. It seems likely that the rat gene also initiates translation at the same AUG as the murine and human genes, although it is a formal possibility that another AUG exists 5' of the cloned region and the rat gene encodes a larger, functionally different protein than the murine and human Ig/EBP genes.

Since the Ig/EBP gene contains one intron (5 and data not shown), we considered the possibility that mRNA encoding a larger protein could be generated by alternative splicing of 5' exons. To test this possibility, a riboprobe spanning the 5' and 3' exons was hybridized to cytoplasmic RNA from several cell lines. Only a single protected fragment, corresponding to the cloned sequence, was observed (data not shown). Thus, there is no evidence for differential splicing and the Ig/EBP gene appears to give rise to mRNA encoding a single 16.4 kDa polypeptide.

The predicted size of Ig/EBP protein was confirmed by immunoblotting of nuclear extracts from 22D6 pre-B cells using polyclonal antiserum raised against bacterially expressed Ig/EBP. These cells have been shown to express high levels of Ig/EBP mRNA (16). A polypeptide of 18.5 kDa was detected with anti-Ig/EBP (Fig. 1B, left lane) but not with pre-immune serum (Fig. 1B, right lane). This size is consistent both with the size of recombinant Ig/EBP when expressed in eukaryotic cell lines (data not shown) as well as with the predicted size of 16.4 kDa.

C/EBP and NF-IL6 have N-terminal regions which have been shown to be transcription activation domains (9,23,37,38). Ig/EBP lacks these regions. In fact, Ig/EBP bears remarkable similarity in size and structure to LIP, a protein with a predicted molecular weight of 15.4 kDa resulting from translation of NF-IL6 mRNA initiated at an internal AUG codon (27) (Fig. 1C). LIP is a negative regulator of NF-IL6 activity in hepatocytes (27) and monocyte/macrophages (39). The structure of murine Ig/EBP suggested that it might not be a transcriptional activator and might have activity similar to that of LIP.

Ig/EBP is neither a transcriptional activator nor a transcriptional repressor

We have previously shown that in preB cell line 18-81 Ig/EBP is the only C/EBP family member whose mRNA is present at detectable levels (40) and all C/EBP site binding activity in nuclear extracts can be removed by antiserum specific for Ig/EBP (41). Therefore, since C/EBP-site-dependent activity in 18-81 is due primarily to Ig/EBP we used 18-81 cells to determine the ability of endogenous Ig/EBP, in the absence of other family members, to activate or repress reporters dependent upon C/EBP sites. First, the ability of endogenous Ig/EBP to stimulate transcription from a minimal promoter containing four C/EBP sites upstream of a minimal alkaline phosphatase TATA element (E4APLuc) was compared to its ability to activate the same reporter lacking C/EBP sites. Expression of the luciferase reporter gene was not increased by addition of C/EBP sites (Fig. 2A), demonstrating that endogenous Ig/EBP does not activate this reporter. We then tested the ability of exogenously expressed

Figure 3. Ig/EBP is not a transcriptional repressor. Comparison of TK200Luc (open bars) and E4TK200Luc (filled bars) reporters cotransfected with increasing amounts of CMV-Ig/EBP expression vector. Activities are expressed as relative light units and represent three or more independent transfections.

Ig/EBP to activate this reporter in 18-81 cells. Cotransfection of a CMV-Ig/EBP expression plasmid also failed to activate the E4APLuc promoter, although an NF-IL6 expression plasmid activated E4APLuc >15-fold (Fig. 2B). In addition, cotransfected Ig/EBP failed to activate the E4APLuc reporter construct in 293T and 3T3 cell lines (data not shown).

We also wished to test the ability of Ig/EBP to activate a C/EBP site in the context of a natural promoter. We chose the immunoglobulin V_H1 promoter. In plasmacytoma cells, which express Ig/EBP and the activator NF-IL6 (40,41), deletion of the C/EBP site in the V_H1 promoter reduces expression nearly 50% upon transient transfection of a rearranged IgM gene dependent upon this promoter (41). However, similar transfection experiments using 18-81 pre-B cells that express only Ig/EBP (41), revealed that deletion of the C/EBP site in the V_H1 promoter of a rearranged Ig gene had no effect on expression (data not shown). This suggests that Ig/EBP is not a transcriptional activator in the context of this natural promoter. From these results we conclude that neither endogenous nor transfected Ig/EBP is capable of activating transcription of promoters containing C/EBP sites.

