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Myofibroblasts possess ultrastructural features intermediate between those of fibroblasts and smooth muscle cells and have been defined by their ability to express contractile proteins, particularly α-smooth muscle actin (α-SMA)3 protein (1–3). Their derivation from fibroblasts can be demonstrated in vitro (4, 5). The myofibroblast differentiation process appears to be a critical event in the pathogenesis of fibrotic diseases such as pulmonary fibrosis (6–11). IL-1β, one of the major cytokines that mediate inflammatory reactions, represses myofibroblast differentiation and α-SMA gene expression (12). Although there is evidence of selective myofibroblast apoptosis in response to IL-1β treatment, the effect on α-SMA gene expression per se has not been specifically addressed, especially with respect to the molecular mechanism.

The basic leucine zipper transcription factor, C/EBPβ, is a trans-acting factor that controls cell differentiation and proliferation in a number cell types (13–17). Two kinds of C/EBPβ isoforms, varying in size from 14–38 kDa, have been reported. One is the 38- and 35-kDa full-length liver-enriched activator protein (LAP) isoforms, and the other is the 21- and 14-kDa truncated liver-enriched inhibitory protein (LIP) isoforms (18–21). The 14-kDa truncated LIP isoform was identified in macrophages (18). Both the p14 and p21 truncated C/EBPβ (LIP) isoforms retain the C-terminal bZIP and DNA binding domains, but lack the N-terminal transactivation domain (20–22). Probably due to its higher affinity for its DNA cognate sequences, LIP can attenuate the transcriptional stimulation by LAP in substoichiometric amounts (20).

Several reports indicate that the ratio of C/EBPβ isoforms (i.e., LAP/LIP ratio) present in a cell can be altered by a variety of cellular conditions (20, 22). This is potentially significant, because evidence indicates that the N-terminal truncated C/EBPβ (LIP) isoforms function as dominant negative inhibitors of the full-length C/EBPβ (LAP) isoform even when present at substoichiometric levels (i.e., LAP:LIP ratio of 5:1) (20, 22).

In this study the roles of C/EBPβ isoforms in the regulation of α-SMA gene expression were analyzed. Western blot analysis showed that C/EBPβ isoforms, 21-kDa LAP as well as the 38- and 35-kDa LAP isoforms, were expressed in rat lung fibroblasts. IL-1β treatment preferentially enhanced the nuclear accumulation of LAP and significantly decreased the LAP/LIP ratio. Transient transfection and gene knockout (KO) analysis demonstrated that LAP stimulated, whereas LIP inhibited, expression of the α-SMA gene. Promoter scanning identified a C/EBPβ binding consensus sequence in the α-SMA promoter that was shown to be important for α-SMA gene expression by site-directed mutagenesis. Binding of C/EBPβ to this promoter was demonstrated by EMSA. Further study showed that IL-1β enhanced the expression of eukaryotic initiation factor-4E (eIF-4E), which is an activator for expression of the C/EBPβ inhibitor isoform LIP. These data taken together strongly suggest an important role for C/EBPβ in mediating the regulation of α-SMA gene expression by IL-1β.
Materials and Methods

Animals and cell culture

C/EBPβ-deficient male mice (−/−) were gifts from Dr. P. F. Johnson (National Cancer Institute, National Institutes of Health, Bethesda, MD) and were generated as described previously (23). They were mated with wild-type C57BL/6 females for generation of heterozygous progeny (+/−), which were genotyped as previously described (23). Then the male and female heterozygous progeny were mated to generate homozygous C/EBPβ (−/−) KO and wild type (+/+) littermate control mice. Fibroblasts were isolated from adult mouse and rat (Fisher 344) lungs as described previously (7). Briefly, animals were killed, and their lungs were perfused with PBS. The lung tissue was digested with trypsin-EDTA solution until the cells were released. The cells were cultured in complete medium, composed of DMEM supplemented with 10% plasma-derived serum (PDS; Cocalico Biologicals, Reamstown, PA), 100 μM penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 1% insulin/transferrin/selenium (Sigma-Aldrich, St. Louis, MO), 5 ng/ml platelet-derived growth factor (PDGF Systems, Minneapolis, MN), and 10 ng/ml EGF (R&D Systems, Minneapolis, MN). These cells were confirmed as fibroblasts on the basis of stellate morphology, production of collagen type I, and ability to propagate in this medium.