To ask if endogenous Ig/EBP is a transcriptional repressor, we compared the activity of a reporter construct with (E4TK200Luc) or without (TK200Luc) four C/EBP sites cloned upstream of a 200 bp fragment of the herpes simplex virus TK promoter. TK200Luc has easily detectable basal activity in 18-81 cells; thus, if endogenous Ig/EBP acts as a repressor, the activity of E4TK200Luc should be decreased (35). However, upon transfection into 18-81 cells TK200Luc and E4TK200Luc gave similar levels of luciferase activity, demonstrating the inability of endogenous Ig/EBP to repress transcription (data not shown). To test the activity of transfected Ig/EBP, 18-81 cells were cotransfected with E4TK200Luc and varying amounts of an Ig/EBP expression vector. The expression of transfected Ig/EBP did not affect the expression of the reporter (Fig. 3). In separate experiments, a co-transfected expression plasmid for NF-IL6 was shown to activate this construct 13-fold over background (data not shown). These results demonstrate that Ig/EBP alone does not actively repress transcription of reporters containing C/EBP sites.

Figure 4. Ig/EBP inhibits C/EBP family activators. (A) Varying amounts of CMV-Ig/EBP expression vector were cotransfected with 5 μ g E_mAPLuc into M12 cells. The activity of the reporter without Ig/EBP was 5396 light units. (B) 25 fMol of CMV-NF-IL6 expression vector and 5 μ g of E_mAPLuc were cotransfected with increasing amounts of CMV-Ig/EBP into M12 cells. (C) 250 fMol of CMV-C/EBP expression vector and 5 μ g of E_mAPLuc were cotransfected with increasing amounts of CMV-Ig/EBP expression vector into 3T3 fibroblasts. [Amounts of CMV-NF-IL6 (LAP) and CMV-C/EBP were chosen to represent the midrange of their linear dose-response curves when cotransfected with the reporter alone]. The mean activity of the reporter alone was defined as 1 and each experiment represents three or more independent transfections.

Ig/EBP inhibits C/EBP family activators

Although when expressed alone Ig/EBP possesses no intrinsic transcriptional activity, we suspected that, when coexpressed with C/EBP family activators, Ig/EBP might inhibit their activity. To test the transcriptional activity of Ig/EBP in the presence of endogenous C/EBP family activators, we performed co-transfec-

Figure 5. Ig/EBP and LIP are transdominant negative regulators of NF-IL6 activity. (A) 3T3 cells were transfected with 0.5 μ g HA-NF-IL6, 20 μ g HA-Ig/EBP or 0.75 μ g HA-LIP expression plasmids. Following transfection whole cell lysates were prepared and samples were separated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and detected using as a primary antibody anti-HA followed by a peroxidase conjugated sheep-anti-mouse polyclonal antibody. Filled arrow indicates HA-NF-IL6 and the open arrow indicates where both HA-Ig/EBP and Ig/LIP migrate. (B) 3T3 cells were cotransfected with 2 μ g/ml of E4APLUC and with expression vectors for HA-NF-IL6, HA-Ig/EBP and/or HA-LIP using the same amounts of expression vectors as (A). pCMV β gal was used to assure that all transfections contained equal amounts of DNA and had the same CMV promoter concentration. Each datum represents the average of duplicate transfections and this experiment is representative of four individual experiments.

tions in B cell lymphoma M12.4.12 cells. These cells have been shown by supershift and RNase protection analyses to express the activator NF-IL6 (40,41). Endogenous C/EBP-binding proteins activate a minimal alkaline phosphatase promoter containing one C/EBP site (EmAPLUC) in M12 cells (S. Artandi and K. Calame, unpublished) and we assume that this is due, at least in part, to NF-IL6. Increasing amounts of cotransfected Ig/EBP expression plasmid reduced the activity of the EmAPLUC reporter >50% (Fig. 4A). These data demonstrate that ectopically expressed Ig/EBP inhibits transcription dependent on endogenous C/EBP activators. Similar results have been obtained using the HIV-1 LTR promoter in U937 promonocyte cells where NF-IL6 is induced upon cell activation. In this system, where C/EBP sites are required for full promoter activity, cotransfection of the Ig/EBP expression plasmid inhibits the HIV-1 LTR promoter (39).

To test the ability of Ig/EBP to inhibit ectopically expressed NF-IL6, increasing amounts of Ig/EBP expression plasmid were cotransfected into M12 cells with a constant amount of an NF-IL6 expression plasmid. Ig/EBP reduced the activation ability of cotransfected NF-IL6 >75% (Fig. 4B). Thus we conclude that Ig/EBP inhibits transcriptional activation by NF-IL6.

We also wished to test the ability of Ig/EBP to inhibit another C/EBP family activator. Therefore, transient cotransfection experiments were performed using Ig/EBP and C/EBP expression plasmids, 3T3 fibroblasts and a C/EBP site-dependent reporter. Ig/EBP reduced the ability of cotransfected C/EBP α to activate transcription >85% (Fig. 4C). These results corroborate the previous results with NF-IL6 and demonstrate that Ig/EBP inhibits activation by two C/EBP family activators, NF-IL6 and C/EBP α .

Ig/EBP and LIP are transdominant negative inhibitors of NF-IL6 activity

The previous experiments showed that Ig/EBP can inhibit cotransfected NF-IL6 and C/EBP α ; however, inhibition required a large molar excess of Ig/EBP plasmid. It was not possible to determine the relative amounts of Ig/EBP, NF-IL6 and C/EBP α protein because each was expressed from a different expression vector and is recognized by a different antiserum. Therefore, to monitor accurately the stoichiometry of the expressed activator and inhibitor proteins, we cloned NF-IL6, LIP and Ig/EBP cDNAs into the pCGN expression vector where they were expressed with an N-terminal influenza virus hemagglutinin epitope tag (HA-tag) (36). Immunoblots of transfected cell extracts probed with a monoclonal antibody recognizing the influenza epitope, allowed us to compare directly amounts of transfected proteins. Thus we were able to compare the inhibiting activities of Ig/EBP and LIP and were also able to determine the activity of equimolar mixtures of cotransfected NF-IL6 and Ig/EBP or LIP.

Different amounts of HA expression plasmids were required to achieve similar protein concentrations *in vivo* (Fig. 5A). The molar excess of HA-Ig/EBP plasmid required suggests decreased stability of HA-Ig/EBP protein or mRNA compared to HA-LIP or HA-NF-IL6. To determine if Ig/EBP can inhibit NF-IL6 activity when these proteins are expressed in equimolar amounts, the E4APLUC reporter was cotransfected into 3T3 fibroblasts along with plasmids expressing HA-NF-IL6 and HA-Ig/EBP. Figure 5B shows that the reporter alone had a low level of activity, due to endogenous C/EBP family activators. Cotransfected HA-NF-IL6 activated the reporter ~3-fold. When HA-Ig/EBP

was present in approximately equimolar amounts with HA-NF-IL6, the reporter activity was the same as when no activator was present. Coexpression of HA-LIP in equimolar amounts with HA-NF-IL6 reduced the activity of the reporter below that caused by endogenous C/EBP proteins alone. Thus we conclude that Ig/EBP and LIP are transdominant negative regulators of NF-IL6 and that LIP is a stronger negative regulator than Ig/EBP in our system.

DISCUSSION

Mechanism of dominant negative action

The studies reported here are the first to evaluate the transcriptional activity of the ubiquitously expressed C/EBP family protein Ig/EBP. Ig/EBP alone acts neither as a transcriptional activator nor a transcriptional repressor but it does act as a transdominant negative inhibitor of C/EBP family activators, a function consistent with its structure. Ig/EBP contains a bZIP region enabling it to bind C/EBP sites and to heterodimerize with other family members including C/EBP α (16) NF-IL6 (20) and ATF4 (20). Unlike NF-IL6 and C/EBP, Ig/EBP lacks an N-terminal activation domain. However, a fusion protein containing a TFE3 activation domain and the bZIP domain from Ig/EBP can activate transcription through C/EBP sites, providing additional evidence that the inability of Ig/EBP to induce transcription is due to the absence of an activation motif (34). Although we have demonstrated that Ig/EBP is a dominant negative regulator of NF-IL6 and C/EBP in fibroblast and B cell lines, the formal possibility remains that in other tissues cofactors might exist which would alter the activity of Ig/EBP.

Two mechanisms, which are not mutually exclusive, are likely to cause dominant negative regulation by Ig/EBP. First, Ig/EBP homodimers, which are transcriptionally inactive, compete with NF-IL6 for C/EBP binding sites by virtue of their similar DNA binding specificities, thereby decreasing the overall amount of C/EBP-site-dependent activation. Secondly, heterodimer formation between Ig/EBP and C/EBP activators such as NF-IL6 appear to generate mixed dimers with reduced activation potential. Inactive or less active heterodimers are likely to be a component of the dominant negative mechanism because our data show that Ig/EBP inhibits NF-IL6 when the two proteins are coexpressed at equimolar protein concentrations (Fig. 5). In addition, we have previously shown that Ig/EBP-NF-IL6 heterodimers form readily *in vitro* and exist *in vivo* (16,40,41). LIP probably inhibits C/EBP site-dependent transcription by similar mechanisms. The reason that LIP was a stronger negative regulator than Ig/EBP in our assay is not clear from these data. It is possible that by virtue of having identical ZIP domains LIP and NF-IL6 may form heterodimers with higher affinity or that LIP-containing heterodimers bind C/EBP sites with greater affinity than Ig/EBP-containing heterodimers.