IL-1β treatment

Cells were cultured overnight in complete medium. They were deprived of growth factors by rinsing twice in PBS and incubating in DMEM containing 0.5% PDS for 24 h. This was followed by addition of IL-1β (R&D Systems) at the indicated concentrations. After 6 and 48 h, cells were harvested for mRNA and protein analyses, respectively, as described below.

α-SMA promoter constructs and cDNA clones

The rat α-SMA promoter was previously cloned by PCR from rat genomic DNA (7). It was inserted into vector pGL3-basic (Promega) at the Smal site to form the α-SMA-luc fusion plasmid pGal3-αSMA, which was used as the template for construction of α-SMA promoter mutants. A C/EBPβ binding consensus, TTTGGGAAG, was identified at −67 to −58 from the transcriptional start site in the rat α-SMA promoter. In accordance with the template for construction of the C/EBPβ binding consensus mutated α-SMA promoter mutant α-SMA-luc-C/EBPmut to confirm the functional importance of the C/EBPβ binding consensus. Primers C (5′-TGCAAAATCTGGCTGCTATCCTGCTATGACTCTACCCACG-3′) and D (5′-GCAAGTGTGCAGAAATGAAAGTTTGCA-3′) were used for construction of the C/EBPβ binding consensus mutated α-SMA promoter mutant α-SMA-luc-homo with a mutation at the C/EBPβ binding consensus. An Eagl or a Povl restriction endonuclease site was created in each mutant, respectively, for screening. All mutants were constructed with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). These mutants were then inserted into vector pGL3-Basic to form fusion plasmids pGal3-αSMA-C/EBPmut and pGal3-αSMA-αSMAControl. Rat C/EBPβ cDNA (35-kDa LAP) expression plasmid pCMV-LAP and C/EBPβ cDNA (21-kDa LAP) expression plasmid pCMV-LIP were gifts from Dr. J. Schwartz (University of Michigan) and were originally obtained from Dr. U. Schübeler (University of Geneva, Geneva, Switzerland) (20).

mRNA analysis

For analysis of α-SMA mRNA levels, real-time PCR was used as previously described (24). Results were expressed as 2−ΔΔCT with GAPDH used as endogenous control (24). For analysis of eIF-4E mRNA expression, total RNA samples were used for RT-PCR analysis. RT-PCR was undertaken with the SuperScript one-step RT-PCR system (Invitrogen Life Technologies, Gaithersburg, MD) and using the following protocol: one cycle each of 50°C for 30 min and 94°C for 2 min, followed by 26 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, and 72°C for 10 min by one cycle of 72°C for 10 min. To normalize the amount of input RNA, amplification of the GAPDH mRNA signal was used as an internal control. The following primers were used: eIF-4E, 5′-CCGAATTCAGCGAGCGAGTTGCGG-3′ and 5′-GAGGCCGCCCTCA GAAGGTGTTCTC; and GAPDH, 5′-GGTCTCTGATGGCGAGTGT AGT-3′ and 5′-TCCAGTGATGACTCTCACCAG-3′. Equal aliquots of each sample were electrophoresed on 1.5% agarose gels. Bands were visualized by ethidium bromide staining.