Biological role of Ig/EBP

Ig/EBP mRNA is expressed in all adult tissues which have been analyzed (16) and it has also been found in many tissues of 14 day mouse embryos (Y. Ren and K. Calame, unpublished). We demonstrate here that Ig/EBP inhibits the transcriptional activities of two proteins, C/EBP α and NF-IL6 (C/EBP β), which profoundly alter cell activation and states of differentiation. NF-IL6 mRNA expression is low in most tissues but is subject to

induction in response to stimulation by cytokines and mitogens, including IL1, TNF α , IL-6 and LPS (2). NF-IL6 protein in turn induces the expression of cytokine regulated genes and is in part responsible for conversion of some cell types to an activated state (2,42). NF-IL6 is also induced during B-cell differentiation (40). C/EBP α is similarly expressed at very low levels and its expression is induced in the differentiation program of hepatocytes and adipocytes (10,22). Ig/EBP is expressed ubiquitously and its levels do not appear to change significantly during activation or differentiation (16,40).

Therefore, in many cells, especially progenitor cells or incompletely differentiated cells, such as pre-B cells, Ig/EBP is expressed in molar excess relative to activators C/EBP or NF-IL6. In this context, Ig/EBP probably acts as a buffer for C/EBP activators, insuring that C/EBP activator activity is kept off. As the C/EBP activator concentration increases during differentiation or cytokine stimulation, the buffering capacity of Ig/EBP is exceeded resulting in C/EBP site-dependent activation. The constant amount of Ig/EBP may result in a threshold phenomenon: C/EBP activator activity may only be detected when protein levels exceed a certain concentration in the nucleus. We know, for example, that the NF-IL6/Ig/EBP ratio increases significantly during B-cell development (40) providing one example where Ig/EBP regulation is likely to play an important biological role.

Other dominant negative regulators in the CEBP family

The ubiquitous expression of Ig/EBP distinguishes it from the two other negative regulators of the C/EBP family. Although the structure and the function of Ig/EBP resemble that of LIP (27), the expression patterns of the two proteins are significantly different. LIP is the product of translational initiation at an internal AUG codon in NF-IL6 mRNA. The mechanism(s) which determine the relative amounts of NF-IL6 or LIP which are translated from NF-IL6 mRNA appear to be regulated (40) but that regulation is not understood. Although it clearly functions to negatively regulate C/EBP family activators in many cell types, LIP is also functionally distinct from Ig/EBP in that it possesses weak intrinsic activation potential in a preB-cell context, where Ig/EBP is functionally inert (40).

CHOP-10, which heterodimerizes but does not bind DNA, negatively regulates C/EBP family activators by preventing them from binding to DNA (28). CHOP-10 is not expressed at significant levels in normal proliferating cells and appears to be induced only upon terminal differentiation or when cells undergo growth arrest or sustain DNA damage (28,43,44). The different expression patterns of Ig/EBP, LIP and CHOP-10 suggest that these three negative regulators may play distinct roles. Ig/EBP may provide general negative regulation, while LIP and CHOP-10 act under more specialized circumstances in response to particular signals.

The multiple dimerization partners for Ig/EBP suggests the existence of a network in which Ig/EBP might buffer C/EBP family inhibitors as well as activators. For example, if Ig/EBP preferentially dimerized with CHOP-10, a cell containing NF-IL6, CHOP-10 and Ig/EBP would have more C/EBP site-dependent activation than a cell with similar amounts of NF-IL6 and CHOP-10 but lacking Ig/EBP. A mechanism such as this may be functional in B cells where previous studies using an *in vitro* transcription system showed that nuclear extracts depleted of Ig/EBP had reduced C/EBP site-dependent promoter activity

(41). An additional function for Ig/EBP is suggested by the recent demonstration that it heterodimerizes efficiently with ATF4, forming a heterodimer with altered DNA binding specificity (20). Ig/EBP may also heterodimerize with other members of the ATF family, since C/EBP and NF-IL6 have been shown to heterodimerize with C/ATF, resulting in heterodimers which bind to cAMP response elements (45). Although the transcriptional activity of ATF/Ig/EBP heterodimers has not been tested, it seems likely that Ig/EBP could negatively regulate activators in this family as well.

The intriguing presence of multiple mechanisms for inhibiting C/EBP family transcriptional activators emphasizes the importance of the C/EBP family for regulation of gene expression in a wide variety of tissues and also suggests that negative regulation of the C/EBP family proteins is critical for their correct function. This study adds to the increasing evidence that negative regulation of transcriptional activators may be an important general mechanism for modulating a variety of transcriptional controls (27,28,46–50)

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