Immunoblotting

Cells (7 × 105/well) were plated in six-well plates and treated with 1μg/l-β and as described above. The cells were prepared in Laemmli sample loading buffer. Equal amount of proteins were electrophoresed through 12% SDS-polyacrylamide gels. Where indicated in the case of analysis of LIP and LAP expression, nuclear extracts were used and prepared as previously described (7). Equal loading was confirmed by probing the blots for β-tubulin using specific Abs (Santa Cruz Biotechnology, Santa Cruz, CA). The separated protein bands were transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 10% nonfat milk (Bio-Rad, Hercules, CA) in 10 mM Tris-buffered saline containing 0.5% Tween 20. α-SMA was detected using anti-α-SMA mAb (Cytosol Biotechnology, Hampshire, U.K.) at a dilution of 1/2000, an anti-mouse IgG linked to HRP (Amersham Biosciences, Piscataway, NJ), and chemiluminescent substrate LumiGLO (New England Biolabs, Beverly, MA). C/EBPβ Ab (sc-7962X) against both C/EBPβ isomers was purchased from Santa Cruz Biotechnology. The blots were exposed to Hyperfilm ECL film (Amersham Biosciences).

Transfection and reporter gene assay

All transient transfections were performed using the FuGENE 6 reagent (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Supercoiled DNA was isolated with an endotoxin-free column kit (Qiagen, Valencia, CA). Unless otherwise indicated, cells were seeded in six-well plates at a density of 105/well in DMEM containing 10% PDS and incubated at 37°C overnight. Two micrograms of DNA of the construct of interest and 1 μg of plasmid pRL-TK control vector (used for normalization) were cotransfected per culture in DMEM containing 0.5% PDS with or without 10ng/ml IL-1β treatment. After 48 h, the cells were harvested and the activity of firefly or Renilla luciferase was measured using the dual luciferase assay system from Promega (Madison, WI). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase. Experiments with each construct were repeated two to four times, and relative activity (fold over promoterless control) was expressed as the mean ± SE.

Nuclear extract preparation

Nuclear extracts were prepared from untreated and IL-1β-treated cultures as previously described (7, 9). Briefly, the cultures were rinsed twice with cold PBS and then with Dignami’s buffer A (10mM HEPES (pH7.9), 10mM KCl, 0.1 mM EDTA, 1mM DTT, 0.5mM PMSF, and 4μg/ml leupeptin). The cells were then scraped into buffer A and washed once with the same buffer. The cell pellet was finally resuspended in buffer A and kept on ice for 15 min before brief extraction in 0.6% Nonidet P-40. The extract was vortexed and centrifuged briefly, and the cytoplasmic extract was removed. The nuclei were further extracted in Dignami’s buffer C (20mM HEPES, 1.5mM MgCl2, 420mM NaCl, 0.2mM EDTA, 25% glycerol, 1mM DTT, 0.5mM PMSF, and 4μg/ml leupeptin) for 10 min on ice. The extracts were centrifuged, and the supernatants were stored at −70°C until used. The protein concentration was tested by the bicinchoninic acid method (Pierce, Rockford, IL).

EMSA

EMSA was conducted as previously described (7). Single-stranded oligonucleotides in the sense and antisense directions were synthesized to detect the binding of C/EBPβ. The sense probe sequence is 5′-TTTGGCTCTTT GTTGGGAAACGGACGTTGGGAGGATGACGACGAC-3′, corresponding to the region between −78 and −36 from the transcription start site of the α-SMA promoter. Another set of primers in which the C/EBPβ binding consensus was altered with the sense sequence 5′-TTTGGCTCTTTT GCGCGCGGAGTTGGGAGGATGACGACGAC-3′ was prepared to confirm the specificity of binding. The oligonucleotides were annealed before labeling with T4 polynucleotide kinase and [γ-32P]ATP to detect dsDNA binding proteins. EMSA reaction mixtures contained 3–5 μg of protein extract, 1.0 μg of poly(dI·dC), 0.1 μg of poly-l-lysine, 0.5–1 ng of labeled probe (20,000–30,000 cpm), and Abs as indicated in a final volume 15 μl of Dignami’s buffer C. Where indicated, the EMSA reaction mixtures were prepared with Abs on ice for 30 min before probe addition and incubation for another 20 min at room temperature. Samples were then analyzed by electrophoresis on 4% nondenaturing polyacrylamide gels at 100 V in 1× 89 mM Tris-borate and 2 mM EDTA, pH 8.0. After electrophoresis, the gels were dried and exposed to x-ray film for 24 h.

Statistical analysis

This was undertaken as before using ANOVA, followed, where appropriate, by post hoc testing using Scheffe’s test (24). A value of p < 0.05 was
used as a criterion for statistical significance in comparisons between any two groups.

**Results**

**Effects of IL-1β on fibroblast C/EBPβ expression**

α-SMA expression is a key phenotypic marker of myofibroblast differentiation from fibroblasts (1–3). The differentiation process is repressed by cytokines such as IL-1β, one of the major cytokines that mediate inflammatory reactions (12). However, the mechanism of this inhibition is unclear. The basic leucine zipper transcription factor C/EBPβ belongs to the C/EBP family and is reported to be induced by IL-1β in rat liver (25). To investigate whether C/EBPβ can play a role in the repression of α-SMA gene expression by IL-1β, the expression of C/EBPβ in rat lung fibroblasts was analyzed by Western blot analysis. The results showed that control untreated cell nuclei contained detectable levels of the LAP forms (35 and 38 kDa) of C/EBPβ, with very low levels of the LIP (21-kDa) isoform (Fig. 1). Treatment with IL-1β increased the nuclear levels of both LAP and LIP isoforms in a dose-dependent manner while concomitantly decreasing α-SMA expression. However in response to the same doses of IL-1β, the increase in LIP (~6.5-fold at 50 ng/ml IL-1β treatment) was greater than that in LAP (~2.5-fold at 50 ng/ml IL-1β treatment), resulting in a decreasing LAP/LIP ratio (from 6.74 in control cells to 2.34 in cells treated with 50 ng/ml IL-1β). Thus, the altered expression ratio between these C/EBPβ isoforms after IL-1β treatment was associated with alterations in α-SMA expression, suggesting a role for these isoforms in mediating the IL-1β signaling in myofibroblast differentiation.

**Effects of C/EBPβ isoforms on α-SMA expression**

To confirm the role of C/EBPβ in the regulation of α-SMA gene expression by IL-1β, LAP and LIP expression plasmids were used to induce their transient expression in rat lung fibroblasts, and the effects on α-SMA expression were analyzed by real-time PCR. As expected, transfection with the LIP plasmid caused a significant increase in nuclear LIP protein levels in control cells as well as in cells treated with IL-1β (Fig. 2A, left panel). However, the LIP plasmid-induced increase in IL-1β-treated cells was less due to the higher baseline levels of LIP in these cells. Transfection with the LAP plasmid did not affect LIP levels in these cells. Similarly, transfection with the LAP plasmid increased nuclear LAP levels, although the increases were smaller than in LIP levels induced by the LIP plasmid (Fig. 2A, right panel). These were probably due to the already higher endogenous levels of LAP in cells transfected with the empty vector only. Transfection with the LAP plasmid also had no effect on nuclear LAP levels. The effects of these alterations in nuclear LAP or LIP levels on α-SMA expression were then analyzed by real-time PCR. The results (Fig. 2B) show that transfection with the LAP expression plasmid caused a significant increase in α-SMA expression in both control and IL-1β-treated rat lung fibroblasts relative to that in cells transfected with the control empty vector. This was in contrast to the significant reduction in cells transfected with the LIP expression plasmid. Western blot analysis showed similar responses in α-SMA protein expression, namely, stimulation by transfection with LAP expression plasmid and inhibition by the LIP expression plasmid, although the stimulation by the LAP plasmid was less in untreated cells than in IL-1β-treated cells (Fig. 3).

To confirm that C/EBPβ regulates α-SMA gene expression at the transcriptional level, the α-SMA gene promoter was inserted into promoterless vector pGL3-Basic to form α-SMA-Luc fusion plasmid where the transcription of the luciferase gene was driven by the α-SMA promoter. This construct was cotransfected into rat lung fibroblasts with the LIP plasmid, the LAP expression plasmid, or the empty expression vector. Luciferase activity was then measured as an indicator of α-SMA promoter activity. As shown in Fig. 4 (left panel), relative to cotransfection with the empty vector, cotransfection with the LAP plasmid stimulated, whereas that with the LIP plasmid inhibited, luciferase activity. These differences remained evident when the cells were treated with IL-1β, although activities were significantly lower than for their respective nontreated counterparts. These results were consistent with the analysis of α-SMA mRNA and protein expression and confirmed that the C/EBPβ isoforms, LAP and LIP, could divergently regulate transcription of the α-SMA gene via the C/EBP-binding consensus sequence present in its promoter. The latter was confirmed by the lack of effect of LAP and LIP plasmids on an α-SMA promoter construct mutated at this C/EBP-binding consensus sequence present in its promoter. To further confirm the importance of C/EBPβ isoforms in the regulation of α-SMA gene expression, lung fibroblasts from C/EBPβ homozygous KO (−/−), heterozygous KO (−/+, or wild-type

![FIGURE 1](http://example.com/fig1.jpg)

**FIGURE 1.** Effects of IL-1β on C/EBPβ and α-SMA expression. Rat lung fibroblasts were treated with the indicated doses of IL-1β for 48 h, then harvested for Western blotting analysis to evaluate expression of α-SMA (upper panel) and C/EBPβ isoforms (lower panel). Equal amounts of protein were loaded for gel electrophoresis before blotting. All samples for detecting α-SMA were from whole-cell lysates, and samples for detecting C/EBPβ were from nuclear extracts. The different isoforms of C/EBPβ were indicated by arrows. The numbers beneath each lane of the α-SMA blot (upper panel) indicate the relative band intensities (as a percentage of the untreated control band intensity) determined by densitometric scanning, with the untreated control set at 100%. The numbers beneath each lane of the blot of C/EBPβ isoforms (lower panel) indicate the relative total LAP or LIP band intensities (as a percentage of LIP band intensity in the untreated control) determined by densitometric scanning, with the untreated LIP control set at 100%. The LAP/LIP ratio is also indicated at the bottom row of numbers beneath each lane. For loading control, the blot for β-tubulin is shown. Representative blots of α-SMA and C/EBPβ isoforms are shown. This experiment was repeated three times with similar results.
mozygous KO mice, which essentially did not respond to IL-1β treatment. These Western blotting results were paralleled by the α-SMA promoter activity analysis. Thus, promoter activity as monitored by luciferase activity was highest in cells from wild-type mice, intermediate from heterozygotes, and least from homozygous KO mice (Fig. 6). IL-1β treatment again suppressed promoter activity relative to their corresponding control cells, except for the cells from homozygous KO mice that failed to respond to IL-1β treatment. These results provided additional support for a key role for C/EBPβ in regulation of α-SMA gene expression, in particular its suppression by IL-1β.

Role of C/EBPβ binding consensus in the α-SMA promoter

In view of the preceding results, the α-SMA gene promoter sequence was scanned for a possible C/EBPβ binding sequence to further confirm direct regulation of the α-SMA promoter by this transcription factor. C/EBPβ is thought to recognize and bind to a TKNNNGA (K = T or G, N = A, T, C, or G) consensus sequence to regulate its target gene expression (26). The scanning results identified such a consensus sequence, TTTGGGAAG, at −66 to −58 upstream of the transcription start site in the rat α-SMA promoter (Fig. 7A). To determine whether this consensus sequence is actually functional or important for α-SMA gene expression, site-directed mutagenesis was used to construct a

(+/+) mice were analyzed for α-SMA expression by Western blotting and α-SMA promoter activity. The results of Western blotting analysis showed that the level of lung fibroblast α-SMA expression correlated with the level of C/EBPβ isoform expression in the various mouse strains (Fig. 5). Thus, the level of α-SMA expression progressively decreased in cells from wild-type to homozygous KO mice, with intermediate expression in cells from heterozygous KO mice. These gradations in α-SMA expression were also noted in cells treated with IL-1β. However, in IL-1β-treated cells, α-SMA expression was decreased relative to their respective untreated control cells, except for the cells from homozygous KO mice, which essentially did not respond to IL-1β treatment.

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C/EBPβ binding consensus mutated α-SMA promoter mutant (α-SMAP-luc) and an α-SMA promoter control mutant with altered sequence outside of the C/EBPβ consensus sequence (α-SMAP-luc-control). These α-SMA promoter mutants and the wild-type α-SMA promoter (α-SMAP-luc) were each inserted into promoterless luciferase vector pGL3-basic to drive the transcription of luciferase in the corresponding constructs (Fig. 7A). Upon transfection into rat lung fibroblasts, the wild-type construct showed expected promoter activity that was suppressed by IL-1β treatment (Fig. 7B). The construct mutated at the C/EBPβ binding consensus sequence (α-SMAP-luc-C/EBPmut) showed markedly reduced α-SMA promoter activity (<40% of wild-type promoter), which was not further affected by IL-1β treatment. The activity of the control mutant promoter construct (α-SMAP-luc-control) was comparable to that for the wild-type control in the absence or the presence of IL-1β treatment. These findings confirmed the importance of the C/EBPβ consensus sequence in regulation of α-SMA gene expression, especially for its inhibition by IL-1β.

Binding of C/EBPβ to the α-SMA promoter C/EBPβ binding consensus

Because C/EBPβ isoforms and the C/EBP binding consensus sequence in the α-SMA promoter appeared to be important for α-SMA gene expression, the possibility of direct binding of this transcription factor to the consensus sequence was examined. To evaluate such a direct interaction of C/EBPβ with the TTTGGGAAG binding consensus in the α-SMA promoter, single-stranded sense and antisense oligonucleotides corresponding to the sequence at −78 to −36 of the α-SMA promoter were synthesized and annealed as the probe used in EMSAs. The results showed that a complex was formed when the probe was incubated with nuclear extracts from either untreated control or IL-1β-treated fibroblasts, although complex formation was dramatically enhanced with extracts from IL-1β-treated cells (Fig. 8). Complex formation was specific because competition with a 100-fold excess of unlabeled probe abolished the shifted band. Furthermore, incubation with anti-C/EBPβ Abs caused the band to supershift, indicative of the presence of C/EBPβ in the DNA-protein complex. Mutation in the binding consensus abrogated the formation of the DNA-protein complex (data not shown). These results indicated that C/EBPβ could bind to the C/EBPβ binding consensus in the α-SMA promoter, which was enhanced by treating cells with IL-1β. Such binding appears to play a key role in regulation of α-SMA gene expression, especially its inhibition by IL-1β.

IL-1β-induced eIF-4E expression

The findings from the above experiments suggested that IL-1β inhibited α-SMA gene expression by altering the ratio of C/EBPβ isoforms that act on the C/EBPβ binding consensus sequence in the α-SMA promoter. eIF-4E, a 25-kDa cap binding protein involved in the initiation of protein translation (27), is known to selectively promote LIP expression (28). To investigate whether IL-1β reduced the LAP/LIP ratio via such a mechanism, the expression of eIF-4E in rat lung fibroblasts was analyzed by RT-PCR and Western blot. The results shown in Fig. 9 indicated that the expression of eIF-4E at both the mRNA and protein levels was stimulated by IL-1β treatment in a dose-dependent manner. As a control, the cellular levels of GAPDH mRNA were found not to be significantly affected by IL-1β. Because eIF-4E is known to selectively activate LIP expression (28), its induction by IL-1β suggests a potential role for this factor in mediating reduction of the LAP/LIP ratio by IL-1β.

Discussion

Myofibroblasts are induced de novo in granulation tissue of contracting wounds and fibroproliferative diseases such as pulmonary fibrosis and are also present in some developing or normal adult...
Wild-type α-SMA promoter construct (pGal3-α-SMAp-luc, also abbreviated as α-SMAP-luc) was transfected into fibroblasts isolated from C/EBPβ KO mice (+/-), heterozygous deficient mice (+/+), or wild-type (++) littermates, then treated with 10 ng/ml IL-1β or buffer only (NONE) for 48 h. The cell extracts were harvested and assayed for luciferase activity. The luciferase activity for each construct was normalized to its respective Renilla luciferase internal control activity and shown as the fold increase over the promoterless activity. Data represent the mean ± SE of triplicate samples. Promoter activity in untreated treated cells from KO mice was significantly lower than that in cells from heterozygous or wild-type mice, whereas heterozygous cells had significantly lower activity than wild-type cells. IL-1β treatment caused a significant reduction in promoter activity only in wild-type and heterozygous cells.

FIGURE 6. Effect of C/EBPβ deficiency on α-SMA promoter activity. A, A representation map of wild-type α-SMA promoter and mutant constructs is shown. The relative locations of the C/EBPβ binding consensus, TTTGGGAAG, and the site for control mutation are indicated with their nucleotide sequences. Mutated bases in the C/EBPβ binding consensus binding sequence were identified with black boxes. Thus, the C/EBPβ binding consensus binding sequence was identified in the α-SMA promoter and found to be important for α-SMA gene expression by site-directed mutagenesis studies. Thus, the C/EBPβ isoforms may play a direct role in regulation of the α-SMA promoter via binding interactions with this consensus binding sequence, which was confirmed by EMSA in combination with supershift analysis. Therefore, the importance of this direct C/EBPβ regulation of the α-SMA promoter to IL-1β regulation of α-SMA gene expression is demonstrated by the lack of responsiveness to IL-1β in C/EBPβ-deficient cells and cells transfected with an α-SMA promoter with a mutated C/EBPβ consensus binding sequence. Thus, the totality of these findings provide adequate proof that C/EBPβ is important in regulation of α-SMA expression, and that...
IL-1β inhibition of such expression is entirely dependent on this transcription factor as mediated by its LIP isoform, perhaps through dominant negative inhibition of LAP activity. LIP is known to be a dominant negative isoform of LAP with the capacity to control LAP activity at substoichiometric concentrations (20, 22). The mechanism of inhibition by LIP is believed to be due to the greater binding capability of LIP to the C/EBPβ binding element or consensus sequence, which is 4-fold higher than that of LAP (20). Although LIP can preferentially bind to this element in the α-SMA promoter at the expense of LAP, it lacks the activation domain present in LAP and is consequently unable to activate the target gene. Consequently, a decreasing LAP/LIP ratio will result in diminution of the activating effects of LAP (20). Hence, the preferential stimulation of LIP by IL-1β represents a key mechanism by which this inflammatory cytokine inhibits α-SMA gene expression.

The mechanism for controlling the LAP/LIP ratio is not fully understood, although there is evidence for eIF-4E-mediated control at the translational level. eIF-4E binds to the 7-methyl guanosine-containing cap of mRNA and facilitates the binding of the eIF-4G and eIF-4A complexes (27–29). These subunits act as an RNA helicase, resulting in the unwinding of the 5′-untranslated regions of mRNA, the binding by the 40S ribosome subunit and the initiation of protein synthesis (30, 31). eIF-4E is the least abundant initiation factor in most cells, and its activity is increased in response to many extracellular stimuli that stimulate cell growth. Thus, overexpression of eIF4E would be expected to preferentially increase the translation of mRNAs that are poorly translated due to presence of extensive 5′ secondary structure. In the case of C/EBPβ, the LAP and LIP isoforms are believed to be translated from the same mRNA, but with different translation initiation sites (20), with eIF-4E enhancing the expression of the truncated isoforms in an upstream (relative to the LIP translation initiation site) open reading frame-dependent manner (28). As for how IL-1β controls the LAP/LIP ratio, our results indicated that eIF-4E may be involved. In this study we showed for the first time the enhancement of eIF-4E expression by IL-1β treatment, which could account for the preferential increase in LIP expression, resulting in the observed reduced LAP/LIP ratio. Further studies are needed to fully delineate the role of eIF-4E and other potential factors in differential regulation of C/EBPβ isoform expression by IL-1β.

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**References**

C/EBPβ REGULATES α-SMA EXPRESSION


